Developing Hybrid Material Interfaces for Microcontact Printing and Molecular Recognition

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

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ABSTRACT

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Abstract

Monomolecular hybrid organic-inorganic interfaces provide opportunities for applications in fields ranging from sensors to electronics. In this thesis, we report our efforts towards (1) developing a universal method for the modification and soft-lithographic patterning of inorganic materials with stable and functional organic systems; and (2) apply our surface fabrication techniques to advance our understanding of molecular recognition force microscopy.

We report the development of a novel bi-layered molecular system that, in conjunction with an inkless catalytic microcontact printing technique, can be used to accurately replicate micro- and nano-scale patterns of chemically distinctive reactive functionalities on virtually any surface, including inorganic semiconductors. Catalytic printing alleviates problems associated with ink diffusion and enables high resolution replication of patterns through specific chemical or biochemical reaction between a functional surface and a stamp-immobilized catalyst. The methodology provides precise control over shape and size of pattern features and provides access to chemically discriminated patterns that can be further functionalized with organic and biological molecules. We demonstrate catalytic printing on both oxide-free silicon and germanium, substrates that do not react readily with organic molecules and have not heretofore been patterned through traditional approaches. Our approach we relies on a stable highly ordered bilayered molecular system that both affords complete protection of all surface-exposed inorganic atoms with stable covalent bonds and supports covalent immobilization of a reactive overlayer, yielding stability and functionality to the surface.
A catalytic acidic stamp was used to achieve pattern-specific hydrolysis of N-hydroxysuccinimide-activated acids immobilized on Si and Ge. Further modification of the chemically discriminated patterns enables chemoselective anchoring of organic molecules and protein.

We demonstrated the utility of the strategy towards a variety of inorganic oxides, including ITO. Utilizing the functionalized bi-layered system on ITO, a single molecular system in combination with different printing approaches can be used to immobilize multiple organic functionalities with exquisite spatial control. The system was used to investigate structure – function relationships of the ordered and functional molecular system on ITO to vertically and laterally control charge injection in organic light emitting diodes (OLEDs).

Finally, we report fabrication of functional hybrid organic-inorganic interfaces for the study of immobilized binding partners, lactose-g3 and complementary ssDNA, in molecular recognition force microscopy (MRFM). We use our system to evaluate the effect of contact force on specific interactions and the effect of dwell time and tether length on the probability of ligand-receptor binding. The methodologies developed enable a reliable evaluation of thermodynamic parameters using MRFM.
Soli Deo Gloria
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List of Abbreviations

AFM – atomic force spectroscopy

APTMS-aminopropyltrimethoxysilanes

Boc – tert-butoxycarbonyl

CLR-collagen-like repeat

CRD-carbohydrate recognition domain

DCM-dichloromethane

DNA – deoxyribonucleic acid

ss-DNA – single stranded deoxyribonucleic acid

ds-DNA – double stranded deoxyribonucleic acid

Exo – exonuclease

$F_c$-contact force

$F_{\text{nom}}$-nominal contact force

$F_{\text{min}}$-Force minimization protocol

$F_{\text{rup}}$-Rupture Force

Fmoc – 9H-fluoren-9-ylmethoxycarbonyl

G3-galectin-3

GFP – green fluorescent protein

His$_6$-hexa-histidine

IC – integrated circuit

IPA – isopropyl alcohol

ITC-isothermal titration calorimetry
KDPG-2-keto-3-deoxy-6-phosphogluconate
MPTMS-mercaptopropyltrimethoxysilane
MRFM-molecular recognition force microscopy
NHS – N-hydroxy-succinimide
NMR – nuclear magnetic resonance
NTA – nitrilotriacetic acid
OEG-oligoethylene glycol
PDMS – polydimethylsiloxane
h-PDMS – hard polydimethylsiloxane
OLED-organic light emitting diode
PEG – polyethylene glycol
PTFE – polytetrafluoroethylene
PUA – polyurethane acrylate
Ra – arithmetic average roughness
Rmax – maximum roughness depth
Rq – root mean square roughness
SAM – self-assembled monolayer
SEM – scanning electron microscopy
SL – soft lithography
TBS – tert-butyl dimethyl silyl
TCEP-tris(carboxyethyl)phosphine
TFA – trifluoroacetic acid
TFT – thin-film transistor
TMS – trimethyl silyl
UV – ultra violet
XPS – X-ray photoelectron spectroscopy
E-beam – electron beam
µCP – microcontact printing
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support and endless encouragement to pursue my academic goals. I will always remember that “those who wait upon the Lord shall renew their strength, they shall mount up with wings like eagles, they shall run, and not be weary, and they shall walk, and not faint.” Isaiah 40:31

Finally, thank you to my devoted husband and best friend, Les, who has shared this journey with me. Your unremitting love, patience and support have sustained me throughout my academic pursuits. Thank you for always believing in me and encouraging me to realize my full potential.
1. Introduction

1.1 Overview: Historical events of molecular printing

Approaches to the storing of information by transferring ink to a substrate – writing –evolved along two distinct paths; serial and parallel writing. The first serial tool, the quill pen, was invented around 2000 BC. This primitive tool served as the principle writing instrument for thousands of years before the invention of the dip pen, the fountain pen and, more recently, the ball point pen. The pen is capable of creating written patterns with high fidelity and accuracy. Ultimately though, the low throughput and poor reproducibility of serial writing tools proved insufficient for rapid high volume archiving and sharing of information. The invention of woodblock printing in China (~200 AD) provided the first parallel method for printing text, images and patterns. Eventually the more sophisticated printing press was developed in 1439 and lithography emerged in 1796, providing rapid methods to transfer high-volume information.[1]

While the development of technology designed to replicate patterns on surfaces was originally motivated by the desire to transfer information – images and words – applications far beyond what could ever have been imagined by the early printing pioneers rapidly emerged. Key among these applications were (1) high-volume production of semiconductors and the need to increase the number of transistors in a given area; (2) the investigation of unique optical properties of nanostructured metallic materials; and (3) biological applications aimed to increase detection limits by using high density surface arrays. As feature size shrinks below the μm level, molecular transport,
self-assembly and intermolecular forces prevail, shifting the art of patterning to the chemist’s domain.\[^{[2]}\]

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<td>Moveable type printing</td>
<td>Ball Point (Loud, 1888)</td>
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**Figure 1. Historical timeline of the development of molecular printing or direct ink transfer to a surface**

The need to pattern materials on surfaces at increasingly smaller scales first emerged in the late 50s, as electrical circuit design necessary to enable computation increased in both size and complexity. Transistors were a promising alternative to vacuum (electron) tubes, offering reduced energy demands, with higher efficiency and reliability. However, scaling simple circuits to the sizes necessary to carry out complex functions rapidly became problematic.\[^{[3]}\] Individual components were soldered and connected manually, and system failures due to faulty connections were widespread. Additionally, the power needed to drive such increasingly complex systems rapidly became impractical. In the early 1960’s, the first integrated circuits containing several transistors and resistors were produced on the surface semiconductor material.\[^{[3-4]}\] All of the necessary components could be constructed simultaneously by depositing, patterning and etching different materials onto a surface. Eventually, the need to position components individually was eliminated, setting the stage for the continuous miniaturization and increasing reliability of modern electronic devices.
Photolithography is the primary process used for generating the initial template of patterns in electronic circuits and remains the patterning method of choice for integrated circuit manufacture. The technique uses light in conjunction with a photo mask to generate patterns on a photosensitive resist. The patterned photoresist can then be used to either spatially control the deposition of conducting materials or to selectively remove materials by acting as an etch mask.[5] The technique has been tremendously successful, driving the miniaturization of integrated circuits. Despite these successes, the resolution of photolithography is limited by optical diffraction. Additionally, photolithography is not compatible with light-sensitive materials or etchants, including many biological materials. As a result, photolithography has found only limited utility in the fabrication of biosensors, hybrid organic-inorganic interfaces and bioelectronics. A technique that could facilitate the rapid, inexpensive fabrication of substrates and pattern more diverse materials was required.

Microcontact printing (µCP) was introduced in 1993 by Whitesides and coworkers as an alternative to photolithography. Microcontact printing is a flexographic technique that relies on the diffusion of molecular inks from an elastomeric stamp to replicate patterns of organic or biological molecules on inorganic substrates.[6] The original goal was to replicate patterns using faster and less expensive methods than photolithography. Because µCP does not rely on light to transfer pattern, its resolution is not limited by optical diffraction, offering the potential that µCP might surpass the resolution of photolithography but at a fraction of the cost. The original embodiment of µCP used alkyl thiols to pattern gold surfaces, and its widespread utility quickly became
apparent. During the past 20 years the concepts of µCP have been expanded to pattern small molecules\cite{7}, metals\cite{8}, polymers\cite{9}, DNA\cite{10}, proteins\cite{11} and cells\cite{12}(Figure 2). Here we review the components and requirements of µCP, discuss the limitations of current variants of µCP and consider unconventional µCP techniques designed to circumvent the limitations of traditional soft lithography.

![Additive parallel process for modifying and patterning surfaces with organic, biological, polymeric and inorganic materials](image)

**Figure 2. Microcontact Printing**

### 1.2 Traditional Microcontact Printing: Components and requirements

Traditional µCP is a remarkably simple and inexpensive method for the modification of surfaces. Similar to conventional printing, µCP requires an ink, a substrate and a stamp. As opposed to the dyes and papers used in conventional printing, µCP inks are printed in monomolecular layers on flat metal, metal oxide or semiconductor surfaces. The technique involves first “inking” a micro-structured stamp with a dilute molecular solution and then bringing the stamp into conformal contact with the substrate surface. The molecular ink diffuses to the surface only in places of stamp contact, forming features identical to the relief structures on the polymeric stamp (Figure 3).
1.2.1 Polymeric Stamps and Templates

The first critical element of µCP is the polymeric stamp. In traditional µCP, a hard inorganic master is used as a template to prepare elastomeric stamps with inverted patterns. UV photolithography is most commonly used to fabricate the master. Typically, a thin, uniform layer of photoresist (an organic polymer sensitive to UV light) is spun onto a silicon wafer. After baking, the photoresist is exposed to UV light through
a metal patterned photo mask, which allows light to pass only in certain areas, thereby transferring the pattern of the photo mask to the underlying polymer. The resist is either retained as a topographic template on silicon for stamp molding, or the unprotected silicon regions are etched and the photoresist stripped, providing a wafer with patterned silicon. The remaining soluble photoresist is washed away, leaving behind a pattern of stable, cross-linked resist on silicon.

Molding a stamp from the hard master template is straightforward. Liquid prepolymer is poured onto the master template and polymerization is initiated thermally or with UV light, providing a solid elastomeric mold. After polymerization, the stamp is carefully peeled away from the master and cut to size based on application. After inking and drying, the stamp is applied to the corresponding surface. Washing the stamp after each printing process removes the remaining molecular inks and allows multiple reuse of a single stamp. Commercially available poly(dimethylsiloxane) (PDMS) possesses nearly ideal properties as a stamp material, and is the most commonly used in μCP. PDMS is both sufficiently flexible to facilitate conformal contact with even rough surfaces and sufficiently stiff to replicate patterns in the micrometer range without stamp collapse or sagging. PDMS is inexpensive, inert and non-toxic. Finally, the optical transparency of PDMS enables penetration of a wide variety of wavelengths.[13]

1.2.2 Molecular Inks and Substrates

The first demonstration of μCP by Kumar and Whitesides transferred alkanethiols to Au-coated silicon substrates (Figure 4). PDMS stamps were first exposed
to dilute solutions of alkyl thiols before being brought into contact with Au coated surfaces. During stamping, molecular ink migrates from the stamp to the surface and forms self-assembled monolayers (SAMs) in areas of stamp-surface contact. During ink transfer the recessed features serve as transport barriers, preventing SAM formation in non-contacted areas.

Figure 4. Traditional microcontact printing

Traditional patterning techniques depend on the spontaneous formation of SAMs at the stamp-substrate interface, and the most common SAM-substrate system involves the formation of thiolate monolayers on gold. Alkanethiols form highly ordered and robust SAMs on gold due to the spontaneous formation of a strong gold-sulfur bond (~44 kcal/mol\cite{14}) and weak attractive van der Waals interactions between alkyl chains. At high surface density, the hydrocarbon tails tilt approximately 30° from the surface normal in an all-
trans configuration that maximizes van der Waals interactions.\cite{15} Long chain (n>6) aliphatic thiols form ordered, crystalline and close-packed SAMs that can withstand harsh environments.\cite{16} This stability allows the patterns to be used as etch masks, resulting in the accurate formation of Au microstructures.
Another common SAM-substrate system utilizes alkylchlorosilanes, alkylalkoxysilanes or akylaminosilanes on oxides surfaces (Figure 6)\textsuperscript{[17]} Organosilanes generally consist of a silicon atom tetrahedrally bound to three similar, kinetically labile functional groups, typically alkoxy groups or halogens, as well as a functional group of interest bonded through a kinetically and thermodynamically stable Si-C bond.\textsuperscript{[18]}

Figure 6. Schematic of organosilane self-assembled monolayer on a SiO\textsubscript{2} substrate
In contrast to the Au-S bond which is charge transfer in nature, silane formation is driven by the \textit{in situ} formation of a polysiloxane network which is connected to the surface silanol groups (-SiOH) via Si-O-Si bonds. Siloxane monolayers have been successfully formed on silicon oxide, aluminum oxide, indium tin oxide, titanium oxide, silicon nitride, and mica. In this instance the packing and ordering of the alkyl chains are determined by the underlying structure of the surface polysiloxane network (Figure 7). Similar to alkanethiol monolayers, organosilanes form robust monolayers and have been used as etch resists to structure inorganic materials. SAMs of silane derivatives, however, are not as straightforward to produce as alkanethiolates on gold. Several AFM studies have shown that the deposition process strongly depends on several parameters including solvent, solution age, water content, deposition time, and temperature.\textsuperscript{[19]}

![Figure 7. Schematic description of the polysiloxane network at the monolayer-substrate interface.](image)
1.3 Traditional Microcontact Printing: Scope and Limitations

The original embodiment of µCP developed by Whitesides provided a facile, inexpensive and experimentally accessible approach to nanofabrication. Soon after the first publication by Kumar and Whitesides reporting patterning on gold, other metals were successfully used as substrates, including Ag, Cu and Pd. The low cost and experimental simplicity inspired widespread application of the technique in chemistry, biology and physics. Several inherent characteristics of the approach, however, limit the resolution of microcontact printing, and the replication of sub-micrometer patterns on diverse substrates remained challenging.

1.3.1 Deformations in elastomeric stamps

PDMS has several attractive properties as a stamp material for µCP. With a Young’s modulus near 1.5 MPa, PDMS affords good conformal contact with substrates while avoiding sag and pairing to replicate micrometer-size patterns with high fidelity. Moreover, the polymer is non-toxic, cheap, chemically inert and simple to polymerize. At dimensions below the µm domain, however, PDMS loses the ability to faithfully transfer patterns.

Although PDMS can be made with relief patterns in the sub-micrometer domain, stamp flexibility precludes high fidelity pattern transfer. Stamp deformations that occur both during removal from the hard master and during stamp-substrate contact provide practical limitations to resolution. The height of a feature divided its length defines the aspect ratio of a pattern. At high aspect ratio, buckling and lateral collapse of the
stamp features occur, while at low aspect ratios, stamp roof collapse or sagging is possible (Figure 8); both events negatively impact the fidelity of pattern transfer and, as a result, resolution. Experience suggests that the aspect ratio of PDMS features should be greater than 0.2 and the feature separation distance ($d$) should be smaller than $20h$ to achieve high fidelity pattern transfer.\textsuperscript{[26]}

![Figure 8. Stamp Deformations](image)

The morphological properties of PDMS stamps can be affected by solvents, providing another impediment to pattern transfer with small stamp features. PDMS swells in most organic solvents, changing the dimensions and shape of relief patterns.\textsuperscript{[27]} Ethanol induces minimal swelling, but is restricted to use with inks soluble in ethanol. Moreover, the hydrophobic nature of PDMS limits its applicability to apolar inks, since water-soluble inks do not effectively wet the surface or permeate the bulk of PDMS stamps. Surface oxidation facilitates the use of polar inks, although this alteration
frequently results in other problems, such as altered mechanical properties and surface cracking. Furthermore, the change in surface polarity appears to be a temporary phenomenon, and hydrophobic recovery occurs over relatively short timeframes.\textsuperscript{[28]}

Together, these limitations limit the applicability of PDMS-based patterning. To broaden patterning applications and achieve sub $\mu$m pattern transfer, mechanical or chemical modification of PDMS is required. Alternatively, a different polymer with a higher Young’s modulus might circumvent some of the limitations associated with PDMS stamps.

\subsection*{1.3.2 Molecular ink diffusion}

Traditional $\mu$CP relies on ink diffusion from an elastomeric stamp to a substrate in regions of conformal contact to form patterned SAMs.\textsuperscript{[29]} Diffusion of molecular inks away from stamp boundaries leads to lateral spreading of ink across the stamp (Figure 9, path 1 and 2). Lateral diffusion significantly diminishes edge resolution and generally precludes the replication of sub-micrometer features.\textsuperscript{[30]} The extent of lateral diffusion depends on both exposure time and the quantity of ink both on and in the stamp.\textsuperscript{[31]}

Furthermore, depending on the vapor pressure of the ink, temperature and humidity, molecular inks are capable of diffusing from the bulk through the vapor phase, depositing ink to the surface where no ink is desired and further degrading both contrast and resolution (Figure 9, path 3).

Much effort has been expended in an attempt to accurately replicate sub-micrometer patterns of alkanethiols on gold. Features as small as 250 nm have been
faithfully replicated, although this resolution was achieved with only specific inks and required tedious optimization of printing conditions (i.e. stamping time and ink concentration). Features below 250 nm have also been replicated, but with significant distortion from the corresponding stamp. It has been demonstrated that diffusion is responsible for at least 50 nm of edge distortion, suggesting that traditional µCP is reliable only for micrometer-size features.\[30\]

![Figure 9. Ink diffusion in traditional µCP along the surface (1, 2) and through the ambient vapor phase (3)](image)

**1.4 Traditional Microcontact Printing: Avoiding Stamp Deformations and Ink Diffusion**

To overcome the limitations associated with traditional µCP, a variety of alternative techniques have been developed. Unconventional printing techniques have focused on (1) modifying the stamp material to avoid deformations; (2) changing the ink properties to avoid diffusion; and (3) developing entirely different patterning approaches (Table 1). Significant work has also been directed towards broadening the
utility of µCP by expanding the range of applicable substrates and inks to enable biological, biochemical and bioengineering applications.

**Table 1. A partial list of alternative µCP strategies.**

<table>
<thead>
<tr>
<th>Avoiding Stamp Deformation</th>
<th>Avoiding Ink Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive µCP</td>
<td>High Speed</td>
</tr>
<tr>
<td>Composite stamps</td>
<td>Submerged µCP</td>
</tr>
<tr>
<td>Hard PDMS</td>
<td>Contact Inking</td>
</tr>
<tr>
<td>Chemically patterned flat stamps</td>
<td>Decal and nano-transfer lithography</td>
</tr>
<tr>
<td>Alternative elastomeric materials</td>
<td>Low Diffusion Inks</td>
</tr>
</tbody>
</table>

**1.4.1 Avoiding Stamp Deformations**

**1.4.1.1 Positive microcontact printing**

Positive µCP was developed by Delamarche and coworkers to overcome problems associated with the mechanical stability of common stamp materials. The approach relies on the formation of two different SAMs, as opposed to with a single monolayer in traditional approaches. In the first step, a labile monolayer is printed (Figure 10 B, SAM2). Delamarche and coworkers use pentaerythritol-tetrakis(3-mercaptopropionate) (PTMP), which forms a stable SAM on gold but which does not provide significant etch resistance. The patterned surface is subsequently backfilled with molecules that form a more protective SAM (Figure 10 B, SAM1). The final wet etching step dissolves SAM from those regions covered by the labile thiol.
Although this method avoids the stamp deformation problems associated with traditional µCP, resolution is still limited by ink diffusion and additionally by molecular exchange between labile and robust SAMs near pattern edges.

1.4.1.2 Composite stamps with rigid back support

To achieve accurate and uniform pattern transfer, two seemingly conflicting stamp characteristics are required: high mechanical stability of the relief structures and sufficient elasticity to ensure conformal contact with the substrate. To improve stamp stability, multilayered stamps composed of different materials have been investigated by several groups. Composite stamp structures have been fabricated with a thin PDMS layer built on a rigid back support, typically a glass plate. The interface of the polymer with the rigid support can be made either by pressing an etched glass slide onto liquid pre-polymer or by chemical adhesion through surface modification. This structure prevents sag and also reduces long range distortions due to external stress.
Using this approach, ultrathin PDMS (200 nm) supported on glass and silicon was capable of replicating sub-micrometer features over large areas.\textsuperscript{[33b]} Composite stamps, however, do not circumvent deformation associated with buckling and collapsing of high aspect ratio features, which are desirable for avoiding molecular ink gas diffusion from the voids between the features.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{composite_stamps.png}
\caption{Architectures of composite stamps: (A) three component and (B) two component.}
\end{figure}

1.4.1.3 Hard PDMS

The elastic modulus of PDMS has been altered in an attempt to increase resolution and support high aspect ratio structures below 500 nm. Schmid and Michel developed a hard PDMS (h-PDMS) with a high modulus (~9 N/mm\textsuperscript{2}), capable of supporting sub-micrometer features.\textsuperscript{[34]} The new stamp material, containing vinlymethyl copolymers and hydrosilane components with additional glass fillers, was capable of replicating structures to 80 nm. However, h-PDMS is brittle and external pressure is required to ensure conformal contact, pressure that induces non-uniform long range distortions in the replicated pattern. Whitesides and coworkers designed a composite
stamp comprising thick and flexible PDMS layers to support a thick layer of h-PDMS to improve the utility of h-PDMS.\textsuperscript{[35]} Thermal curing, however, induced shrinking which altered the supported pattern. Choi and Rogers developed a UV-curable h-PDMS to overcome problems associated with thermal shrinking.\textsuperscript{[36]} This material demonstrated reduced shrinking and was able to replicate dense, submicron patterns. However, feature distortion related to ink diffusion could not be avoided.

1.4.1.4 Printing with chemically patterned flat stamps

The use of flat featureless stamps obviates stamp deformation: with a flat stamp buckling and sagging are no longer possible, and the lack of void spaces eliminates vapor transfer of molecular inks. In this approach, a chemical pattern is formed on a featureless PDMS stamp by inking the elastomer with a patterned inker pad (Figure 12). Effective application of the approach then requires: (1) chemical barriers on the stamp surface capable of suppressing ink diffusion, and (2) a means to selectively ink PDMS surfaces with the desired molecules. Diffusive inks such as alkanethiols are too mobile to remain confined to specific areas of the flat stamp. The technique does, however, simplify stamp fabrication and has been successfully used to replicate protein micropatterns.\textsuperscript{[37]}

![Figure 12. Patterned SAM formation with flat, featureless stamps](image-url)
A more elaborate approach was developed to stabilize ink patterns on PDMS. Huskens and coworkers exposed a flat elastomer surface to a patterned shadowmask to facilitate selective oxidation and then immediately reacted the stamp with a perfluorinated silane, which reacts only in oxidized regions. The stamp was subsequently inked with thiols which wet only the unmodified PDMS regions, leading to patterned ink transfer (Figure 13). In this approach, perfluorinated silane acts as a lateral ink barrier while still allowing vertical ink diffusion from unmodified regions of the stamp to the gold surface. This technique was expanded to pattern polar inks by locally grafting aminosilanes in the non-fluorinated areas. The approach offers a flexible method for patterning hydrophilic or hydrophobic molecules without lateral ink diffusion across the stamp surface. The technique obviates both stamp deformation and vapor diffusion of the ink. The technique does not, however, prevent lateral diffusion of ink across the surface and thus has a limit of resolution on the order of 500 nm.
Figure 13. Printing alkanethiols with flat stamps: the perfluorinated silane layer acts as an ink barrier and the non-covered PDMS transfers the thiol to the gold substrate.

1.4.1.5 PDMS alternatives

Although most µCP uses PDMS stamps, several alternative stamp materials overcome the limitations of PDMS, in particular poor mechanical stability and low polarity. The commercial block copolymers poly(styrene-block-buta diene-block-styrene and poly(styrene-block-ethylene-co-butylene-block-styrene were introduced by Trimbach et al. for printing thiols on gold. The resulting monolayers were well ordered and sufficiently dense to act as etch resists. The polyolefin stamps offer higher mechanical strength than PDMS, making them more suitable for effective printing with a wide range of aspect ratios. Polyolefin plastomers (POP), which combines qualities of elastomers and plastics, also offer increased mechanical stability, and have been used to print fibrinogen patterns at 100 nm resolution.
Unmodified PDMS is hydrophobic and useful only for patterning non-polar inks. Several hydrophilic polymeric stamps have been employed for printing polar inks. Hydrogels incorporate polar units in the polymer structure and as a result are highly hydrophilic. Coq and coworkers used hydrogel stamps to pattern proteins and antibodies without loss in biochemical activity.\textsuperscript{[42]} On the other hand, the ratio of components dictates the mechanical strength and porosity and hydrogel stamps require tedious optimization. Agarose, a linear polysaccharide of galactose and 3,6-anhydrogalactose, is another hydrophilic stamp material that has found utility in \( \mu \)CP.\textsuperscript{[43]} More commonly used in electrophoresis, agarose was adapted for \( \mu \)CP in 2004.\textsuperscript{[43a]} A significant advantage of agarose stamps is their ability to serve as ink reservoir, limiting the need for stamp re-ink. Furthermore, very low ink concentrations have been necessary due to favorable ink-stamp interactions. Agarose stamps have been successfully used to print protein, DNA, cells and bacteria.

UV-curable polyurethane acrylate (PUA) material has also been used for replica molding and \( \mu \)CP.\textsuperscript{[44]} The mechanical properties of this acrylate system are easily tailored by variation of the acrylate modulator chain length during cross linking and by varying the ratio of high- and low-molecular weight acrylates. This modulation can be utilized to obtain the appropriate balance between the rigidity and elasticity. Finally, the surface energy of the stamp can be adjusted by modifying the acrylate components. Overall, this PUA system is suitable elastomer for a wide spectrum of patterning applications.
1.4.2 Avoiding Ink Diffusion

1.4.2.1 High Speed µCP

The extent of lateral ink diffusion and the resulting diminished resolution in traditional µCP is determined by ink mobility and stamp-substrate contact time. Wolf and coworkers demonstrated that uniformity and reproducibility of transferred patterns increased when contact times were reduced from seconds to milliseconds.\textsuperscript{[45]} An automated piezoelectric actuator on a motorized stage was used to control stamp position, contact time and retraction rate. This system was capable of replicating 1 µm features of alkanethiols on gold with sufficient density and uniformity to be etch resistant. The decreased contact time diminishes the resolution problems associated with lateral ink diffusion and vapor diffusion from stamp voids. Shorter contact times, however, ultimately adversely affect the density and organization of the transferred monolayer.\textsuperscript{[18]}

1.4.2.2 Submerged µCP

Printing in a liquid medium, or submerged µCP, has been successfully used to increase µCP resolution by decreasing vapor transport of molecular inks. Ink volatility also leads to ink evaporation and transport to regions of the surface outside of those in contact with the stamp. Xia and Whitesides demonstrated that the transport of long-chain hydrophobic thiols is inhibited by submersion in water, permitting sub-micrometer feature transfer to gold.\textsuperscript{[46]} The advantages of submerged µCP are due to the incompressibility of water and the immiscibility of the molecular ink. Decreased resolution by diffusion or evaporation can be avoided by choosing a liquid in which the
molecular ink is insoluble. In addition to limiting ink diffusion, stamp stability is improved in a liquid medium, facilitating the use of 15:1 feature aspect ratios, dimensions not possible with traditional µCP.\textsuperscript{[47]} The low coefficient of compressibility of water, due to extensive hydrogen bonding, allows water to withstand the applied force and supports the PDMS stamp during printing. Although the technique improved resolution, it is limited to hydrophobic inks.

1.4.2.3 Contact Inking

Vapor phase transport can also be limited with a contact inking approach, a solvent-free alternative to traditional µCP.\textsuperscript{[48]} In this approach a patterned stamp is brought into contact with a flat PDMS-based inking pad: ink is transferred only to protruding features on the stamp and does not impregnate the voids. The absence of ink in the recessed features limits vapor phase diffusion that would otherwise compromise pattern resolution. Delamarche and coworkers successfully employed contact inking to transfer thiol-based patterns down to 100 nm.\textsuperscript{[48]}

1.4.2.4 Low diffusion inks

An obvious solution to ink diffusion is the use of low diffusivity molecular inks. Inks in traditional µCP are typically long chain thiols that create densely packed, ordered monolayers capable of protecting the underlying gold from reactive species. High molecular weight inks or inks bearing multiple attachment points reduce lateral ink diffusion but degrade the order of the resulting monolayers.\textsuperscript{[49]}
Low diffusivity inks have been successfully used in positive microcontact printing. This technique relies on the formation of two SAMs, one of which is not a robust etch resistant monolayer. Low diffusivity ink is used as the first, lower stability SAM followed by the formation of the second traditional alkanethiol based monolayer. The first monolayer facilitates high resolution pattern transfer, whereas the second ordered monolayer provides highly stable etch resistant regions. Poly(propyleneimine) dendrimers with thioether head groups are effective low diffusivity inks for use in positive µCP. Even at long contact times the high molecular weight dendrimers showed virtually no surface spreading, achieving high resolution printing down to 100 nm.\textsuperscript{[50]}

1.5 Unconventional Microcontact Printing Techniques

Traditional µCP exploits the spontaneous adsorption of organic molecules to form SAMs on unmodified inorganic substrates. The technique is restricted to surfaces that undergo rapid reaction with organic inks under ambient conditions (Table 2). Traditional patterning approaches have been used to transfer patterns in thiol/metal and silane/oxide systems but are not effective with less reactive passivated surfaces such as oxide-free silicon or germanium. The formation of SAMs on such surfaces requires long reaction times and harsh environments (i.e. high temperature, inert gas, UV light, and high pressure) that are incompatible with traditional patterning conditions. To extend the utility of µCP beyond noble metals and metal oxides, several approaches toward soft lithographic pattern replication on pre-formed self-assembled monolayers have been developed. Rather than relying on direct reaction between inks and an
inorganic substrate, these techniques replicate patterns *via* chemical or biological reaction between functional groups affixed to the SAM and the reactive moieties adsorbed on the stamp.

**Table 2. Self-assembled monolayers in traditional µCP.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ligand</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>Thiols: RSH</td>
<td>[51]</td>
</tr>
<tr>
<td>Au</td>
<td>Disulfides: RSSR'</td>
<td>[51b, 52]</td>
</tr>
<tr>
<td>Au</td>
<td>Sulfides: RSR'</td>
<td>[51b, 53]</td>
</tr>
<tr>
<td>Ag</td>
<td>Thiols: RSH</td>
<td>[51]</td>
</tr>
<tr>
<td>Ag</td>
<td>Disulfides: RSSR'</td>
<td>[51b, 54]</td>
</tr>
<tr>
<td>Cu</td>
<td>Thiols: RSH</td>
<td>[51]</td>
</tr>
<tr>
<td>Pd</td>
<td>Thiols: RSH</td>
<td>[51b, 55]</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>RSiCl$_3$, RSi(OR')$_3$</td>
<td>[17, 19]</td>
</tr>
<tr>
<td>In$_2$O$_3$/SnO$_2$ (ITO)</td>
<td>RPO$_2$H$_2$, RSiCl$_2$, RSi(OR')$_3$</td>
<td>[51b, 56]</td>
</tr>
<tr>
<td>Al$_2$O$_3$</td>
<td>RCOOH</td>
<td>[51b, 58]</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>RCOOH, RPO$_2$H</td>
<td>[51b, 59]</td>
</tr>
</tbody>
</table>

**1.5.1 Formation of Self-Assembled Monolayers**

The spontaneous organization of molecules into complex structures is well known in biology, chemistry and physics. In surface chemistry, SAMs are formed by the exposure of a surface to molecules bearing chemical reactivities with strong affinities for the surface. The resulting order in the structure is a function of interactions between surface and ink as well as on intermolecular interactions between ink molecules. Binding to the surface is a result of either physisorption ($\Delta H < 10 \text{ kcal/mol}$) or chemisorption ($\Delta H > 10 \text{ kcal/mol}$). Additional interactions between molecules and substrates or intermolecular interactions between the molecules of the monolayer (i.e. hydrogen bonding, donor-acceptor and/or ion pairing) make the assemblies more stable. As previously noted, the best studied monolayers involve silanes on oxides and alkanethiols on several metals.[$^{60}$]
The simplicity of SAM formation makes them attractive for surface engineering. In contrast to amorphous ultrathin films, SAMs are highly ordered, oriented and capable of incorporating multiple groups in the alkyl chain and diverse functionality at the termini. SAMs enable the chemical tuning of surfaces for wide range of applications, from corrosion prevention and wear protection to chemical and biochemical sensing. The micro- and nanoscale patterning of organic monolayers on semiconductors enables myriad applications, including ultra-thin etch resists and pH, inorganic and bio-inorganic sensors. The continuous decrease in feature size offers interesting possibilities for the integration of organic molecular systems with inorganic microelectronic materials. SAMs have been investigated as efficient passivating layers that can serve as effective dielectric materials in transistors. Clearly, controlling the organic/inorganic interface with SAMs will be crucial for future generation devices.

Numerous reports have demonstrated the facile formation of well-ordered SAMs of organic molecules on noble metal and inorganic oxides. These substrates undergo rapid, low activation barrier reactions with organic thiols, organosilanes, and phosphonic acids leading to the formation of well-defined monolayers. Organic thiols, silanes and phosphonic acids can all be easily patterned on untreated inorganic materials using traditional μCP. In contrast, the formation of ordered and stable SAMs on inorganic materials such as group IV semiconductors (diamond, Si, Ge, and SiN) remains problematic and usually requires overcoming significant activation barriers to form Si-C, Ge-C or C-C bonds. In the following sub-chapter, we review reactions capable of forming self-assembled monolayers on semiconductor materials.
1.5.1.1 Formation of organic monolayers on semiconductor surfaces

Organic monolayers on semiconductor surfaces can be formed using a wide range of ligands (Table 3). Monolayer formation on semiconductors is significantly more complex than the formation of SAMs on gold. Before the monolayers can be formed, the surface is passivated by removing the native oxide layer. Passivation can be achieved using simple monatomic agents to coordinately saturate all surface bonds. This step ensures that all surface atoms exist with essentially ideal coordination, reducing reactivity towards oxygen present in air and aqueous solution. The most commonly used passivating agent is hydrogen, but halogens including chlorine and iodine have also been used. Silicon can be H-terminated by immersion in dilute solutions of HF. Fluoride removes the oxide layer efficiently but does not quickly etch the underlying silicon. Typically dilute (2-10%) HF is used to passivate Si(001) and ~40% NH₄F is used for the (111) facet.

Germanium can be passivated as any of three terminating layers: sulfide-, chloride-, and hydride-terminated germanium. Sulfide termination creates a more stable passivating layer, whereas chloride and hydride termination allow further chemical functionalization. Lu and coworkers demonstrated that Ge (111) could be passivated by immersing the surface in a dilute solution of HCl. Chlorination resulted in a well ordered, atomically flat surface that is stable to air oxidation for hours. Chlorination resulted in a well ordered, atomically flat surface that is stable to air oxidation for hours. Similar to chloride termination, hydride surface termination creates a passivated surface with each hydrogen atom bound to a single Ge atom. Dilute HF is most commonly used as an etchant to produce Ge-H. Unlike the Cl-terminated surface, the Ge-H is a kinetic
product: the Ge-F bond (116 kcal/mol) is thermodynamically favored over the Ge-H bond (77 kcal/mol).\textsuperscript{[67]} HF etching does not, however, produce a chemically uniform surface, yielding instead multiple hydride phases. As a result, hydride termination is less desirable than is chloride termination.\textsuperscript{[64]}

**Table 3. Self-assembled monolayers on semiconductors**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ligand</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GaAs</td>
<td>Thiols: RSH</td>
<td>\textsuperscript{[68]}</td>
</tr>
<tr>
<td>InP</td>
<td>Thiols: RSH</td>
<td>\textsuperscript{[69]}</td>
</tr>
<tr>
<td>Ge-H</td>
<td>Thiols: RSH</td>
<td>\textsuperscript{[64]}</td>
</tr>
<tr>
<td>Ge-H</td>
<td>Alkenes: RCH=CH\textsubscript{2}</td>
<td>\textsuperscript{[64, 70]}</td>
</tr>
<tr>
<td>Ge-Cl</td>
<td>RMgX</td>
<td>\textsuperscript{[64, 70]}</td>
</tr>
<tr>
<td>Si-H</td>
<td>Alkenes: RCH=CH\textsubscript{2}</td>
<td>\textsuperscript{[62-63, 70]}</td>
</tr>
<tr>
<td>Si-Cl</td>
<td>RLi, RMgX</td>
<td>\textsuperscript{[70]}</td>
</tr>
</tbody>
</table>

Following passivation, the semiconductor surface can be alkylated with unsaturated organic compounds or organic carbanions (Figure 14). Reactions with alkenes or alkynes typically require some form of activation, analogous to classical hydrosilylation reactions employing Lewis acid catalysts such as EtAlCl\textsubscript{2}. The reaction of H-terminated surfaces with simple alkenes can be initiated with diacyl peroxides. These agents decompose thermally to form radicals that abstract H atoms from the surface and exposing unsaturated dangling bonds that react rapidly with alkenes or alkynes. Thermal activation can also be used to promote radical hydrosilylation. Typically, H-terminated surfaces immersed in the alkene must be heated for extended periods (i.e. hours to days at $\sim$200°C). Photochemical activation with UV light (254 nm) has also been successfully utilized.
Figure 14. Common methods to form organic monolayers on semiconductor surfaces

Whereas H-terminated semiconductor surfaces are relatively unreactive, Cl-terminated surfaces undergo rapid chemical functionalization. Lewis and coworkers prepared organic monolayers on Cl-terminated silicon using Grignard and organolithium reagents.\(^{[71]}\) A two-step halogenation/alkylation procedure was used to generate alkyl-terminated silicon surfaces; these monolayers exhibit exceptional stability, with lower oxidation rates than H-terminated silicon. Alkylation of Si with propenyl Grignard yielded nearly complete surface coverage, with a propenyl moiety bound to nearly every silicon atom.\(^{[72]}\) Such densely packed covalent organic layers prevent diffusion of oxygen and water into the monolayer/surface interface.

The formation of a covalently bound alkyl surface generates a thermodynamically and kinetically stable monolayer that is thermally and chemically robust.\(^{[73]}\) Alkoxy monolayers on silicon (Si-O-C linked), on the other hand, show poor
chemical stability and are susceptible to hydrolysis.\textsuperscript{[74]} Similarly, Ge-C bonds are stable and alkyl monolayers on Ge eliminate sensitivities to ambient moisture.\textsuperscript{[75]}

More recently several researchers have reported efforts to functionalize various carbon surfaces, including diamond and diamond-like carbon, glassy carbon, graphite and graphene. These surfaces are ideal for the formation of biological interfaces due to their biocompatibility, mechanical hardness, and chemical strength.\textsuperscript{[76]} Hamers and coworkers have shown that photo-induced hydrosilylation of alkenes provides a robust method by which to link organic molecules to carbon surfaces.\textsuperscript{[76b, 77]} The formation of C-C bonds results in excellent monolayer stability, even at elevated temperatures.\textsuperscript{[78]}

1.5.1.2 Formation of functional monolayers

Monolayers comprising simple alkyl chains can act as impervious barriers to organic and aqueous solutions due to their highly ordered structure and hydrophobic nature. SAMs with terminal functionality, however, are required for further surface manipulation either by wet chemical functionalization or by microcontact printing. Reactive, functional SAMs directly attached to inorganic surfaces are generally ineffective in protecting the underlying interface from degradation. Steric constraints introduced by bulky, terminal functional groups result in relatively disordered monolayers.\textsuperscript{[18, 51b]} The packing density and degree of order can be improved by backfilling the grafted SAMs with shorter filler molecules. Alternatively, co-adsorption from mixed organic solutions containing both functional and filler species has been used to increase order. Both methods have limitations; backfilling can cause desorption of the grafted functional SAMs and mixed monolayer deposition can result in phase segregated
domains over varying distance scales. In addition, the composition of the mixed monolayer does not necessarily reflect the relative concentrations of the two components in solution, and the distribution of functional components within the monolayer is difficult to resolve. It has been suggested that the components form small clusters (< 1000 Å across) to minimize their free energy, similar to their behavior in solution.\cite{79} Furthermore, both mixed and backfilled SAMs have been limited to gold and silicon oxide substrates.

Bilayered SAMs overcome the limitations of mixed and backfilled SAMs. Bilayered SAMs are composed of a highly ordered primary SAM with a secondary reactive overlayer, simultaneously achieving surface passivation and terminal functionalization (Figure 15).\cite{72} Ideally, the initial primary SAM achieves complete reaction of all surface exposed atoms, forming a close-packed molecular system capable of protecting the interface against degradation. The secondary overlayer contains terminal functional moieties whose reactivity can be masked or enhanced via additional chemical manipulations. The introduction of terminal functional groups on an ordered, protective monolayer facilitates subsequent patterning applications on pre-formed, stable, and reactive SAMs on virtually any surface.
1.5.1.3 Characterization of organic monolayers

The characterization of organic monolayers typically involves a combination of techniques. Contact angle goniometry is a simple and sensitive tool used to characterize monolayer structure, composition and surface energy over large areas. The contact angle formed by a droplet of liquid on a monolayer-modified surface can be easily quantified by measuring the angle, $\Theta$, between the tangent of the drop where it meets the surface and the surface itself; this angle is determined by the interfacial surface free energy, which is in turn determined by the chemical nature of the surface and the probe liquid. Different liquids can be used to probe specific molecular forces; water senses polar functional groups whereas hydrocarbons respond to dispersive forces. Three angles are typically measured: a static angle, an advancing angle and a receding angle, measured as a drop is advanced or withdrawn across the surface. The advancing contact angle is predominately determined by the polarity of the outer 0.5 nm of the monolayer. The receding angle for the decreasing drop volume is lower than the advancing angle. The difference between the advancing and receding angles (hysteresis)
for well-ordered SAMs is around 10° but can increase to 30° or more with disordered layers. Hysteresis is usually interpreted in terms of SAM disorder, surface roughness or polarity. Methyl terminated monolayers result in very high water contact angles (>110°), whereas hydroxyl or carboxylate terminated monolayers produce wetting.\[60\]

More detailed information regarding the nature of the surface can be determined with spectroscopic approaches, especially grazing angle Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). The frequencies and intensities of the vibrational modes probed by FTIR provide detailed information on the functional groups present at the surface. FTIR can also be used to identify (1) the molecular orientations on the surface, (2) the tilt of longer-chain molecules and (3) the extent to which the organic layer has adopted an all-trans configuration.\[63\] It is mainly used in three spectroscopy modes: attenuated total reflection, grazing angle, and Brewster angle.\[19, 80\]

XPS provides a quantitative approach to surface analysis.\[80\] The surface in question is irradiated with monochromatic X-rays, leading to the emission of core electrons. An analyzer collects the escaping photoelectrons and measures abundance as a function of kinetic energy. The calculated binding energy provides information on the surface bound elements and their oxidation state. Low-energy resolution provides elemental analysis of the surface species, while higher energy resolution provides additional chemical information through the core-level shifts. Surface bound molecules undergo shifts in core-level binding energies due to the presence of electron withdrawing or donating groups. By measuring the ratio of a core-level atom in the
organic layer (e.g. C 1s) and another atom in the bulk (e.g. 2p) the number of molecules per unit area can be calculated. XPS analyzes only 1-10 nm of the surface, making it ideal for SAM analysis. Replacing the X-ray source with UV excitation yields ultraviolet photoemission spectroscopy (UPS), which provides information valence electronic states; specifically the binding energies of the highest occupied molecular orbitals and vacuum level positions. \[63, 81\]

1.5.2 Patterning pre-formed SAMs

Traditional µCP relies on the spontaneous diffusive SAM formation from stamp to surface and is thus limited to metals and oxides. Furthermore, diffusive SAM formation suffers the inherent limitation of lateral ink diffusion that limits pattern resolution. In order to expand the types of substrates that can be patterned by µCP and improve pattern resolution, techniques that rely on patterning pre-formed SAMs have been developed. Unconventional µCP printing techniques, including reactive, catalytic and supramolecular printing, have been developed to overcome the liabilities of conventional µCP, specifically the inability of traditional µCP to pattern substrates that do not undergo rapid reaction with organic inks and the limits to resolution attributable to molecular diffusion.

1.5.2.1 Reactive µCP

Reactive microcontact printing is an alternative to the direct patterned SAM formation by the diffusion of molecular ink from an elastomeric stamp. The technique relies on the formation of a reactive SAM on a substrate followed by µCP with an
elastomeric stamp that locally transfers reagents to the SAM, inducing chemical reaction in regions of stamp-substrate conformal contact (Figure 16). In addition to patterning surfaces that are otherwise incompatible with traditional µCP, this method facilitates the formation of patterned bifunctional SAMs without an additional backfilling step.

Whitesides and coworkers reported the first example of reactive µCP. A reactive SAM on silver or gold bearing carboxylic anhydrides was brought into conformal contact with a PDMS stamped inked in \( n \)-hexadecylamine. In regions of contact anhydride/amine reaction resulted in the formation of a mixed SAM comprising \( N \)-alkyl amides and carboxylic acids. Remaining anhydride groups were reacted with a fluorinated amine to give a patterned bifunctional SAM. The approach was extended to pattern ligands that resist nonspecific protein adsorption for biosensor applications. Following these initial reports, patterned immobilization of a wide variety of amines has been demonstrated, including simple amines, DNA, dendrimers, proteins, and fluorophores. Inverse patterning of active esters on amine modified SAMs has also been reported. The high effective concentration of ink at the stamp-substrate interface in combination with geometric pre-organization of the reactants in the monolayer is thought to facilitate rapid formation of covalent bonds.
In recent years, azide-alkyne cycloaddition has been adapted for surface patterning in reactive µCP. Cycloaddition is biocompatible and orthogonal to many functional groups, leading to its extensive application in materials science. The first report by Rozkiewicz et al. used PDMS stamps to immobilize n-octadecyne and lissamine rhodamine alkyne on an azido-terminated SAM on silicon oxide. Ravoo and Michel showed that “click” chemistry can be used to prepare carbohydrate arrays on silicon oxide. In this instance, reactive µCP was used to pattern a carbohydrate alkyne on an azido-terminated SAM such that the immobilized carbohydrates retained their characteristic selectivity toward lectins.

More recently, reactive µCP was extended to technologically relevant oxide-free silicon. As previously noted, traditional µCP is limited to metals and oxides capable of spontaneously forming SAMs in regions of conformal contact. Not only does reactive printing expand the types of patterned functional groups that can be immobilized, but it
also permits pattern formation on virtually any substrate that bears a reactive molecular layer, since the metal-organic layer is produced prior to the pattern-forming step. Zuilhof and coworkers demonstrated patterning of primary amines on oxide-free silicon bearing a pre-formed, reactive acid fluoride monolayer.[87] This method permitted the preservation of the oxide-free interface, an important property for molecular electronics.

Although reactive μCP has successfully expanded the range of applications accessible through microcontact printing, it still suffers from limitations associated with molecular ink diffusion. The diffusive limitations of traditional μCP can only be avoided by methods that entirely eliminate the use of molecular inks for pattern replication.

1.5.2.2 Supramolecular μCP

Supramolecular μCP uses reversible host-guest association to transfer pattern. Such systems play key roles in biology due to their highly specific and reversible binding nature, properties that also make them ideal candidates for the controlled positioning of organic and bio-organic molecules on solid substrates. In a typical patterning experiment, a pre-functionalized stamp is used to selectively adsorb species from a solution and then transfer that species to a solid support displaying the complementary binding moieties (Figure 17). Delamarche and coworkers reported the first example of this method. They used stamps functionalized with protein antigens (entire immunoglobulin G) to selectively capture and adsorb proteins from a crude biological solution and transfer them in a site specific manner.[88] A similar approach was also used to transfer specific single stranded DNA to complementary ss-DNA anchored to a
Stamps pre-functionalized with ssDNA were used to adsorb complementary ssDNA. Once in conformal contact with the surface, the pre-formed dsDNA on the stamp dissociated and formed a new complementary dsDNA at the surface. Reinhoudt and coworkers have used this technique extensively to print a variety of molecules on SAMs functionalized with receptor groups (molecular printboards). More specifically, this group has applied the approach towards kinetic and thermodynamic studies of mono- and multivalent interactions at surfaces.\cite{Reinhoudt2000}

**Figure 17. Supramolecular µCP**

Compared to traditional µCP, supramolecular µCP involves several complicating features, including the requirement for a stamp that can selectively adsorb components of a complex mixtures and a surface with sufficient reactivity and specificity to bind the ink from the stamp. The specificity and selectivity of the method, however, provides a useful tool for the fabrication of biological-based arrays and for the investigation of intermolecular interactions.
1.5.2.3 Catalytic µCP

Catalytic µCP is an inkless printing technique that relies on the action of an immobilized catalyst on a stamp surface to transfer pattern to a surface bearing complementary reactivity. Because the approach does not rely on the transfer of ink across a stamp-substrate interface, it completely eliminates the diffusive limitations of both traditional and reactive printing. In catalytic µCP, the surface of the stamp is catalytically active towards a functionalized substrate. Pattern replication is achieved through a specific chemical or biochemical reaction between the stamp material and the substrate. Because all reactive components are either covalently or specifically immobilized, lateral and gas phase diffusion is circumvented, and the resolution of the replicated pattern is determined solely by deformations or movement of the elastomeric stamp.

Figure 18. Schematic representation of catalytic µCP

Reinhoudt and coworkers reported the first example of catalytic µCP, using an oxidized PDMS stamp to modify pre-formed SAMs of bis(ω-
trimethylsiloxyundecyl)disulfide or bis(ω-tert-butyldimethylsiloxy-undecyl)disulfide on gold. The oxidized elastomer was sufficiently acidic to catalyze the hydrolysis of the TMS- and TBS-protected alcohols in places of conformal contact (Figure 19). Because all components of the reaction were either covalently linked to the substrate or incorporated in the bulk stamp material, this inkless method was able replicate sub-micrometer features with ~50 nm edge resolution. XPS analysis, however, showed only approximately 30% deprotection of the TMS groups even after 30 minutes of stamping. Oxidized stamps underwent rapid hydrophobic recovery resulting in decreased catalytic efficiency. Finally, exposure of PDMS to oxidative environments leads to changes in feature morphology and induces stamp defects including cracks. Together, these properties significantly limit the utility of the approach.

**Figure 19. Catalytic µCP with oxidized PDMS**
Another catalytic µCP approach reported by the Reinhoudt group relied on copper-modified PDMS to catalyze the functionalization of an azide-terminated surface. In this work, a cured PDMS stamp was used to support plasma vapor (PVD) deposited Ti (as an adhesive) followed by copper. Surprisingly, the metal-coated stamps retained their elasticity and were capable of making conformal contact with the reactive surface. Copper-modified stamps promoted spatially selective reaction between immobilized azide on and pendant alkynes. Electrochemical measurements estimated that the reaction achieves nearly complete conversion to the corresponding triazole ring.

Another transition metal-based catalytic µCP approach, based on palladium nanoparticles, was reported by Mizuno and Buriak. In this approach, a patterned array of Pd nanoparticles on flat PDMS was used to catalyze alkene and alkyne hydrosilylation to H-terminated silicon. Absent of stamp deformation and ink diffusion, sub-100 nm pattern replication was achieved. A similar approach was used to print aldehydes on H-terminated silicon and aryl iodides to alkene terminated SAMs via the Heck reaction. Because pattern transfer relies on the interaction between immobilized catalytic nanoparticles and a solid-substrate, the conformal contact is diminished relative to truly elastomeric stamps, a limitation that will diminish reaction efficiency and pattern density.

A radically different approach towards inkless µCP that exploits biochemical catalysis was developed in the Toone laboratory. Here, pattern replication via a biocatalytic reaction between an immobilized substrate and an enzyme modified stamp was used to transfer pattern. Modification of polyacrylamide-based stamps with
nitriloacetic acid groups allowed for the reversible attachment of hexahistidine-tagged enzymes through Ni$^{2+}$ chelation. The method permits attachment of virtually any histidine-tagged enzyme, providing tremendous diversity of reactivity and rates of catalysis. The applicability of the approach was demonstrated using exonuclease 1-modified stamps and single-stranded DNA immobilized on glass and gold. In places of conformal contact, the enzyme catalyzed the degradation of the surface bound DNA. Good pattern transfer was achieved although resolution was limited by stamp deformations: polyacrylamide lacks the mechanical integrity necessary to support and transfer sub-micrometer features. Still, the patterning approach is fundamentally different from other modes of pattern transfer. First, it is completely free of any molecular inks; rather, a preformed monolayer is modified by a stamp immobilized catalyst in the absence of diffusion. Second, it offers almost unlimited versatility, given the broad range of chemistries available through biological catalysis. Recent work by Cathala and coworkers used biocatalytic method to site selectively pattern a layer of poly-L-lysine (PLL) on a solid substrate.[94] In this work, trypsin was immobilized on PDMS facilitating the enzymatic degradation of PLL in regions of conformal contact. Fluorescence and atomic force microscopy (AFM) analysis showed well-defined patterns with accurate reproduction of the master. The inkless catalytic method eliminates problems associated with lateral ink diffusion and allowed continuous use of the stamp without re-inking.

Recent work in our laboratory has focused on inkless chemical (i.e. abiological) catalytic methods. The first chemical catalytic µCP protocol used an alkaline chemical
catalyst bound to a mechanically stable polyurethane acrylate (PUA) stamp. The approach reproduces patterns by catalyzing the deprotection of Fmoc-modified SAMs by a piperidine catalyst covalently tethered to the PUA polymer (Figure 20). 9-Fluoroenylmethoxycarbonyl (Fmoc) group is a frequently used amine-protecting group. The Fmoc group is easily installed by reaction of an amine with 9-fluoroenylmethyl chloroformate (Fmoc-Cl) or 9-fluoroenylmethyl-N-hydroxysuccinimide (Fmoc-NHS) and is selectively cleaved under mildly basic conditions using aliphatic amines such as piperidine. Although this inkless approach significantly improved on the traditional patterning approach by obviating the diffusive resolution limitation, it required prolonged reaction times and elevated temperatures to achieve complete deprotection.

Figure 20. (A) Catalytic µCP on Fmoc-protected SAMs on gold with a piperidine functionalized stamp. (B) Components of reactive polyurethane acrylate (PUA).
To reduce reaction times, we next considered acidic stamps. Fast and complete pattern replication on SAMs of Boc-and TBS-protected thiols on gold was achieved using PUA functionalized with covalently bound sulfonic acids. Similar to Fmoc, both tert-butyl carbamate- (Boc) and tert-butyldimethylsilyl- (TBS-) are common amine and hydroxyl protecting groups that can be removed under acidic conditions. Conformal contact between the acidic PUA stamp and SAMs of Boc- and TBS-protected thiols resulted in accurate and complete pattern transfer. Moreover, this method achieved complete protecting group ablation at room temperature after just one minute of stamping time and produced sub-200 nm size structures of chemically distinct SAMs.

The chemical catalytic patterning methods developed in the Toone group offer several advantages over traditional µCP. First, the approaches do not rely on diffusive pattern transfer; rather, pattern transfer is achieved by catalytic reaction between covalently modified stamps and pre-formed reactive SAMs. As a result, limitations associated with ink diffusion are completely eliminated. Second, the use of the PUA polymer, which was recently used to make highly accurate patterned molds with high aspect ratio features, eliminates many of the stamp deformation problems associated with PDMS. Finally, the technique provides a functional, patterned template that can be further modified with various organic or bio-organic moieties.

1.6 µCP Applications

Since the first report by Kumar and Whitesides, µCP evolved enormously. The main concept remains the same; a pattern is transferred from an elastomeric stamp to a
hard substrate via conformal contact. But the method of effecting that contact and the components used have evolved continuously over the last 20 years. Novel printing techniques were developed to avoid mechanical deformations and ink diffusion; new materials were investigated to expand the applications of µCP. The development of non-diffusive inks has made sub-micrometer pattern resolution possible, while catalytic printing completely eliminated the problems associated with ink diffusion. Although originally developed for application in microelectronics, the method has inspired innovative applications in many fields including chemistry, physics and biology; highlighting its power and versatility. In the following sections, we briefly review some significant applications of µCP.

1.6.1 Patterning biological materials

Artificial biological surfaces adorned with specific biomolecules find application in fields as diverse as biochips, biosensors, tissue engineering, drug screening and fundamental studies in molecular and cell biology.\textsuperscript{[95]} SAM based approaches using simple alkanethiolates on gold provide a useful template for fabricating biological interfaces. µCP is not, however, compatible with traditional micromachining techniques and has yet to find widespread application. In many cases substrates must be manually manipulated through multiple steps to achieve the desired functionality, pattern design, and resolution, severely limiting throughput. Furthermore, to effectively replicate the complexity of \textit{in vivo} biological surfaces, multifunctional SAMs that can rapidly interrogate a wide range of samples are necessary.\textsuperscript{[96]}
Microcontact printing provides an inexpensive and simple approach to spatially pattern specific biomolecules including DNA, proteins, and antibodies, on a single surface. It provides the ability to both position distinct biomolecules with well-defined feature size, shape and spacing, while retaining their native biological properties and to resist non-specific adsorption on non-patterned regions of the substrate. These capabilities have been used to model and study the properties of the extracellular matrix (ECM). Cells interact with the complex and heterogeneous ECM to both support cell adhesion and transfer biochemical signals; deconvoluting this complexity is astonishingly difficult. The generation of screening platforms that simplify this complexity by isolating specific biomolecular events (i.e. cell-protein, protein-protein, protein-ligand interactions) would enable a fundamental understanding of ECM-cell interactions in ways that would lead to advancements in cell biology, tissue engineering, and biosensors. Rozkiewicz et al. used µCP to pattern collagen-type protein. They constructed pre-formed monolayers on gold and silicon oxide bearing terminal aldehyde functional groups; conformal contact between elastomer and surface formed patterns of protein covalently bound to the surface. Backfilling the patterned surface with amino-poly-(ethylene glycol) (PEG) formed a cell-adhesion resistant area. Human malignant carcinoma (HeLa) cells were then incubated over the patterned surface, adhering only to the protein. The size and shape of the cells could be controlled, and the effect of cell morphology on growth and protein secretion was studied.
To increase throughput of the production of biological surfaces, Hlady and coworkers recently reported multiprotein microcontact printing method.\textsuperscript{[102]} Using an inverted microscope, they sequentially deposited multiple proteins on a single surface with micrometer precision by mounting an elastomer on the microscope stage and the surface to be patterned on the objective. This geometry facilitates visualization and alignment of multiple protein patterns in real time.

µCP has also been successfully applied to DNA patterning for array fabrication.\textsuperscript{[10]} DNA arrays offer the ability to simultaneously monitor the expression levels of multiple genes in real time. DNA microarrays were printed using a spotter to ink an elastomeric stamp; the loaded stamp could be used multiple times to pattern DNA without re-inking.\textsuperscript{[95]} Printed DNA arrays show greater sensitivity and higher binding efficiencies than do traditional adsorbed surface spots. A printing approach reduces both time and required DNA, making µCP attractive for DNA fabrication.\textsuperscript{[7]}

### 1.6.2 Patterning inorganic materials

Significant progress has been made in the solution phase production of nanoparticles with controlled size, composition, shape, dispersity and surface character.\textsuperscript{[103]} An important remaining challenge is the development of effective methods to self-assemble these nanoscale components into larger structures for applications in molecular electronics. Andres and Santhanam extended traditional µCP methods for patterning alkanethiol-coated gold nanoparticles on a gold substrate.\textsuperscript{[104]} Using PDMS as the elastomeric stamp and the uniform nanoparticles as the “ink,”
particle arrays with ~3 µm lines and ~10 µm squares were prepared. Calame and coworkers used a similar method to fabricate networks of molecular junctions.\textsuperscript{105} This group patterned dodecanethiol-capped nanoparticles on silicon oxide and studied electrical transport through the resulting arrays. Patterning produced stable molecular circuit devices, permitting the simultaneous preparation of hundreds of devices on a single chip. More recently, µCP was used to form gold nanoparticle arrays for applications in flash memory performance. By patterning a close-packed Au nanoparticle monolayer between Al\textsubscript{2}O\textsubscript{3}, multilevel data storage could be controlled by an external gate bias. µCP enabled an increase in trapping sites and reduced lateral leakage. In addition, the device is mechanically stable, suggesting potential application for µCP based fabrication in the construction of flash memory devices.

Colloidal quantum dots (QDs) assembled into large area arrays are useful for a variety of applications including photonics\textsuperscript{106} and light emitting devices,\textsuperscript{107} and photovoltaics.\textsuperscript{108} A major challenge in the construction of quantum dot (QD) based photovoltaic (PV) devices is the assembly of QDs into multi-layer or multi-junction structures with precise control of the topological and interfacial properties of the assembly. Transfer printing of QD thin films with patterned polymeric stamps provides a manufacturing process that is inexpensive, fast, and scalable to large areas. Novel µCP strategies are currently being explored to pattern QDs with spatial precision and orientation on the nanometer scale in order to improve charge generation and transport properties. Current challenges facing adaptation of traditional soft lithographic (SL) methods to the additive manufacture of QD thin-films include: 1)
adapting contact printing methods, originally designed to pattern monolayers of organic molecules, to thin films of inorganic nanocrystals; 2) identifying polymers for with surface energies tuned to enable transfer of QD thin films from one material to another without affecting their bulk properties; and 3) optimizing the Young’s moduli of transfer polymers to increase printing resolution and enable roll-to-plate and roll-to-roll processes.

Arango et al. demonstrate a bilayered QD-based photovoltaic architecture using two device fabrication steps. Frist, they deposit a thin QD film (~20 nm) onto an organic hole transport layer using µCP. Second, a thin film of ITO is deposited onto the QD layer as a transparent electrode. The resulting device can (1) accommodate different QD sizes, (2) produce large open circuit voltages and (3) yield high internal quantum efficiency. More recently, the first demonstration of a large-area, full-color quantum dot display using optimized quantum dot films on glass substrates was reported using solvent-free transfer printing. The printed quantum dot films exhibit excellent morphology, well-ordered quantum dot structure and clearly defined interfaces. Furthermore, they demonstrated significant enhancements in charge transport/balance in the QD layer that improves electroluminescent performance. Their results suggest routes towards creating large-scale optoelectronic devices in displays, solid-state lighting and photovoltaics and flexible bio-imaging devices.

Rogers and coworkers use patterned PDMS to design large area, stretchable GaAs photovoltaic systems. They exploit the elastomer with surface relief structures to confine strain away from the brittle GaAs solar cells. The mechanics allow
deformations such as bending or twisting that do not affect the patterned inorganic devices. The structural design is simple; they use microstructured PDMS to print the entire device containing interconnected GaAs solar microcells. This cheap and scalable structuring method can easily be applied to other stretchable semiconductor device technologies.

1.6.3 Patterning organic electronics

Organic electronic and optoelectronic device fabrication has been an active research area in materials science during the last 20 years. Nearly all components of organic electronic devices must be laterally structured and interconnected, and patterning is a key determinate of device performance, pattern density and accuracy, and cost.\textsuperscript{112} Traditional silicon-based technologies, such as photolithography and shadow-mask deposition, are both area-limited and cost prohibitive for large-area manufacturing of organic electronics. Moreover, these approaches are not compatible with soft materials, and their application towards flexible organic electronics has yet to be demonstrated. Additive µCP might offer a practical approach to scalable manufacturing of inexpensive organic electronic devices. The process is simple, efficient and suitable for large area device production and can be carried out in the ambient, and is adaptable to roll-to-roll fabrication.\textsuperscript{112}

µCP has been used extensively in recent years to structure polymer light emitting diodes (PLEDs) and organic light emitting diodes (OLEDs). Several reports of patterning SAMs to control the charge injection and light emission with micrometer scale
resolution have appeared.\textsuperscript{[113]} Charge injection from a metal contact into an organic semiconductor is primarily determined by the energy difference between the metal work function and the highest occupied molecular orbital (HOMO) for hole injection, and structuring SAMs is an important determinant of device performance. A more detailed discussion on the application of patterning in OLED manufacturing is included in the following chapter.
2. Catalytic microcontact printing

2.1 Overview

Microcontact printing (µCP) is a powerful and versatile tool for patterning self-assembled monolayers (SAMs) on a wide variety of surfaces. By combining high throughput, low cost, and operational simplicity, µCP has become the method of choice for high fidelity patterning in diverse fields including microelectronics, (bio)chemical sensing, organic photovoltaic devices, tissue engineering and cell signaling, and DNA/protein array fabrication. Although traditional µCP is routinely used to create arrays of microscopic objects, limitations associated with ink diffusion and stamp deformation constrained its use for the replication of sub-300 nm features. Furthermore, the vast majority of all known µCP techniques were limited to less technologically relevant metal and oxide surfaces and could not be easily applied to substrates such as oxide-free silicon and germanium and indium tin oxide which do not react specifically with organic materials.

Patterning these substrates with organic or bio-organic molecules can be achieved by relying on µCP techniques that modify preformed functional SAMs instead of a bare inorganic interface. Catalytic µCP, which relies on the action of a chemical catalyst bound to a polymeric stamp to transfer patterns to preformed functionalized SAMs, not only expands the utility of µCP to a virtually any substrate capable of supporting an organic SAM, but also offers a much higher resolution than traditional µCP by relying on diffusion-free processes. Past efforts in our lab were targeted...
towards (1) proof-of-concept demonstrations of different catalytic µCP systems and (2) overcoming diffusion and deformation limitations of traditional printing.

In this work we shift the focus of our soft-lithographic studies to demonstrate the powerful utility of catalytic printing in real-life applications. As such, we focus on (1) demonstrating the versatility of catalytic µCP in patterning diverse classes of inorganic materials by relying on the same universal bi-layered molecular system; (2) adapting the techniques to produce chemically and physically stable patterns by protecting the underlying surface-SAM interface from degradation; (3) demonstrating chemoselective functionalization of patterns with a variety or organic and biological molecules; and (4) demonstrating the application of the printed functional patterns in controlling interfacial charge-transport in OLED devices.

2.2 Working Principle and Requirements

Catalytic µCP shares some general requirements with traditional printing; namely (1) a patterned elastomeric stamp and (2) a corresponding reactive substrate to transfer the molecular pattern. However, because it relies on a completely different mechanism of pattern transfer (interfacial reaction vs. molecular diffusion) the specific requirements for the stamp-substrate system are substantially different from traditional µCP. As such, it needs a stamp polymer capable of supporting covalently or specifically attached catalytic groups and pre-functionalized substrates bearing corresponding substrate units.
2.2.1 Stamp Materials

To facilitate a catalytic patterning method that can cover a range of chemistries, we required a stamping material suitable for broad functionalization. Lee and coworkers reported an oligoethylene glycol acrylate urethane copolymer that is capable of high resolution pattern transfer (Figure 21). Moreover, the rigidity and surface energy can be easily tuned through the choice of glycol component and the mole fraction of the components. In addition, the acrylate moiety provides a handle to covalently attach the catalyst of choice. Photocuring (λ=365 nm) the pre-polymer cast against a Si/SiO₂ master provides a micro or nano-structured elastomer. In our studies we modify this polymeric system with a covalently-bound catalyst to pattern pre-formed, functional SAMs on technologically relevant substrates.
Figure 21. Components of polyurethane acrylate (PUA) corresponding stamps bearing 45 nm dots or 16 µm hexagons.

2.2.2 Substrate: Functional SAM formation

Catalytic µCP achieves pattern reproduction via a specific chemical reaction between stamp and surface. The reagent immobilized to the stamp catalyzes a reaction with functional entities on a flat substrate. As such, this method requires a substrate bearing a stable, reactive pre-formed SAM in order to replicate patterns. The controlled assembly of organic molecules into organized and stable, yet reactive and functional
monomolecular structures cannot be effectively realized by traditional approaches, such as the formation of mixed and backfilled SAMs, because of the problems associated with the desorption and phase separation of dissimilar molecules.\cite{18} This problem can be resolved by relying on a bilayered interfacial system that comprises two covalently linked monolayers of organic molecules that grant stability and functionality to the inorganic substrate supporting them (Figure 22). In this system the primary monolayer is composed of highly organized and chemically inert molecules that protect the interface by chemically passivating the inorganic material and by restricting the diffusion of corrosive species. The secondary overlayer is affixed to the primary monolayer with stable C-C bonds via carbene insertion chemistry. This overlayer comprises densely spaced functional organic molecules that provide chemical reactivity or desired physical properties to the interface. The stability, molecular order, reactivity, and functional group density of this system can all be controlled and adjusted via organic synthesis and surface reactions. In addition, the bi-layered system can be chemically modified with functional molecules with desired physical, biological, chemical, electronic, electrostatic or HOMO/LUMO properties, permitting facile incorporation and specific geometrical positioning of reactive chemical species, active enzymes, charge-attracting and transferring groups within the inorganic-organic interface.
2.2.3 Stamp-substrate reactions

During the stamp-substrate reaction, the catalyst immobilized to the stamp modifies the substrate in places of conformal contact. Ideally, this interaction should be specific to avoid unwanted side reactions and should proceed quickly at ambient conditions. Moreover, the reaction should undergo a complete, irreversible reaction and form stable species. Because catalytic µCP achieves pattern replication through specific chemical reactions, it offers several advantages over traditional patterning methods. It can achieve higher resolution and accuracy with more diverse substrates and can quickly generate chemoselectively functionalized substrates in a pattern specific manner. Previous work in our laboratory demonstrated that a sulfonic acid modified PUA stamp can effectively transfer micro and nano-scale patterns to Boc-modified SAMs on gold and oxide-free silicon.[121] Here, we utilized an identical stamp to selectively deprotect NHS-modified SAMs on oxide-free silicon and germanium and indium tin oxide, generating patterns containing protected and free amino regions that could be further manipulated.
2.3 Catalytic µCP on oxide-free silicon

2.3.1 Introduction

Previous work demonstrated the proof-of-concept for transferring high resolution patterns on gold and oxide-free silicon with catalytic µCP. Here, we extend the initial methodology to develop a functional printing approach to generate high resolution protein modified oxide-free silicon for sensing and diagnostic applications. We required (1) a stable interface that could withstand exposure to high temperatures and water, (2) a functional template that could be used to attach and pattern any biomolecule and (3) a universal modification method that could be applied to virtually any semiconductor.

The development of hybrid bioelectronic devices relies in large part on the integration of (bio) organic materials and inorganic semiconductors through a stable interface that permits efficient charge transport while protecting underlying substrates from oxidative degradation. Interfaces composed of ordered organic molecules covalently bound to inorganic semiconductors offer unique advantages in terms of stability and charge transfer over traditional conjugated polymers physisorbed on inorganic materials. Group IV semiconductors can be effectively protected with highly-ordered SAMs composed of simple alkyl chains that act as impervious barriers to both organic and aqueous solutions. Several groups have reported that alkylation of Si-H substrates with both Grignard reagents and organo-lithium compounds results in

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1 The following work was done in collaboration with Dr. Alex Shestopalov and was published in Langmuir in 2011, vol. 27, pages 6478-6485. For additional experimental detail see experimental section 4.1
complete functionalization of all exposed Si atoms with chemically stable Si-C bonds.\textsuperscript{72, 124} Alternatively, hydrosilylation with alkenes or alkynes results in substitution of only 50\% of the exposed Si-H sites, leaving remaining atoms susceptible to oxidation.\textsuperscript{70, 125} Nonetheless long-chain alkenes and alkynes can too effectively protect passivated silicon via hydrophobic chain-chain interaction similar to thiolated SAMs on gold.\textsuperscript{17, 126} Simple alkyl SAMs, however, are inert and not amenable to traditional functionalization protocols.

Although carbanion-based covalent monolayer integration provides a durable, robust surface, it is not amenable to traditional soft lithographic techniques, and robust methods for patterning traditional inorganic semiconductors are largely lacking. Microcontact printing has not been well developed for pattern transfer to technologically important substrates such as oxide-free silicon, and its application remains limited largely to noble metal surfaces. Furthermore, the resolution of traditional \( \mu \text{CP} \) is near 1 \( \mu \text{m} \), limited primarily by ink diffusion.\textsuperscript{26, 29a, 30-31, 48, 127}

Recent reports of soft-lithographic approaches towards Si-H patterning and functionalization have appeared. Buriak and co-workers utilized polymer-imbedded Pd nanodots to replicate patterns of organic SAMs on Si-H.\textsuperscript{92} The technique relies on a Pd-catalyzed hydrosilylation between surface Si-H sites and alkyne molecules physisorbed on the Pd surface. Although the approach replicates nanodot patterns at the 100 nm scale, it does not protect unreacted Si-H areas from oxidation, and it cannot replicate features of arbitrary geometry. In another study, Zuilhof and co-workers patterned functionalized silicon via reactive \( \mu \text{CP} \) with amine nucleophiles.\textsuperscript{87} The method
functionalizes Si with various organic species and replicates features in the ~5 µm domain. Because the approach relies on diffusive pattern-transfer, edge resolution of printed features is limited to ~300 nm. Moreover, the technique utilizes reactive functionalized SAMs grafted directly to Si that normally do not protect the Si-SAM interface from degradation.\textsuperscript{18, 51b, 82, 128} Such monolayers cannot be stabilized via hydrophobic chain-chain interactions, because of the presence of large terminal functional group, permitting access by destructive agents to the underlying surface.\textsuperscript{63, 123-124, 126b}

In contrast to traditional reactive printing, inkless µCP transfers pattern using the specific reaction between a surface-immobilized substrate and a stamp-bound catalyst, without relying on diffusive SAM formation. The technique significantly expands the diversity of patternable surfaces and obviates the feature size limitations imposed by molecular diffusion, facilitating replication of very small (<200 nm) features.\textsuperscript{91, 120, 129} Until now, however, inkless µCP has been used only for the patterning of relatively disordered molecular systems, which do not protect underlying surfaces from degradation.

We developed a simple, reliable high-throughput method for patterning passivated silicon with reactive organic monolayers and demonstrate selective functionalization of the patterned substrates with both small molecules and proteins. The technique utilizes a preformed, uniform bilayered NHS-modified silicon substrate, which is hydrolyzed in a pattern-specific manner with a sulfonic acid-modified acrylate stamp to produce chemically distinct patterns of NHS-activated and free carboxylic
acids. The approach completely protects silicon from chemical oxidation, provides precise control over the shape and size of the patterned features in the 100 nm domain, and gives rapid, ready access to chemically discriminated patterns that can be further functionalized with both organic and biological molecules.

Figure 23. Catalytic µCP on passivated semiconductors. a: Passivated oxide-free semiconductor. b: The primary SAM forms stable covalent bonds with the substrate, resulting in complete termination of all surface-exposed atoms and yielding a chemically inert, closely-packed system that does not degrade in harsh environments. c: The reactive overlayer forms stable C-C bonds with the primary SAM. d: The elastomeric stamp makes conformal contact with the substrate and catalyst modifies the reactive overlayer in areas of conformal contact. e: An acrylate stamp contains covalently bound catalyst. The mechanical rigidity of the stamp supports sub-100 nm high- and low-aspect ratio features.
2.3.2 SAM Components

Our protocol, shown schematically in Figure 23, is a form of inkless \( \mu \)CP. In this approach, a stamp-immobilized catalyst is brought into contact with a surface bearing functional groups with reactivity cognate to that of the immobilized catalyst; pattern is transferred through catalysis in areas of conformal contact. Because pattern transfer does not rely on ink transfer from stamp to surface the diffusive resolution limitation of traditional and reactive \( \mu \)CP is obviated, permitting routine transfer of objects in the 100-nm domain. To protect underlying silicon from oxidation damage and/or electronic degradation, we required a highly ordered molecular system that provides complete functionalization of all surface-exposed silicon atoms with stable carbon-silicon bonds. Concurrently, to facilitate \( \mu \)CP we required a system that supports immobilization of sterically bulky reactive groups. To simultaneously satisfy both requirements a bilayered system was used, composed of an ordered protective primary SAM upon which was built a secondary reactive overlayer; together the system achieves both protection and functionalization (Figure 23). H-terminated silicon (2) was first chlorinated with \( \text{PCl}_5 \) and then alkylated with allyl Grignard, forming a highly stable, closely-packed propylene-terminated SAM (4, Figure 24). The propenyl-functionalized surface was next reacted with a diazirine carbene precursor containing NHS-activated carboxylic acids, providing modified surface 5. NHS esters were chosen for the surface overlay because of their hydrolytic reactivity to acid catalysts and facile electrophilic reactivity towards amine nucleophiles, permitting sequential patterning and elaboration. Surface 5, comprising an inert closely-packed primary monolayer and a reactive NHS-terminated overlayer,
simultaneously protects silicon from oxidation and SAM degradation while providing latent functionality that both facilitates µCP using acid catalysts and permits covalent attachment of both organic and biological molecules following stamping. The protocol achieves complete termination of all surface-exposed Si atoms with Si-C bonds, preventing oxygen and water migration to the Si-SAM interface.\textsuperscript{72}

Figure 24. Formation of NHS-modified substrates and subsequent chemical modifications \(a\): XPS spectra of 5. \(b\): C 1s XPS spectra of substrates 5,6,and7.
AFM analysis showed both the primary monolayer 4 and reactive overlayer 5 were uniform and with very low roughness (Rq=0.366 nm, Ra=0.287 nm, Z range=3.34 nm, Rq=0.320 nm, Ra=0.256 nm, Z range=2.42 nm, respectively). Surface 4 showed minimal contact angle hysteresis (2°) consistent with the formation of a highly ordered densely-packed molecular surface.\textsuperscript{[51b]} Formation of the reactive overlayer to yield surface 5 resulted in a significant increase in contact angle hysteresis (22°), consistent with the formation of a loosely-packed overlayer and/or NHS-ester hydrolysis during goniometry. XPS analysis revealed the presence of fluorine on surface 5, confirming carbene insertion to the primary protective SAM. The XPS Si 2p spectrum of the NHS-modified substrate showed no observable oxide, demonstrating the stability of the bilayer system even under oxidative environments (UV light, air; Figure 24 A).

2.3.3 Chemical Functionalization of the NHS-modified SAMs

To demonstrate the reactivity of NHS-modified surfaces towards organic molecules, we exposed a freshly prepared NHS-SAM substrate to mono Boc-protected ethylenediamine (Figure 24). The XPS C1s signal of the resulting Boc-protected ethylenediamine-modified surface showed an additional sharp peak at 286.5 eV, corresponding to the presence of C-C, C-O, and C-N bonds in the surface-grafted SAM (Figure 24 B).\textsuperscript{[130]} Concomitantly, the intensity of this secondary peak in the initial NHS-modified substrate, which has fewer sp3-hybridized carbons, was significantly diminished. Deprotection of the Boc-modified substrate with 25% TFA in dichloromethane resulted in reduction of the sp3 C1s peak intensity, consistent with
selective hydrolysis of the Boc-moiety. These secondary modifications of the NHS-modified SAMs were accomplished with no detectable silicon oxidation, demonstrating complete passivation of the underlying Si-SAM interface by the primary protective SAM, even towards strong acids. Additional stability tests on the Boc-modified substrate (high temperature, basic and acidic solutions, and prolonged exposure to air) produced neither oxidation of the silicon substrate nor monolayer desorption. These results emphasize the remarkable stability of the primary SAMs toward degradation and desorption.
2.3.4 Catalytic PUA Stamps

The formation of stable reactive patterns on oxide-free silicon relies on a hydrolytic reaction between NHS-functionalized SAM (5) and a sulfonic acid-modified catalytic stamp.\textsuperscript{[120b]} We have previously utilized oligo(ethyleneglycol)-modified polyurethane/acrylate (PUA) stamps for this process. These materials offer both the
opportunity for ready functionalization with a range of catalytic moieties and the ability to carefully control the modulus of elasticity. This latter property facilitates the creation of high aspect ratio features necessary for nanoscale pattern transfer.

Patterned and flat sulfonic acid-modified catalytic PUA stamps were prepared by reacting the components of a pre-polymeric mixture with 2-mercaptoethanesulfonic acid at 50°C for 5 minutes, followed by the degassing under vacuum at room temperature (Figure 25). The resulting mixture was cast against a Si/SiO₂ master, covered with a transparent glass slide, and polymerized under UV light at room temperature for 2 hours. Following polymerization, the stamp was peeled off from the master, washed extensively with ethanol and water, dried under a stream of filtered nitrogen, and kept at ambient temperature. Featureless stamps were prepared in a similar manner by polymerizing pre-polymeric mixture between two flat glass slides. Catalytically inactive stamps were prepared by polymerizing initial PUA mixtures without 2-mercaptoethanesulfonic acid.
2.3.5 Hydrolyzing efficiency of the catalytic PUA stamps

To determine the efficiency of NHS hydrolysis, we reacted featureless catalytic and inactive control stamps with NHS-terminated monolayers at room temperature for various times. Sulfonic acid stamp-treated surfaces show a carbon XPS signal diminished to the same extent as that produced by 1 M HCl (Figure 26), indicative of complete and selective NHS hydrolysis. Inactive stamps produced no changes in SAM composition visible by XPS. The fluorine signal remained constant under all conditions, suggesting that transformations induced by both sulfonic acid stamps and HCl solution were specific to the NHS groups and did not affect other components of the bilayered molecular system.
Figure 27. Hydrolysis of NHS substrates with featureless catalytic stamps: secondary reactions.

The hydrolysis efficiency of the sulfonic acid PUA stamp was also evaluated via secondary reactions of the NHS-modified SAMs with perfluorinated alkyl amines (Figure 27). Stamp and HCl-hydrolyzed samples and unmodified NHS-SAM were reacted with a dichloromethane solution of pentadecafluoroctan-1-amine for 2 hours at room temperature, and carbon and fluorine concentrations of the resulting surfaces were analyzed by XPS. Figures 26 and 27 show that stamp- and HCl-modified SAMs showed essentially identical carbon and fluorine concentrations before and after reaction with perfluorinated amine, whereas the NHS-SAM surfaces showed a significant increase in both fluorine and carbon signal intensities after the reaction with the fluorinated amine (Figure 27). These results not only confirm that the sulfonic acid PUA stamp induces equivalent changes in the NHS-SAMs as does HCl solution, but also that neither stamp-
or HCl-hydrolyzed free acid SAMs react with primary amines without coupling reagents. The observed difference in chemical reactivities of free acid- and NHS-terminated SAMs can be exploited to selectively functionalize patterned NHS-substrates with heterobifunctional linkers and biomolecules, providing a simple and efficient method for patterning and functionalizing passivated oxide-free silicon with a variety of organic and biological molecules.

![SEM images of NHS-patterned SAMs on oxide-free silicon.](image)

**Figure 28.** SEM images of NHS-patterned SAMs on oxide-free silicon.

### 2.3.6 Nano-features on passivated silicon

Our goal was to develop an effective approach to pattern passivated silicon through soft lithographic techniques. Because our method does not rely on diffusive pattern transfer, it can achieve resolutions near those of such instrument-intensive techniques as photolithography and e-beam lithography. To demonstrate the utility of our approach for the creation of chemoselective patterns on oxide-free silicon, surface 5 was reacted for 4 minutes at room temperature with a patterned polyurethane-acrylate stamp bearing covalently bound sulfonic acid moieties. The catalytic stamp hydrolyzed NHS moieties in areas of conformal contact, yielding a patterned bifunctional substrate.

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Nano patterning was performed in collaboration Dr. Briana N. Vogen.
(9) bearing regions of NHS activated and free carboxylic acids. Figure 28 demonstrates the efficiency of the technique across the entire substrate surface, yielding 125 nm features. To the best of our knowledge, this pattern contains one of the smallest SAM features replicated through soft lithography. The catalytic stamp can be used repeatedly without losing efficiency. Control acid-free stamps failed to produce any discernible change in the NHS-modified SAM.

Figure 29. Soft-lithographic patterning and functionalization of passivated silicon with organic and biological molecules. a: SEM image of the patterned NHS-modified substrate 9. b: Fluorescent micrograph of GFP modified substrate 12.
2.3.7 Secondary functionalization of the patterned substrates

With an effective approach for patterning passivated silicon at 100 nm length scales in hand, we turned to functionalization of silicon surfaces with (bio)organic materials. By exploiting the differential reactivities of activated and free carboxylic acids, we affixed nitrilotriacetic acid-terminated (NTA) heterobifunctional linkers to the NHS-functionalized regions of surface (9). A 20 mM solution of lysine-N,N-diadic acid and 100 mM solution of triethylamine in a 1:1 DMF-water mixture was reacted for 1 hour at room temperature with NHS-patterned substrate (9). Following the reaction, the substrate was thoroughly rinsed with water, DMF, and ethanol, and dried under a stream of filtered nitrogen. The resulting NTA-patterned surface was used as a template for the selective attachment of hexa-histidine-tagged GFP. Fluorescence microscopy clearly shows differential fluorescence intensity between GFP-modified and hydrolyzed free carboxylic acid regions (Figure 29 B). The size and shape of the replicated features are consistent between both NHS patterned surface (9) (Figure 29 A) and GFP-modified surface (12) (Figure 29 B). That pattern integrity is completely conserved even after multiple surface modifications further demonstrates the remarkable stability of carbon passivated surfaces and the selectivity of the stamping approach. Specifically bound His-tagged protein can be removed from the surface with imidazole, allowing reversible immobilization of multiple proteins on the same substrate. The protocol is obviously not limited to His-tagged protein, and can be used to pattern other biomolecules including DNA and antibodies.
In conclusion, this approach offers a universal method for patterning semiconductor surfaces with a broad range of inorganic, organic, biological and polymeric materials. Precise control over terminal functional groups is achieved by varying both heterobifunctional linkers and carbene donors in the reactive overlayer, while the underlying inorganic surface is robustly passivated with a highly ordered inert carbonaceous monolayer. The ability to create patterned organic-semiconductor interfaces without expensive, complex instrumentation offers myriad opportunities in fields such as electronics, nanotechnology, biochemistry and biophysics, and will facilitate an understanding of fundamental properties of (bio)organic and inorganic interfaces. We have continued to demonstrate the broad applicability of this catalytic lithographic method to other technologically-relevant semiconductor surfaces.

2.4 Catalytic µCP on oxide-free germanium

2.4.1 Introduction

As we mentioned in the previous chapter, the integration of organic and biological molecules on traditional semiconductor materials is an increasingly important area of research for the development of hybrid bioelectronics devices.\textsuperscript{[63, 122a, b]} Semiconductor materials such as silicon (Si), gallium arsenide (GaAs) and germanium (Ge) play ubiquitous roles as the most technologically important materials today.\textsuperscript{[62]} At the same time, recent investigations into the integration of these substrates with organic self-assembled monolayers (SAMs) have provided new and promising

\textsuperscript{3} The following work was published in Langmuir in 2011, vol. 27, pages 6486-6489. For additional experimental detail see experimental section 4.2.
approaches towards the development of hybrid bioelectronics devices that utilize the properties and features of both components.\textsuperscript{62, 122a} Such hybrid semiconductor substrates functionalized with biological or organic molecules can be used in a variety of sensing applications, where the molecular response to biological or chemical stimuli can be coupled to the semiconductor surface and processed using traditional microelectronic methods generating a chip-based chemical or biological sensor.\textsuperscript{62}

For more than 40 years, silicon has served as the semiconductor of choice due to the facile formation of a stable oxide layer. On the other hand, germanium forms an unstable, water soluble oxide layer, precluding its widespread use in many applications. Replacing silicon with a higher mobility material, however, offers many advantages, and the development of facile approaches to hybrid devices based on substrate materials other than silicon is an area of great interest.\textsuperscript{64} Germanium has attracted significant interest in this regard, offering bulk electron and hole mobilities three and four times higher, respectively, than those of silicon.\textsuperscript{131} In addition, germanium is easily integrated with existing silicon-based devices for the construction of high-speed electronic and optoelectronic devices. The increased interest in germanium has prompted the development of robust chemistries to passivate and protect against chemical oxidation.\textsuperscript{70}

As we demonstrated in the previous chapter, sulfonic acid modified polyurethane-acrylate (PUA) stamp can be used to pattern oxide-free silicon by hydrolyzing NHS-activated monolayers.\textsuperscript{121} Here, we extend this approach to pattern functionalized SAMs on germanium. Our approach utilizes a bilayered molecular system
on germanium composed of a primary ordered protective monolayer and a secondary reactive overlayer that can be patterned using an acidic PUA stamp.

Several methods exist for the passivation of germanium through the addition of simple alkane monolayers: such ordered SAMs passivate the germanium surface and protect it from oxidation.\cite{132} On the other hand, the incorporation of organic and biological components into a hybrid organic/germanium semiconductor device requires new methods that functionalize germanium stably with functional molecular systems: no such method for the creation of such systems currently exists.\cite{63} Moreover, despite numerous examples of patterned SAMs on various metals and metal oxide surfaces, no reports have appeared of patterning germanium with organic or biological molecules.

Native germanium surfaces are highly sensitive to oxygen and other reactive species. SAMs covalently bound to germanium were shown to impart excellent surface stability by passivating and protecting the underlying surface from chemical oxidation.\cite{132b} Therefore, in order to protect the electronic properties of germanium substrates, patterning must be performed on a stable, preformed SAM. Because catalytic µCP patterns pre-formed monolayers it provides an excellent opportunity to transfer pattern to oxide-free germanium surfaces; a task not possible with traditional µCP.\cite{91}
Figure 30. Structure of bi-layered molecular system on Ge. (1) Primary alkyl monolayer forms stable Ge-C bonds (~56 kcal/mol) with the substrate and provides a chemically inert and close packed system that protects the underlying surface from degradation. (2) Secondary overlayer forms stable C-C bonds (~82 kcal/mol) with primary protective layer and provides terminal functional groups.

In order to form patterned monolayers on germanium using catalytic µCP, we required a SAM bearing functionality cognate to that of the stamp-immobilized acid catalyst and sufficiently stable to survive the patterning step. Numerous methods exist for the immobilization of functional organic molecules on metals and silicon oxide. Specifically, SAMs with terminal functional groups including amino, mercapto and carboxyl moieties have been immobilized using alkysilanes monolayers on native oxide.\[17, 19\] Such monolayers, however, cannot be supported by germanium due to the soluble nature of its native oxide. Recently, several reports have appeared describing the formation of stable highly-ordered alkane SAMs on hydride-terminated germanium using Grignard, hydrogermylation and thiolation reactions.\[67, 132b\] Because of the higher Ge-C bond strength relative to the Ge-S bond, Grignard and hydrogermylation approaches form more stable molecular systems than the corresponding thiol
However, despite the stability of such simple alkyl monolayers, they do not provide the terminal reactivity required for catalytic μCP. To take advantage of the stability imparted by alkyl monolayers and provide the reactivity required for catalytic μCP, we applied the bi-layered functionalization strategy that utilizes an initial stable organic monolayer as a support for a functional reactive overlayer (Figure 30). The overlayer, which serves as a functional substrate for catalytic μCP, ultimately supports attachment of a variety of organic and biological molecules, providing a facile approach to the creation of germanium-based functionalized semiconductor devices. This approach is similar to the catalytic method we used to achieve chemoselectively anchored organic molecules and proteins on oxide-free silicon.
2.4.2 SAM Components

The protocol for formation of a bilayered, patternable molecular system on germanium is summarized in Figure 31. The functionalization protocol begins from a native germanium surface (1). XPS analysis of this substrate reveals a germanium 3d signal containing a peak indicative of native oxide. This native surface was chlorinated...
by treatment with 10% aqueous HCl, generating an air stable Ge-Cl surface that can be manipulated in ambient atmosphere for up to 2 hours.\textsuperscript{[65]} XPS analysis revealed complete loss of the oxide peak, confirming removal of the oxide layer.

We previously reported a protocol for catalytic μCP on native silicon that generates an initial stabilizing underlayer with allyl Grignard. Such an approach is not feasible here.\textsuperscript{[121]} The greater Ge-Ge spacing relative to silicon renders alkyl Ge-C bonds kinetically susceptible to scission by adventitious solvent and/or oxygen when the monolayer is prepared using short (C2) chains. On the other hand, longer alkyl chains provide sufficient flexibility to form tightly packed monolayers, affording effective exclusion of oxygen from the surface, and previous reports have shown that long chain alkyl monolayers impart significant stability against oxidation.\textsuperscript{[132b, 133]} Accordingly, an octyl stabilizing alkyl layer was utilized.

Exposure of the chloro-terminated surface to octyl Grignard for 48 hours at 130 °C produced the corresponding alkyl-terminated surface (3). The C1s/Ge3d intensity ratio is greater for surface 3 than for 2, consistent with an increase in carbon at the surface during SAM formation. AFM analysis (Rq=0.77 nm) and goniometry in water (Θ先进=82°, Θ回收=71°) showed low surface roughness, consistent with the formation of a uniform SAM. Following formation of a stable primary alkyl monolayer, the alkyl-Ge surface was functionalized with a reactive overlayer. This secondary overlayer was formed by reacting methyl-terminated substrate 3 with an NHS-diazirine-derived carbene, producing a reactive surface containing N-hydroxysuccinimide activated acids (4). XPS analysis revealed a F 1s signal, consistent with the successful insertion of the
diazirine-derived carbene to the methyl terminated SAMs. The primary alkyl monolayer provides sufficient stability to prevent formation of an oxide layer throughout the entire functionalization procedure. Remarkably, the surface was resistant to oxidation even during exposure to UV light, a strongly oxidative environment.

To demonstrate the reactivity of the NHS-activated surface, we modified surface 4 with pentadecafluorooctylamine. Comparison of the F1s/Ge3d XPS signal ratios between surfaces 4 and 5 confirmed reaction, with the ratio increasing from 0.56 to 0.67. The ability to modify the reactive NHS terminated overlayer via specific chemical reaction affords the ability to utilize this approach for the immobilization of a variety of organic and biological molecules on oxide-free germanium.
2.3.3 Patterning NHS-Modified SAMs on Germanium

Stable reactive SAMs capable of supporting functional organic and biological molecules have a wide range of applications, from molecular electronics to biosensors.\textsuperscript{[62, 124d]} The majority of such applications depend on an ability to pattern these surfaces with sub-micrometer resolution. Such high-resolution patterning of biomolecules on semiconductor surfaces will not only facilitate miniaturization of the individual components of the hybrid bioelectronics devices, but can also provide a mechanism with which to control the interfacial properties of semiconducting materials. Such applications typically require robust attachment of species to the surface, and
patterned molecular systems should rely on covalent attachment of individual components rather than on sequential physisorption of multiple biological or organic films. The approach reported here offers a simple and efficient method for patterning such SAMs on germanium.

Our patterning approach utilizes an elastomeric stamp bearing covalently bound sulfonic acids to achieve pattern-specific hydrolysis of NHS-functionalized SAMs (Figure 31). Catalytic patterned and flat stamps were prepared using previously published protocols. Briefly, a pre-polymeric polyurethane/acyrlate mixture was reacted with 2-mercaptoethanesulfonic acid for 5 minutes and deoxygenated under vacuum. The mixture was then cast between a patterned Si/SiO$_2$ master and a flat glass slide and polymerized by exposure to UV light for 2 hours. Polymerized stamps were removed from the corresponding support, rinsed with water and ethanol and dried under filtered argon. Catalytically inactive stamps were prepared in a similar fashion but in the absence of 2-mercaptoethanesulfonic acid.

Patterned SAMs were formed by bringing a freshly prepared NHS-substrate (4) in conformal contact with a patterned sulfonic acid PUA stamp containing 16 µm hexagons for 2 minutes at room temperature. The treated surface was rinsed with ethanol, dried under argon and analyzed by scanning electron microscopy (SEM) and contact mode lateral atomic force microscopy (AFM) (Figure 32). During the stamp-surface reaction, the stamp-bound sulfonic acids hydrolyzed terminal NHS groups in the secondary reactive overlayer in regions of conformal contact. The acid catalyzed reaction between stamp and surface 4 produced patterns containing both NHS-activated and free
carboxylic acid groups. The efficiency of the sulfonic acid stamp in the hydrolysis of NHS-terminated SAMs was previously determined to be essentially quantitative. Both SEM and AFM images showed uniform patterns with no evidence of edge distortion. The patterns showed friction differences of ~9.5 mV and ~15.4 mV between NHS-activated and free carboxylic acid regions, confirming successful chemical modification of the NHS-monolayer with catalytic stamp. The size and shape of the replicated patterns were identical to the features on the corresponding Si/SiO$_2$ master and catalytic PUA stamp, again demonstrating the advantages of diffusion-free pattern transfer.

Figure 33. SEM and AFM friction images of patterned SAMs on Ge with a catalytic PUA stamp.
To further demonstrate the selectivity of the acidic PUA stamp in the site-specific hydrolysis of NHS-functionalized germanium, we performed stamping experiments with featureless sulfonic acid stamps and catalytically inactive stamps (Figure 33). To demonstrate that sulfonic acid stamps selectively hydrolyze NHS-modified SAMs but produce no additional changes to the bilayered system, we first reacted a flat catalytic stamp with freshly prepared NHS-substrate, producing surface 7. The XPS C1s/Ge signal ratio of hydrolyzed substrate 2 decreased from 0.77 to 0.64, indicating selective hydrolysis of NHS groups by the catalytic stamp. In a subsequent experiment a portion of a patterned NHS-substrate was reacted with a flat catalytic stamp. The SEM image of the resulting substrate (6) clearly demonstrates that the flat catalytic stamp completely ablated the initially patterned surface, confirming that the pattern was composed of NHS-activated and free carboxylic acid groups. Finally, control experiments utilizing patterned stamps lacking sulfonic acid catalyst and freshly prepared NHS-substrates produced no pattern on the resulting substrate (8), further demonstrating the selectivity of the acid modified stamps in the catalytic pattern transfer.

In conclusion, we have demonstrated a simple reliable protocol for the formation of functional, patterned monolayers on germanium. The technique begins with formation of a stable, chemically inert primary alkyl monolayer which serves as an effective barrier to oxide formation. Construction of a secondary reactive overlayer provides terminal NHS functional groups that serve as attachment points for a variety of chemical and biological species. This stable bilayered molecular system was subsequently patterned using the catalytic µCP approach we have previously reported.
on other surfaces. A sulfonic acid bound PUA stamp was used to transfer chemically
distinct patterns of NHS activated and free carboxylic acid groups. The ability to pattern
stable functional SAMs on germanium enables myriad applications in various areas,
including microelectronics and sensing.

2.5 Multicomponent Patterning on Indium Tin Oxide

2.5.1 Introduction

Here, we extend our bilayered patterning approach to affix multiple
functionalities on an oxide semiconductor, indium tin oxide (ITO). The low electrical
resistance, outstanding conductive properties, and optical transparency make indium tin
oxide (ITO) an important substrate in materials science, chemistry, physics, and chemical
engineering. ITO has found use as an electrode or a substrate in photovoltaic, optical,
and electroluminescent devices, in sensors and DNA chips. ITO is an especially
important substrate for organic semiconducting materials (e.g. organic-light emitting
diodes (OLEDs)) and organic photovoltaics (OPV)), due to a tunable work function and
high conductivity. These devices often require patterned architectures; therefore
the ability to design robust ITO interfaces that can support multiple patterned
functionalities is desirable.

The current generation of organic semiconductors relies primarily on direct
coating of ITO with conducting polymers and/or small molecules. Several studies have
indicated that well-ordered and stable self-assembled monolayers (SAMs) both improve

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4 The following work was published in ACS Applied Materials and Interfaces in 2012, vol. 4, pages 3932-
3937. For additional experimental detail see experimental section 4.3.
charge transfer efficiency between the polymer and ITO and enhance the structuring and nano-morphology of the applied polymer.\textsuperscript{[138]} Such SAMs can be formed on ITO using organosilanes, carboxylic acids, phosphonic acids and alkanethiols.\textsuperscript{[139]} Each of these functionalities form relatively stable ordered films on ITO, with long-chain alkylsilanes forming the most stable and robust monolayers.\textsuperscript{[57, 140]}

Simple alkyl monolayers, however, are of only limited utility. The modification of work functions requires the introduction of a wide range of functionalities of varying polarity and polarizability, while the robust immobilization of biomolecules requires chemical “handles” that can be selectively accessed under mild conditions.\textsuperscript{[141]} Surprisingly, few immobilization strategies exist for the modification of ITO surfaces with diverse functionality, and no simple method exists for patterning ITO with functional monolayers. Techniques such as microcontact printing (\(\mu\)CP) and dip pen lithography, which rely on diffusive ink transfer from a polymeric stamp or AFM tip, have not been routinely successful for patterning ITO. Such diffusion-based methods require long reaction times and high reaction temperatures due to the high kinetic barrier to Si-O-In/Sn bond formation.\textsuperscript{[142]} An alternative to direct patterned SAM formation is afforded by the initial formation of a reactive SAM followed by catalytic or reactive \(\mu\)CP to introduce new functionality through chemical reaction in regions of substrate-stamp conformal contact.\textsuperscript{[114a]}

Here we report a patterning strategy for ITO substrates through both catalytic and reactive \(\mu\)CP techniques, passivated with simple alkyl SAMs, using a variety of organic molecules (Figure 34). The primary advantages of the approach are its ability to
(1) form stable and functional monolayers on ITO; (2) pattern ITO with virtually any functionality with precise spatial control at the microscale; (3) accurately form multicomponent patterns with organic and biological species on a single ITO substrate. We demonstrate the versatility of the approach through reactive and catalytic µCP, as well as a novel photocatalytic µCP. This approach is especially suitable for ITO, providing a simple and convenient method to passivate, functionalize, and pattern ITO with diverse molecules, overlaying a variety of organic and biological functional groups on a robust alkyl substratum.

Figure 34. Schematic description of the molecular systems and printing techniques used to functionalize and pattern ITO. (a) Catalytic inkless µCP with a sulfonic acid stamp produces patterns of activated and free carboxylic acid. (b) Reactive transfer µCP with an amine modified stamp produces patterns with two distinct functionalities. (c) Photoreactive transfer µCP allows site-specific immobilization of C60. (d) Combining catalytic and reactive µCP techniques facilitates the formation of three distinct functionalities on a single substrate.
2.5.2 SAM Components

In order to pattern orthogonally reactive SAMs on ITO, we required a functional monolayer that is both cognate to the reactive features on an elastomeric stamp and sufficiently stable to survive the patterning step. SAMs are typically formed on ITO using organosilanes, carboxylic acids, phosphonic acids or thiols. Neither carboxylic acids nor thiols bind strongly to ITO, and monolayers of these species are labile.\textsuperscript{143} Silanes, on the other hand, bind strongly to ITO, forming homogenous, oriented monolayers.\textsuperscript{57} Alkylsilane monolayers have been successfully used as low-energy self-developing e-beam resists.\textsuperscript{140} We exploited the stability and order imparted by silanes to create a primary monolayer on ITO. The subsequent incorporation of a reactive overlayer provides a functional moiety cognate to the reactivity borne by the elastomeric stamp. The approach allows for facile, site-specific immobilization of multiple organic molecules.

The formation of a bilayered molecular system on ITO begins from a native ITO surface (1) (Figure 35). The surface was oxidized in a plasma asher, generating surface hydroxyl groups (2).\textsuperscript{144} Exposure of the hydroxyl-terminated surface to trimethoxyoctylsilane for 18 hours under high vacuum produced the corresponding alkyl-terminated surface (2). The C1s/In3d intensity ratio in the XPS spectrum is greater for surface 3 than 2, consistent with the deposition of carbon at the surface during SAM formation. Goniometry analysis using water (Theta\textsubscript{adv.}=95°, Theta\textsubscript{rec}=90°) suggests a homogenous, well packed aliphatic SAM.\textsuperscript{139} Following the formation of the primary silane monolayer, surface 3 was reacted with an NHS-diazirine-derived carbene,
producing a reactive surface displaying N-hydroxysuccinimide (NHS) activated acids at the surface (4). XPS analysis revealed an F 1s signal, indicative of successful UV-catalyzed insertion of the diazirine-derived carbene to the methyl terminated SAM. Goniometry analysis, again using water ($\Theta_{\text{adv}}=86^\circ$, $\Theta_{\text{rec}}=72^\circ$), indicated an increase in the hydrophilicity of the surface due the presence of the NHS moiety. A contact angle hysteresis of $14^\circ$ is presumably indicative of a loosely-packed overlayer.

Surface 4 exposes reactive head groups patternable by both catalytic and reactive microcontact printing. In contrast to traditional µCP, both catalytic and reactive printings rely on modification of a preformed, functional monolayer (Figure 34), facilitating patterning of substrates normally incompatible with fast SAM formation.

Figure 35. Functionalization scheme for the preparation of a bilayered molecular system on ITO and corresponding XPS spectra.
2.5.3 Catalytic µCP on ITO

Our patterning approach utilizes an elastomeric stamp bearing covalently bound sulfonic acids to achieve catalytic pattern-specific hydrolysis of NHS-functionalized SAMs (Figure 36). Patterned SAMs on ITO were formed by bringing a freshly prepared NHS-terminated ITO surface (4) in to conformal contact with a sulfonic acid stamp bearing 20 µm hexagons for 1 minute at room temperature. Following removal of the stamp, the surface was rinsed with isopropanol, dried under argon and analyzed by scanning electron microscopy (SEM) (Figure 36). The acid catalyzed reaction between stamp and surface 4P produced patterns containing both NHS-activated and free carboxylic acid groups; the size and shape of the replicated patterns were identical to those of the stamp, demonstrating high fidelity pattern transfer.
2.5.4 Reactive µCP on ITO

In contrast to catalytic printing, reactive microcontact printing relies on the diffusion of a reactive species adsorbed on a stamp to a surface bearing cognate reactive functionality (Figure 34). To demonstrate the suitability of NHS-terminated surfaces for reactive µCP, we employed azido-dPEG₃-amine as a reactive ink. To verify the amine molecule was reactive towards surface 4, we initially exposed the NHS-terminated SAM to a 2 mM solution of azido-dPEG₃-amine in anhydrous dichloromethane (DCM) for 1.5 hours at room temperature (Figure 4a). XPS analysis
revealed a significant increase in the N1s/In3d intensity ratio from surface 4 to 5, consistent with an increase in nitrogen at the surface as the result of reaction.

Patterned azido- and NHS-terminated SAMs on ITO were formed by bringing a freshly prepared NHS-terminated ITO surface (4) in conformal contact with an azide-inked PUA stamp bearing 20 µm hexagons for 1 minute at room temperature. Upon removal of the reactive stamp, surface 5P was immediately rinse with isopropanol, dried under argon and analyzed by SEM (Figure 37). The reaction between the azide-inked stamp and surface 4 produced patterns containing both NHS-activated and azido functional groups. As was the case for catalytic patterning, the size and shape of the surface features were identical to those of the stamp, again demonstrating high fidelity pattern transfer.
To demonstrate the power and versatility of the approach, we achieved pattern-specific functionalization of ITO using three different organic moieties. NHS-terminated substrate 4 was successively treated with a reactive stamp bearing aminoazide ink, followed by a catalytic stamp bearing sulfonic acid moieties. The initial reactive printing step produced a pattern of azido-terminated molecules in the form of hexagonal rings (Figure 37), while the following catalytic step hydrolyzes unreacted NHS-groups to yield carboxylic acid-terminated areas. To facilitate analysis of the final surface, the catalytic stamp contained features different than those of the reactive stamp, producing a surface with two distinct sets of features. Figure 37 demonstrates that the initial reactive printing step produced uniform, clearly defined hexagonal rings across the
entire substrate area. The subsequent catalytic printing step produced a second set of features; namely 8 µM hexagons (Figure 38). This second set of features was replicated only in NHS-terminated areas without intrusion into azido-terminated space, consistent with the stability of the amide bond formed during the reactive µCP step.

Figure 38. Preparation protocol for multicomponent ITO with reactive and catalytic µCP techniques.

2.5.5 Photoreactive µCP on ITO

To further demonstrate the remarkable simplicity and versatility of the approach, we created a diazirine-terminated surface that facilitates photoreactive µCP. The formation of the required photoreactive bilayered molecular system on ITO begins with a native ITO surface (1) (Figure 39), which was oxidized in a plasma ashier to generate reactive hydroxyl groups (2). Exposure of the hydroxyl-terminated surface to aminopropyltrimethoxysilanes for 18 hours under argon produced the corresponding
amino-terminated surface 7. XPS analysis revealed the presence of a N1s peak, consistent with the generation of an amino-terminated substrate. Surface 7 was reacted with an NHS-diazirine-derived carbene. In contrast to the previous bilayered system, the NHS activated acid reacted at the amino-terminated surface, producing the photo-reactive carbene precursor-terminated surface 8. XPS analysis revealed a F 1s signal, indicating successful reaction between the NHS activated acid and the amino-terminated substrate.

Surface 8 contains a photoreactive moiety capable of cyclopropanation reaction with olefins. To demonstrate the photoreactivity of the monolayer, we reacted it with Buckminsterfullerene (C60), which readily undergoes carbene insertion. C60 is of considerable interest in chemistry, physics and materials science due to its unusual structure and extended π-electron system.\textsuperscript{[146]} C60 is an essential component of many OPV devices and the unique electronic properties of C60 can be used to modify the electronic and optical properties of ITO.\textsuperscript{[146-147]}

Diazirine-terminated surface 8 was exposed to a solution of C60 in benzene for 1 hour in the presence of UV light. UV-Vis analysis suggested successful C60 modification, and the absorption spectra presented in Figure 6 agree with previously reported spectra for a C60-bearing SAM.\textsuperscript{[148]}
Figure 39. Functionalization scheme for the preparation of C60 modified ITO and corresponding XPS and UV/Vis spectra.

To spatially resolve C60 on ITO we used photoreactive microcontact printing. Employing C60 as the photoreactive ink, conformal contact of diazirine-modified surface 8 with a stamp bearing photoreactive molecules in the presence of UV light allows for a direct carbene insertion to the surface (Figure 39). The elastomeric stamp was “inked” in a 7 mM solution of C60 in benzene for 1 minute, dried under argon and applied to surface 8. The stamp was held in conformal contact with the surface for 2 minutes while UV light (254 nm) was irradiated through the transparent glass/ITO surface. The surface was immediately rinsed with filtered isopropanol, dried with filtered argon, and
analyzed by SEM. The UV-catalyzed reaction between stamp and surface 8 produced patterns containing C60. SEM analysis shows uniform and high fidelity pattern transfer (Figure 40). As a control, we performed the same stamping protocol in the absence of UV light. SEM analysis in this instance revealed no pattern transfer. As an additional control, we applied a patterned stamp lacking C60 to a freshly prepared carbene-terminated layer in the presence of UV light. The absence of C60 precluded pattern transfer, further demonstrating the selectivity of the C60 modified stamps in the photo-reactive pattern protocol.

![Figure 40. SEM image of patterned SAMs on ITO using reactive µCP and C60 as the photoreactive ink.](image)

The overarching goal of this work was to develop high efficient, operationally simple approaches to pattern a wide variety of technologically relevant materials. Here, we demonstrated a simple and reliable protocol for patterning multiple functional moieties on ITO. The technique begins with the formation of a stable, well-ordered primary monolayer. Construction of a secondary reactive overlayer using an NHS-diazirine-derived carbene provides a terminal functionality that can be further modified...
to incorporate a wide range of surface groups. The functional bilayered molecular system was subsequently patterned using three different printing approaches. In catalytic printing, a sulfonic acid bound PUA stamp was used to transfer chemically distinct patterns of NHS activated and free carboxylic acid groups. Following catalytic pattern transfer, reactive µCP was used to transfer patterns bearing azido functional groups. The resulting surface contained three chemically distinct groups (NHS activated acid, carboxylic acid, azide) site-specifically patterned on ITO. Finally, we demonstrated photoreactive microcontact printing of C60 in the presence of UV light. The ability to easily pattern multiple functional groups on ITO using a tunable bilayered molecular system enables myriad applications, including micro- and optoelectronics and sensing.

2.6 Tunable Control over Hole-Injection in OLEDs via Organic Monomolecular Systems

2.6.1 Introduction

We have previously demonstrated a universal bilayered functionalization/patterning technique. The approach completely protects the underlying inorganic-organic interface and permits ordered attachment and patterning of virtually any species on semiconductor and metal oxide surfaces (Figure 41). Here we use this approach to (1) investigate structure/function relationships of ordered, functional molecular systems on ITO that act as organic light emitting diodes (OLEDs) and (2) demonstrate the powerful ability of catalytic lithography to quickly generate functional patterns in practical devices.
Figure 41. Stable, functional mono-molecular system on ITO. (a). Formation of protected interface that retains properties under harsh conditions: dense and ordered monomolecular layer that prevents diffusion of corrosive materials. (b). Formation of a secondary reactive overlayer: bridges the material interface to tunable functional groups (c). Formation of a terminal functional layer: desired chemical, physical or electronic properties.

Organic electronics offer the promise of lighter, more flexible and less expensive devices than those based on more traditional silicon-based technologies. Combined with large-area deposition and printing, thin-film organic electronics have the potential to substantially lower the production cost of light emitting diode (LED) and photovoltaic
(PV) devices. Several drawbacks, such as higher power consumption and reduced stability associated with molecular degradation of multilayered organic materials and their interfaces, must be overcome in order for the full potential of organic electronics to be realized.

Organic electronic devices, such as organic thin-film transistors (OTFTs), OLEDs and organic photovoltaics (OPVs), comprise a charge-injection interface between an inorganic electrode and organic semiconductor layer and precise control over the interface is essential for device performance. Thus, for example, large differences between the work function of the inorganic anode and the highest occupied molecular orbital (HOMO) level of the organic semiconductor limit hole-injection in OLEDs and lead to high turn-on voltage and low brightness and efficiency. Several approaches for modifying ITO to facilitate efficient hole-injection have been investigated, including, plasma and UV/ozone treatment, surface energy modification, insertion of organic and inorganic charge-injection thin films and incorporation of field gradients across the inorganic/organic interface. Such approaches, however, are typically inconsistent and offer only limited control over charge-transfer. Moreover, all approaches typically rely on complex deposition or surface-treatment techniques.

Monomolecular-based electronic devices composed of ordered organic monolayers (SAMs) promise control over the rate and mechanism of charge transfer through electronic interfaces. Such organic monolayers covalently immobilized on inorganic substrates represent a promising pathway towards robust inorganic-organic
interfaces with charge injecting or blocking capabilities. SAMs yield highly ordered and stable structures on inorganic surfaces by forming strong covalent bonds, and offer superior modification capability by matching the surface energy of the inorganic material to that of the organic thin film. Moreover, such systems permit excellent control over the interface work function by incorporating organic moieties with tunable HOMO/LUMO levels, making them particularly well suited for OLED applications. Recent work has focused on investigating traditional self-assembled monolayers (SAMs) of aliphatic and fluorinated organic molecules as hole-injecting layers.\textsuperscript{[113b]} Organic silanes and phosphonic acids improve the performance of ITO-based OLEDs by forming stable ordered monomolecular aggregates and by matching surface energy with the organic hole-transporting layer (e.g. NPB).\textsuperscript{[153d, 155]} Moreover, the dipole moments of individual molecules, the structure of the monolayer, and dipole depolarization in the SAM all affect charge injection and are capable of enhancing work functions.\textsuperscript{[151a, 156]} Schwartz et al. demonstrated that variation of the dipolar properties of surface-bound molecules alters the work function of an ITO electrode.\textsuperscript{[157]} Studies reported to date, however, are limited to simple aliphatic and perfluorinated SAM-forming molecules, and lack an obvious mechanism by which to incorporate functional molecules with tunable HOMO/LUMO levels.

Here, we use our previously described bi-layered strategy to devise functional organic structures on ITO with vertically and laterally controllable charge injection for OLEDs. We use as demonstration a typical OLED device that relies on hole injection from an ITO electrode to a charge-transport layer (Figure 42). The structure is composed of an
anode (ITO), a hole transport layer (HTL), an emissive layer (EL), an electron transport layer (ETL), and a cathode (Al). When a voltage is applied across the device, holes are either injected from the anode to the HOMO of the HTL either directly or through a thin hole injecting layer whose function is to facilitate hole transport by matching the work function of ITO with the HOMO of the HTL. Similarly, electrons are injected in the opposite direction from Al to the LUMO of the ETL via a thin electron injecting layer. When holes and electrons recombine in the EL, they release energy as light, functioning as an OLED.

By utilizing the functionalized bi-layered system on ITO as a hole-injecting layer, we demonstrate that:

(1) the interface between ITO and the HTL (NPB) can be stabilized with a highly ordered protective primary monolayer directly bound to the ITO surface;

(2) the driving voltage, efficiency and the luminance of the OLED are easily controlled through a secondary functional overlayer covalently bound to the primary SAM; and

(3) lateral control over hole-injection on a micrometer scale can be achieved by soft-lithographic patterning of hole-blocking and hole-injecting molecules.

Such precise control over device function using a single molecular system supports the notion that control over orientation, chemical properties and surface density of interfacial self-assembled structures enhances their electronic properties, rendering them superior to traditional inorganic electronic materials for the construction of luminescent devices.
Figure 42. OLED architecture: (Left). Electron and hole movement from the cathode and anode into the light emitting layer. (Right) OLED configuration.

Molecular self-assembly has emerged as a powerful pathway for the integration of inorganic and organic surfaces. The spontaneous formation of highly ordered SAMs in solution or in the gas phase on numerous inorganic materials is by now well established.\[51b, 63\] The flexibility inherent to organic chemistry provides a unique opportunity to manipulate physical and electronic properties at inorganic/organic interfaces. Several studies have demonstrated that anodic hole-injection in OLED devices can be controlled with organic SAMs on ITO. Existing approaches focus primarily on two groups of SAM-forming species: short aromatic organosilanes or phosphonic acids, and long-chain aliphatic and perfluorinated alkyl-silanes and phosphonic acids.\[155b\] Both groups display properties that limit the stability and/or the electronic tunability of the modified interface. HOMO/LUMO energies of aromatic molecules are controllable synthetically, providing a facile approach for matching the ITO work function to the HOMO of the organic charge-transporting layer. Small aromatic
molecules, however, form disordered monolayers that provide low surface coverage and no more than limited stability to the underlying surface. Aliphatic and perfluorinated alkyl silanes and phosphonic acids, on the other hand, form extremely robust and ordered structures on many inorganic oxides. On the other hand, electronic energy levels in simple aliphatic molecules cannot be modified synthetically without the introduction of bulky functional groups that impair surface coverage.

A multilayered interface design that permits formation of both highly ordered and functional molecular systems offers the attractive attributes of both groups, offering at once dense, uniform surface coverage and tunability of redox/electronic properties. The ability to incorporate diverse functionality with predictable physical and chemical properties into highly ordered and stable inorganic/organic interfaces will facilitate broader application of SAM-modified electronic interfaces and lead to the development of new organic electronic devices.

2.6.2 Surface Functionalization and Characterization

The multilayer molecular system relies on the formation of a stable, well ordered primary monolayer that protects ITO from degradation, and the subsequent attachment of a secondary overlayer that serves as a reactive template.\textsuperscript{[121, 145]} SAMs are typically formed on ITO using organosilanes, carboxylic acids, phosphonic acids or thiols. Neither carboxylic acids nor thiols bind strongly to ITO, and monolayers of these species are labile.\textsuperscript{[143]} Organosilanes and phosphonic acids, however, bind strongly to ITO, forming homogenous, oriented monolayers.\textsuperscript{[140, 158]} We took advantage of the stability and order
imparted by both organosilanes and phosphonic acids to create primary monolayers on ITO (Figure 43).

**Figure 43. Functionalization scheme for the preparation of the primary monolayer on ITO and the corresponding XPS spectra**

Primary silane monolayers were formed by sequentially sonicating ITO substrates in filtered water and IPA for 5 minutes followed by oxidation in an oxygen plasma, generating a dense monolayer of surface hydroxyl groups. The hydroxyl terminated surfaces were then exposed to trimethoxyoctadecylsilanes for 18 hours under high vacuum, producing an alkyl-terminated surface (P1). The C1s/In 3d intensity ratio in the XPS spectrum is greater in substrate P1 than in native ITO, consistent with the deposition of carbon during SAM formation. Additionally, the presence of Si2p peaks suggests successful alkylsilane deposition. The phosphonic acid head-group can
protonate both bridging oxides and hydroxyl-groups making exposure to a plasma ash unnecessary.\textsuperscript{[113b]} To form phosphonic acid SAMs, cleaned ITO was exposed to a 10 mM solution of octadecylphosphonic acid for 30 minutes or overnight (16-18 hours) producing alkyl-terminated surface P2. XPS analysis shows the presence of a P2p peak while the C1s/In 3d intensity ratio in the XPS spectrum is greater than native ITO, both observations consistent with successful SAM deposition.

Following formation of the primary protective monolayers, the alkyl modified surfaces (P1, P2) were reacted with an NHS-diazirine-derived carbene, producing a secondary reactive overlayer bearing N-hydroxysuccinimide (NHS) activated acids (S1, S2; Figure 44). XPS analysis revealed an F1s signal, indicating the successful UV catalyzed insertion of the NHS-diazirine to the methyl terminated surfaces.\textsuperscript{[121, 145]} This molecular architecture provides a general template that can be further modified to provide functionality. Specifically, the incorporation of a secondary overlayer provides a reactive NHS-group to which additional species displaying specific physical and/or electronic properties can be covalently bound. Extensive literature suggests that dipolar SAMs on ITO improves OLED performance compared to untreated ITO. Consequently, we incorporated two species with opposing dipole moments (Figure 44). NHS terminated surfaces S1 and S2 were exposed to a solution of either 10 mM 4-(aminomethyl) piperidine (F1, F2) or 10 mM 1H, 1H-perfluorohexylamine in IPA to produce surfaces F3 and F4. The N1s/In3d ratio in the XPS spectrum increases on conversion of surfaces S1 and S2 to surfaces F1 and F2, consistent with successful incorporation of 4-
(aminomethyl) piperidine into the NHS-terminated ITO. Similarly, the F1s/In 3d ratio in the XPS spectrum increases from surfaces S1 and S2 to F3 and F4.

**Figure 44.** Functionalization scheme and XPS spectra for the formation of the secondary reactive overlayer and terminal functional layers

To assess the performance of the designed molecular systems in facilitating or inhibiting anodic hole-injection in OLEDs, we compared them with traditional inorganic
and organic hole-injecting layers (Figure 45). Clean ITO (C1) and molybdenum oxide-modified ITO (C2) were selected as control substrates, while fluorinated silane and phosphonic acid surfaces C3 and C4 were chosen as comparison with previously described SAM-ITO hole injection systems. Finally, substrate C4, bearing densely populated amino groups, was selected as a hole-blocking monolayer. Substrate C2 was prepared by evaporating 5 nm of MoOx onto a clean ITO surface. Fluorinated silane (C3) and phosphonic acid (C4) were deposited on clean ITO as described above. Amino-terminated monolayer C5 was formed by exposing clean ITO to vapor phase amino-propyltrimethoxy silane at room temperature and atmospheric pressure for 16 hours.

![Figure 45. Control surfaces](image)

### 2.6.3 Controlling Hole Injection

The hole injection barrier at the ITO interface can be influenced through modification of the local work function of the ITO anode. More specifically, the barrier to hole injection can be significantly reduced by changing the work function of ITO using surface modification techniques.\[^{113c, 159}\] Increasing the work function of ITO should
enhance hole injection by reducing the energy barrier between the ITO anode and the HOMO of the NPB hole transporting layer.

Extensive work suggests that molecular structure, including molecular dipole and aliphatic chain length, plays a crucial role in determining the charge injection properties of the SAM-modified ITO\textsuperscript{138a, 155a, 160}. The work function, $\phi$, of ITO is defined as the difference between the vacuum level and the Fermi level and is equivalent to the energy required to move an electron from the Fermi level to vacuum. Modifying ITO with molecules with their dipole moment oriented towards the ITO anode shifts the vacuum level outside of the anode to a higher level; this higher vacuum level implies a higher effective work function, since the Fermi level remains unchanged. Thus, polar molecules can be used to increase or decrease the anode work function by appropriate design of dipole moments. The thickness of the SAM (i.e the length of the alkyl spacer) also plays a role in charge injection\textsuperscript{138a}. Specifically, longer alkyl chains lead to higher turn-on voltages and lower efficiency at equivalent current.

Since work functions are sensitive to molecular structure, we expected our modified ITO substrates to exhibit different work function values compared to untreated ITO and ITO bearing simple alkyl SAMs. Work functions were measured using ultraviolet photoelectron spectroscopy (UPS), and results are shown in Table 4. Work function values were calculated according to equation 1\textsuperscript{161}. The low photoelectron kinetic energy ($E_{\text{min}}$) defines the lowest energy electrons able to overcome the work function of the surface and the high kinetic energy ($E_{\text{max}}$) reflects the electron population around the Fermi level. The energy ($h\nu$) provided to the electrons was 21.2
eV. Overall, our data shown in Table 4 suggests that work functions can be modified significantly through modification of the ITO interface, ranging from 3.77 to 5.12 eV.

Table 4. Work function measurements

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E_{min}</th>
<th>E_{max}</th>
<th>Work Function (eV)</th>
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<tbody>
<tr>
<td>P1</td>
<td>9.43</td>
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<td>4.43</td>
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<td>P2</td>
<td>9.3</td>
<td>26.2</td>
<td>4.35</td>
</tr>
<tr>
<td>P3</td>
<td>9.63</td>
<td>26.2</td>
<td>4.63</td>
</tr>
<tr>
<td>S1</td>
<td>9.93</td>
<td>26.2</td>
<td>4.93</td>
</tr>
<tr>
<td>S2</td>
<td>9.63</td>
<td>26.2</td>
<td>4.8</td>
</tr>
<tr>
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<td>10.0</td>
<td>26.2</td>
<td>5.0</td>
</tr>
<tr>
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<tr>
<td>C4</td>
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<td>26.2</td>
<td>5.12</td>
</tr>
<tr>
<td>C5</td>
<td>9.63</td>
<td>26.2</td>
<td>4.08</td>
</tr>
</tbody>
</table>

**Work Function = E_{min} + hv – E_{Max} (1)**

The behavior of the modified ITO substrates demonstrate that varying degrees of electron-withdrawing character result in different dipole moments, and that different chain lengths result in varying work functions. Figure 46 shows the effective dipole moment and aliphatic chain lengths of each modified ITO substrate. The magnitudes of the dipole moments and molecular lengths agree well with the work function results.
shown in Table 4. Both properties influence work function values and are important design features in the construction of bilayered molecular systems. Specifically, a combination of short alkyl spacers and high dipole moments led to higher work functions. Substrate S3 shows the highest work function of any bilayered substrate, at 5.0 eV (Δϕ = 0.55). The work function change, Δϕ, from clean ITO (C1) is comparable to that of the control substrates C3 and C4, substrates modified with fluorinated alkyl chains commonly used to improve hole injection. While S3 does not display the highest dipole moment (45.89 D), it is the shortest bilayered molecular system considered (11.49 Å). Substrate F3 incorporates the highest dipole moment of any bilayered substrates (102.1 D) but also incorporates longer alkyl spacers compared to the other substrates, leading to a significantly lower work function (4.62 eV). Bilayered substrates F1 and F2 were modified to suppress hole injection. In both cases a terminal functional group decreases the magnitude of the overall dipole moment by adding electron donating properties (Figure 46). These substrates show the lowest work function values and are comparable to control surface C5 (4.08 eV) which also incorporates electron donating properties and a diminished dipole moment.
2.6.4 Device Fabrication and Performance

Bilayered molecular substrates were also incorporated into OLED devices. Device fabrication was carried out in the custom-built vacuum chamber with a base pressure of $\sim 3 \times 10^{-7}$ Torr. The OLED device was structured as follows (shown schematically in Figure 48): ITO/anode buffer layer (P, S, F, or C)/N, N’-dinaphthalene-1-yl-N, N’-diphenyl-benzidine (NPB) /Alq3/bathophenanthroline (Bphen)/LiF/Al. The deposition rate for organic layers was roughly 4 Å/s. The deposition rates for MoOx, LiF and Ag were typically 2, 0.5 and 6 Å/s, respectively. All thin film depositions were monitored by an Inficon IC5 controller. The device active area was 0.1 cm$^2$, which was defined by the overlapping area of the ITO and Al electrodes.
Device performance results are shown in Figure 48. As expected, device performance correlates with the work function measurements. Substrate S3 showed both largest work function and the lowest driving voltage ($V_{dr}$) at an applied current of 20 mA/cm$^2$. $V_{dr}$ is reduced from 10.3 V (clean ITO) to 5.9 V by grafting an electron-withdrawing NHS-diazirine moiety on a C8 monolayer. By contrast, functionalizing the substrate with an electron-donating amino-methylpiperidine moiety results in an increase of $V_{dr}$ to 14.6 V (F1) or 15.1 V (F2). Although substrates F3 and F4 contain more electron withdrawing species and therefore have a higher dipole moment, $V_{dr}$ remains above that observed for S3. This result is again in accord with work function measurements. Together, these results suggest that molecular length in combination with the dipole magnitude influence hole injection and device performance in a
predictable fashion. Clearly, the bilayered molecular system is an effective method by which to tune device performance.

Figure 48. Device Performance
2.6.5 Patterned electroluminescence

Modification of the ITO anode with functional organic molecules provides a simple means by which to affect device performance. Implementation of monolayer-based architectures in OLED devices requires large-area patterning of high-density patterns of meso- and micro-structures, and key processing parameters such as scalability and cost must be considered for manufacturing. Flexibility in pattern design is also crucial; feature geometry dictated by the device architecture and its total size must be accommodated. Traditional technologies such as photolithography and shadow mask deposition rely on expensive and area-limited methods to produce patterned thin-films. Moreover, these techniques are largely restricted to vacuum-based deposition of thin films and cannot be easily adapted to solution-processed materials.

Catalytic and reactive printing techniques are fast, accurate, inexpensive approaches to pattern preformed functional monolayers on diverse inorganic substrates. We have demonstrated the patterned attachment of organic and biological functionalities to a robust, albeit reactive, molecular system immobilized on a variety of substrates, including oxide-free silicon, germanium and ITO.\cite{120-121, 129b, 145, 162} SAM-based hole-injecting layers in OLEDs represent an ideal substrate system for such patterning technology. Such layers can be chemically patterned to define charge-blocking and injecting layers, eliminating the need to structure OLED components through shadow-mask deposition. As a demonstration of this promise, both catalytic and reactive printing techniques were used to demonstrate large-area micro-patterning
Catalytic microcontact printing was used to pattern ITO with molecules containing NHS-activated and free carboxylic acid groups. Such patterns were formed by bringing freshly prepared NHS-terminated ITO surfaces (P1, P2) into conformal contact with a catalytic stamp bearing 20 μm hexagonal features. The stamp, bearing covalently bound sulfonic acids, initiated pattern-specific hydrolysis of NHS-groups in regions of conformal contact, converting them into free carboxylic acids. After 1 minute, the stamp was removed and the surface was rinsed with isopropanol, dried under argon and analyzed by scanning electron microscopy (SEM) (Figure 49, top left). The size and shape of the replicated patterns were identical to those of the stamp, demonstrating high fidelity pattern transfer.
In contrast to catalytic printing, reactive microcontact printing relies on the diffusion of a reactive species adsorbed on a stamp to a surface bearing cognate reactive functionality (Figure 49, bottom left). This method was used to site-specifically immobilize molecules with different dipole moment values on ITO. Molecules were patterned that maximized both hole-injection blocking (4-(aminomethyl) piperidine: F1, F2) and enhancement (1H, 1H-perfluorohexylamine: F3, F4) into the hole-transporting layer. Piperidine- and NHS-terminated SAMs on ITO were first formed by bringing freshly prepared NHS-terminated ITO surfaces (S1, S2) into conformal contact with a 4-(aminomethyl)piperidine-inked PUA stamp bearing 20 µm hexagonal features for 1 minute at room temperature. Upon removal of the reactive stamp, surfaces (RA1, RA2)
were immediately rinsed with isopropanol and dried under argon. Surfaces bearing patterns of piperidine- and NHS-terminated SAMs were then reacted with a 10 mM solution of 1H, 1H-perfluorohexylamine in IPA for 30 minutes, producing substrates (RB1, RB2) with chemically distinct patterns, bearing molecules with opposing dipole moments.

Following catalytic and reactive patterning of hole-injecting layers, patterned substrates were used to prepare Alq3-based OLED devices (Figure 42). In these devices spatially segregated moieties with differing electronic structures create areas with different hole-injection efficiencies on the ITO electrode. These areas determine the extent of charge injection into the hole-transmitting (NPB) and light-emitting (Alq3) layers, creating patterns of bright and dim electroluminescence in the otherwise uniform Alq3 layer (Figure 49, right). Pattern contrast depends on hole injection efficiencies and should increase as differences in efficiency increase. This prediction was borne out by experiment (Figure 48). Substrate RB2, which contained a pattern of electron-donating and electron-withdrawing molecules with opposite dipole moments, showed much higher electroluminescent contrast than did sample CB1, which bore a pattern of electronically different molecules with aligned dipole moments. Our results demonstrate that contrast in patterned OLEDs is easily and predictably adjusted through modification of hole-injecting layers without the need for complex device structures and patterning techniques.
2.6.6 Conclusions

We have demonstrated that a bi-layered functionalization strategy is a promising approach towards the construction of tailored robust inorganic-organic interfaces with charge injecting or blocking capabilities. By tuning the hole injecting capability via the nature of a secondary reactive overlayer, we demonstrated that the efficiency and the luminance of a typical OLED device can be readily controlled with a monomolecular layer covalently attached to ITO. Moreover, the technique provides a simple route towards micro-patterned anodes with diverse hole-injecting or blocking character. The method is scalable and inexpensive and can be directly implemented in the production of low-cost illuminated signs and displays. Moreover, the flexibility of the soft-lithographic approach facilitates compliance to non-rigid or non-planar substrates for novel applications such as OLEDs on myriad surfaces. *In toto*, the approach offers a simple, inexpensive, and efficient route towards micro-structuring large areas on organic electronic devices, enabling more elaborate pixilation schemes.
3. Investigating non-covalent molecular associations using single molecule force microscopy

3.1 Overview

We previously demonstrated the formation of functional organic monolayers for applications in catalytic patterning, chemical and biological functionalization, and interfacial charge transfer. Here we extend our work on functional monolayers to study the thermodynamic parameters of molecular recognition events using atomic force microscopy (AFM). Non-covalent molecular association in aqueous milieu controls virtually all biological events. Specific recognition events between receptors and their cognate ligands include complementary strands of DNA, enzyme and substrate, antigen and antibody, lectin and carbohydrate, and ligands and cell surface receptors. These interactions facilitate the control genome replication and transcription, enzymatic activities, initiation of infection and immune, and many other biological responses. The specificity and selectivity of such molecular recognition events have become the basis for many bioanalytical and biomedical applications.

As a result, there is a need to understand the structure and function of receptor-ligand complexes at the single molecule level. Numerous biological systems interact through simultaneous molecular contacts. Such multiple interactions have unique collective properties, which are qualitatively different from properties of their monovalent constituents. The importance of the polyvalent interactions is most prominent in the interaction at biological interfaces when polyvalent ligands in solutions interact with surface-immobilized receptors (binding of cells to polyvalent molecules)
and when biological interfaces functionalized with ligand-receptor pairs adhere to each other (virus-cell, bacterium-cell, and cell-cell adhesion). The majority of studies on polyvalent and/or surface immobilized interactions rely on such techniques as isothermal titration calorimetry (ITC), fluorescence titration, stopped-flow fluorescence and circular dichroism (SFF, SFCD), surface-plasmon resonance (SPR), and atomic force microscopy (AFM). Although the solution-based techniques (ITC, SFF, SFDCD) have been extremely successful in investigating monovalent interactions, they are fundamentally flawed by the intramolecular contacts and formation of multimolecular aggregated when dealing with polyvalent systems. SPR spectroscopy represents a unique and widespread approach for studying interactions between surface-immobilized substrates and solution-phase ligands. However, due to its reliance on the dissociation off-rates and unique materials and optical requirements it is still somewhat limited in its generality, sensitivity and selectivity. Atomic force microscopy represents the only broadly recognized method for mimicking surface-surface adhesion and studying non-covalent interactions in surface-immobilized systems. The ability to spatially isolate and measure forces between individual molecules makes AFM an ideal technique for probing molecular recognition between two surfaces and has been widely applied towards studying non-covalent rupture forces in ligand-receptor associations in aqueous environments. [166]
3.1.1 Principles of AFM

3.1.1.1 Applications in imaging and molecular recognition

AFM provides a method to image, spatially manipulate, and measure forces between immobilized molecules. AFM is widely used as an imaging tool for surface characterization; providing unprecedented resolution with minimal sample preparation.\textsuperscript{[167]} Meyer and coworkers reported sub-nanometer resolution was achieved with observation of individual atoms and bonds within a single pentacene molecule.\textsuperscript{[168]}

AFM force spectroscopy allows measurement of piconewton ($10^{-12}$) forces associated with single molecules. Advances in instrument design and sample preparation have enabled the spatial manipulation of immobilized molecules with sub-nanometer accuracy. The effect of force on proteins and polymers has been investigated by anchoring one end of the molecule to the tip and the other end to the surface. Retraction of the surface or tip applies force to protein or polymer. This technique has been used to reversibly unfold proteins,\textsuperscript{[169]} break covalent bonds in polymers\textsuperscript{[170]} and even monitor the electrocyclic ring opening of a gem-dibromocyclopropane (gDBC).\textsuperscript{[171]}

Another application of force spectroscopy is the investigation of force driven dissociation of non-covalently bound complexes, called molecular recognition force microscopy (MRFM). This approach has provided fundamental insight into the molecular basis of biological processes and properties including DNA mechanics and cell adhesion.\textsuperscript{[164]} Recent work in our laboratory reported the development of an AFM based method for the surface evaluation of solution phase binding constants using galectin-3
(G3), a carbohydrate binding protein, and lactose as a model system.\[172\] The AFM approach is based on the competitive binding of immobilized G3 to immobilized lactose in the presence of increasing concentrations of soluble lactose. A binding isotherm described by a binding polynomial provided information on binding constants for the complexation of immobilized protein and immobilized ligand.

3.1.1.2 Instrument Design

The main components of the AFM are the cantilever and tip, the sample stage, and the optical deflection system consisting of a laser diode and a photodetector. AFM images are created by scanning in the x and y direction using a sharp tip mounted on a soft cantilever. The interaction forces between the tip and the sample provides a three dimensional topography of the surface. Force spectroscopy relies on measuring piconewton forces as the sample approaches the tip in the z-direction and retracts from it. In a typical force microscopy experiment, each element of a cognate ligand-receptor pair is immobilized on an AFM tip, or surface (Figure 50). The surface mounted on a piezoelectric scanner is driven towards the tip until a pre-determined contact force is achieved. The tip and surface are held in this position for a defined period of time (dwell time). Retraction of the surface applies force to the molecular assembly causing cantilever deflection and eventual force-driven dissociation (rupture) of the ligand-receptor complex. We refer to this approach-retract cycle as a “pull.” The cantilever deflection is detected by a laser beam focused on the tip of the cantilever and reflected to a position sensitive photodiode. A plot of cantilever deflection versus retraction distance is generated from which rupture forces and rupture lengths can be
determined. The extent of cantilever deflection is directly proportional to the force and can be converted using Hooke’s Law (F= -kd).

**Figure 50. Schematic representation of an AFM**

3.1.1.2 Experimental design: contact force and dwell time

The lifetime of the functionalized tip and surface can be affected by tip contamination or damage leading to a loss of sample activity and unreliable data collection. As a result, the data collection routine must be designed to preserve the activity of the fragile biomolecules. Achieving close proximity between the functionalized probe and surface is necessary to establish non-covalent interactions between the relevant species. Interaction between receptor-ligand pairs is enabled by
positioning the tip and sample at a separation distance less than the combined length of the tethered pair. Currently, many automated AFM instruments move the piezoelectric scanner back and forth between two positions without regard to tip-sample contact force. Too much applied contact force, however, can cause sample denaturation and non-specific adsorption onto the tip (Figure 51). These events lead to the formation of non-specific interactions that result in cantilever movement suggestive of a specific unbinding event. Efforts have been made to avoid the complications resulting from sample degradation through the use of ‘compression-free’ force spectroscopy, in which close proximity is achieved without tip-sample contact.\textsuperscript{[173]} A systematic study of the effect of compressive forces on the probability of binding, the specificity of binding, and observed rupture forces and length is described in chapter 3.2.

Figure 51. Schematic representation of potential results of tip-sample proximity

Another important experimental parameter is dwell time. Immobilized ligand-receptor pairs must be kept in contact long enough to facilitate binding. Berry et al.
reported increases in binding probability and adhesion energy of ocular mucins as dwell times increased under massive (nN scale) contact forces. Serpe et al. also reported an increase in the probability of reversible DNA polymer bridging with increasing dwell time.

3.1.1.2 Preparation of AFM tips and samples

Molecular recognition studies required functionalization of AFM tips and samples with the appropriate molecules. It is essential to immobilize each binding partner to the surface such that they can be physically manipulated and detected. Furthermore, the quality and reproducibility of the surface preparation techniques is important for acquiring accurate, reliable and reproducible data. The following criteria must be considered when designing an immobilization strategy: (1) a stable surface-bound molecular layer that can withstand exposure to aqueous environments and high temperatures, (2) appropriate molecular orientation and mobility to facilitate binding and (3) suitable surface density to observe sufficient interactions to be statistically relevant yet are indicative of single receptor-ligand forces.

The stability of the immobilized binding partners is an important consideration. Immobilization protocols generally require multiple procedures involving exposure to aqueous and organic solutions and potentially high temperatures and pressures. The immobilized species should be sufficiently robust to withstand the conjugation protocol. Furthermore, the binding of the molecules to the surface should be stronger than the intermolecular forces being studied. This can be achieved by using covalent bonds (1-4 nN) which are typically at least ten times stronger than the non-covalent
associations between the ligand-receptor pair.\textsuperscript{170, 174} Traditional surface chemistry techniques including chemisorption of thiols on gold or silanes on oxides are predominately used in MRFM. While these methods alleviate many drawbacks associated with non-covalent physisorption, they suffer from limited functionality and uniformity as discussed in chapter one. More notably, the Au-S bond is less stable than many other bonds including covalent C-C (607 kJ/mol) and even ionic NaCl (242.6 kJ/mol). Whereas Si-O bonds are thermodynamically stable (498.4 kJ/mol) they are susceptible to hydrolysis. Therefore reliable immobilization protocols for the stable, covalent functionalization of tips and surfaces is required.

Molecules immobilized at the tip-surface interface must retain the ability to form a bound complex. The immobilization method must allow the functional molecules to orient properly for binding; site directed coupling in which the molecule has a defined orientation is desired. Furthermore, the bound species should retain sufficient mobility so that they can freely interact with the complementary a molecule, which is achieved by attaching the molecules on the surface via a flexible molecular spacer. Poly (ethylene glycol) (PEG) spacers have proven to be suitable crosslinkers for force spectroscopy. These moderately hydrophilic polymers offer a high degree of conformational flexibility in aqueous media and unlike alkane linkers they are likely to remain extended, rather than folded in aqueous solution.\textsuperscript{175} Moreover, PEG based monolayer confer inert, non-fouling properties to the surface and are effective at resisting non-specific adsorption of proteins from solution for applications in MRFM.\textsuperscript{176}
The dependence of affinity on molecular orientation and linker length is well documented. A report from Vijayendran and Leckband suggested that the orientation of immobilized antibodies affects the binding affinity.[177] Another report demonstrated that homogenously oriented antibodies result in reproducible, reliable data when compared to randomly oriented antibodies. Several studies have reported on the effect linker length has on binding affinity in MRFM. Tethered molecules are restricted from freely sampling all space within the solvent; instead they are constricted near a surface to volumes defined by the effective radius of the molecule and its tether.[178] As a result, tethering and immobilization may cause the activity of the biomolecules to deviate from ideality.[163] Long, flexible tethers, however, alleviate the problems associated with tethering by allowing the binding partners to freely orient in the hemispherical, nano-confined environment.[179] A report from Hinterdorfer and coworkers demonstrated the immobilization of biologically active antibodies on AFM tips via PEG linkers. The flexible PEG tether was shown to influence the effective reach of the ligand-receptor interaction and therefore affect the on-rate for the binding event.[166d, 180] Antibodies have also shown higher antigen capture affinities when immobilized with longer, flexible PEG spacers.[181] This observation is attributed to greater availability of the antigen binding site due to the conformational mobility of longer spacers.[176a,176c]

In chapter 3.2 and 3.3 we demonstrate (1) the development of robust, reliable AFM data collection routines capable of isolating and characterizing specific bound interactions, (2) the effect of contact force, contact (dwell) time, and linker length on the observed interactions and (3) the development of tip and surface functionalization
strategies to achieve high density, oriented and stable receptor-ligand binding partners using lectin-carbohydrate and complementary DNA molecules.

### 3.2 The effect of compressive force, dwell time, and linker length on the unbinding profiles of specific protein-ligand complexes

Molecular recognition events are the basis for many biosensing technologies and considerable effort has been devoted to understanding the underlying thermodynamics characterizing non-covalent recognition events.[163, 182] Molecular recognition force microscopy (MRFM) has been widely applied to the study of protein unfolding and biomolecular association, both in aqueous and non-aqueous environments.[164, 169, 183] Non-covalent rupture forces between biotin and avidin,[166a, 184] complementary DNA strands,[166c, 185] antibody-antigen,[166d, 186] and protein-carbohydrate[166e, 172] complexes have been studied using AFM-based methods. Although the goal of MRFM is to study specific biomolecular interactions, the shape of unbinding profiles, the probability of binding during an approach-retract cycle, and degree of non-specific adhesion are highly dependent on a set of experimental variables including, but not limited to, the specific biomolecules used, [166a, 166c-e, 169, 172, 184-185] the nature and geometry of immobilization,[187] and composition of the solution in which molecular interactions take place.[188]

Specific interactions in MRFM arise from cognate ligand-receptor attractive forces between tip and surface. Non-specific interactions or adhesion arise from other attractive forces for which no true binding equilibrium expression can be written.

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5 The following work is under review at The Journal of Biophysical Chemistry B. For additional experimental detail see experimental section 4.5
Specifically, the introduction of soluble ligand should not drive the system toward the unbound state. Conversely, because specific interactions can be described by an equilibrium expression, the addition of soluble ligand should push the equilibrium toward the unbound state. Sufficiently high concentrations of soluble ligand should effectively block all specific tip-surface interactions.

An important goal of our research is to develop AFM methodologies that facilitate evaluation of thermodynamic parameters by characterizing specific non-covalent interactions between individual immobilized molecules. Such methodologies will facilitate both evaluation and interpretation of thermodynamic parameters and will allow us to better understand the behavior of interacting species, both at surfaces and in solution.

Our approach to the evaluation of surface-based thermodynamic parameters measures the fractional probability of the formation of bound complex between surface- and tip-immobilized binding partners during an approach – retract cycle: at equilibrium this probability is related to the free energy of binding. The determination of bound complex in turn relies on use of a force signature, derived from a force vs. extension plot, to differentiate bound complex from no complex during an approach-retract cycle; the formation of non-specific complexes during approach-retract complicates this analysis, obfuscating the true fractional probability of complex formation and resulting in an incorrect free energy. This differentiation is not straightforward: the forces required to rupture non-covalent interactions are weak (on the order of $10^{-1}$-10$^{-2}$ pN) but of similar magnitude to specific non-covalent forces,
and, operative over the same length scales as specific interactions. Clearly, the effective interpretation of data from molecular recognition force spectroscopy relies exquisitely on experimental protocols that both minimize the formation of non-specific complexes and effectively discriminate between specific and non-specific complexes.

In a typical MRFM experiment, the elements of a cognate ligand-receptor pair are immobilized to a cantilever tip and sample.\textsuperscript{164, 176b, 189} In the most commonly used configuration, the sample is driven toward the tip until a pre-determined contact force (pN to nN scale) is achieved. Retraction of the sample applies force to the molecular assembly causing cantilever deflection and, ultimately, force-driven dissociation (rupture) of the complex. A plot of force versus extension provides a force curve from which rupture force and length can be determined.

Although achieving physical proximity of receptor and ligand is obviously essential to facilitate binding, excessive force between immobilized species confounds data interpretation. Specifically, compressive strain has been reported to cause protein denaturation,\textsuperscript{190} and tip fouling.\textsuperscript{191} We also consider the effect of contact or dwell time between the immobilized ligand and receptor and the influence the linker has on their ability to bind. To date, no systematic study has been conducted on the effect of compressive force, dwell time, and linker length on binding probability and blocking efficiency. Here we use a specific lactose-G3 system to investigate the effect these parameters have on the probability of observing a rupture event, the normalized number of blockable rupture events per pull, and rupture force and length distribution.
in force *versus* extension plots. Two control systems with no known affinity, mannose-G3 and lactose-2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, were also studied.

### 3.2.1 Immobilization of lactose and mannose on silicon nitride AFM tips

Aminofunctionalization of silicon nitride AFM tips was accomplished using the method of Ebner *et al.*[192] Briefly, plasma-cleaned tips were subject to vapor phase deposition of 3-aminopropyltrimethoxy silane (APTMS). The resulting amine-terminated surface was conjugated to a heterobifunctional (oligoethylene) glycol linker ((Mal-PEG$_x$-NHS ester (x=2, 6, or 12) Quanta BioDesign)). Thiopentyl lactoside (2) and thiopentyl mannoside (3) were covalently bound to the surface by conjugate addition to the reactive maleimide functionality (Figure 51).[172] Thiopentyl mannoside, a molecule for which galectin-3 has no known affinity, was used as a negative control.

### 3.2.2 Immobilization of galectin-3 and KDPG aldolase on silicon substrates

Vapor deposition of freshly distilled 3-mercaptopropyltrimethoxy silane (MPTMS) onto plasma-oxidized silicon <111> samples provided thiol-terminated surfaces. Conjugate addition of surface thiols to maleimide 1[172] provided a chelation site for Ni$^{2+}$-mediated immobilization of His$_6$-G3 and his$_6$-KDPG aldolase via His$_6$-Ni$^{2+}$-NTA chelation. The terminal His$_6$ tag, routinely used for affinity purification of galectin-3 and KDPG aldolase, does not significantly affect solution phase affinities of either biomolecule.[172]

Figure 52 describes the molecular assemblies used for this investigation. The surface-immobilized carbohydrate binding protein, galectin-3, binds immobilized lactose
with an affinity of $\sim 6400 \text{ M}^{-1}$,\textsuperscript{172} and displays no significant affinity for mannose. At the C-terminus, a 137 amino acid carbohydrate recognition domain (CRD), is oriented away from the surface and a disordered N-terminal 120 amino acid collagen-like-repeat (CLR) is anchored by an N-terminal His$_6$ tag. Chelation-based immobilization of proteins has previously been utilized to anchor other His$_6$ tagged proteins to NTA terminated monolayers for AFM binding studies.\textsuperscript{164, 193} In crystalline form the G3 CRD is $\sim 4.5$ nm across its longest axis; in fully extended form, the CLR is $\sim 44$ nm in length. The total estimated tip to surface length of immobilized lactose bound to a properly folded CRD with fully extended CLR and linkers is $\sim 60$ nm.

Hexa-histidine tagged KDPG aldolase, a protein with no measurable affinity for hemiacetal forms of glucose, galactose, or lactose, was used as a negative control. KDPG aldolase binds the open chain form of 2-keto-3-deoxy-6-phosphogluconate and related straight-chain aldehydes.\textsuperscript{193b} Active KDPG aldolase exists in solution as a trimeric assembly of identical subunits 3.9 nm in length.\textsuperscript{194} The total estimated length, from tip to sample, of the KDPG aldolase construct is $\sim 16$ nm. Because of its decreased molecular length and lack of affinity for lactose, no specific interactions between KDPG aldolase and tip-immobilized lactose were expected.
Figure 52. Molecular assemblies for pulling experiments. Thiopentyl lactoside (2) and thiopentyl mannoside (3) were conjugated to an aminosilanized silicon nitride tip through a bifunctional poly ethylene glycol linker (NHS-PEGxMal). His$_6$-galectin-3 and His$_6$-KDPG aldolase were immobilized via N-terminal-His$_6$-Ni$^{2+}$ coordination to a covalently anchored NTA linker 1 on mercaptosilanized silicon <111>. A crystal structure of the G3 C-terminal CRD is shown. The disordered N-terminal G3 CLR with His$_6$ tag is also represented (red line).

3.1.3 Data Collection Routines

Our goal was to gain insight into the effects of compressive forces and dwell time on the data produced during molecular recognition force microscopy experiments. To achieve this goal we evaluated complex formation using three pulling routines. Minimal contact forces were achieved using a routine designated force minimization ($F_{\text{min}}$). This custom pulling routine uses the data recorded during the previous approach-retraction cycle to change the position of the sample surface so that minimal contact force is exerted on the tip during the next approach.$^{[195]}$ Initially, the surface was manually brought into close proximity with the tip by driving the samples towards the tip in 5 nm increments until observing the first sign of contact; tip deflection. This position determined the initial Z contact. The Z contact point was adjusted throughout the experiment to obtain average contact forces below 100 pN (Figure 53). To achieve
specific predetermined contact forces a force trigger routine was utilized. The sample was driven towards the surface until nominal contact force ($F_{\text{cnom}}$) trigger of 250 pN or 1000 pN was achieved, triggering first a one-second pause in sample movement then retraction of the sample at a rate of 200 nm s$^{-1}$. Dependence of binding probability on contact duration or dwell time was measured at contact times ranging from 0 to 20 seconds at the same approach and retract velocity (200 nm s$^{-1}$). In a typical experiment, 250 force curves were generated at 25 different locations on the sample surface.

![Histogram of observed contact forces ($F_c$) for the lactose-G3 system using the $F_{\text{min}}$ data collection routine to complete 250 approach-retraction cycles.](image)

**Figure 53.** Histogram of observed contact forces ($F_c$) for the lactose-G3 system using the $F_{\text{min}}$ data collection routine to complete 250 approach-retraction cycles.

### 3.2.4 Data analysis

The force curves generated during approach-retract cycles provided rupture forces and lengths for each bound interaction. The RMS of noise input of the system is 17 pN, therefore all rupture forces >35 pN were considered to be resolved above the noise. Unbinding events at extensions below 10 nm could not be defined by a binding
equilibrium and were considered non-specific adhesion. Removal of the collagen-like repeat domain has no effect on the binding behavior of the G3 carbohydrate recognition domain\textsuperscript{[196]}, and we assume that lactose-G3 affinity is not affected by extension of the CLR. Unbinding beyond the length of the fully unfolded system implies compression-induced processes that extend the overall length of the interacting system. It is conceivable that high Fc results in a “snow plow” effect as the cantilever tip is driven into and rasters across the surface. A likely result from this process is ablation of protein from the surface and tip fouling, through transfer of protein aggregates\textsuperscript{[197]} from sample to tip. It was also assumed that specific lactose-G3 binding is not possible if the CRD is denatured or partially unfolded.

In summary, force signatures at extensions between 10 and 70 nm at least potentially originate from the rupture of specific complexes while events at extensions >70 nm must originate from non-specific association.

3.2.5 The Probability of observing a rupture and blocking efficiency

The probability of observing a rupture ($\rho_{\text{bind}} = \text{number of rupture-containing pulls/total pulls}$) increased with increasing Fc, as did the number of rupture events per pull for the lactose-G3 system (Figure 54A). Under minimal compressive forces, single rupture events predominated with $\rho_{\text{bind}} = 0.33$. After blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4), $\rho_{\text{bind}}$ decreased to 0.09, a 73% blocking efficiency. Based on immobilized lactose-G3 affinity,\textsuperscript{[172]} 98% of surface sites should be blocked by soluble lactose. A compressive force of 250 pN resulted in $\rho_{\text{bind}} = 0.57$ for the unblocked
configuration. This probability was diminished to $\rho_{\text{bind}} = 0.44$ upon blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4); a 23% blocking efficiency.

Under the maximum compressive force used for this study (1000 pN) a significant increase in both probability of binding $\rho_{\text{bind}} = 0.66$) and the number of multiple ruptures were observed for the unblocked system. This probability was diminished to $\rho_{\text{bind}} = 0.62$ upon blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4); a 6% blocking efficiency.

In order to further investigate the effect of compressive forces on the integrity of the lactose-modified tip, a freshly prepared tip and sample were subjected to 50 approach-retract cycles using a force trigger of 1000 pN. The tip was then moved several micrometers laterally, and the $F_{\text{min}}$ pulling protocol was utilized to generate 250 force curves. This probability of binding – $\rho_{\text{bind}} = 0.09$ – was significantly lower than those measured in a typical $F_{\text{min}}$ experiment $\rho_{\text{bind}} = 0.33$), suggesting that repeated application of compressive forces on the order of 1000 pN degrades immobilized protein, rendering it unable to form specific complexes.
Figure 54. The probability of observing an unbinding event for the (A) lactose-G3, (B) mannose-G3, and (C) lactose-KDPG aldolase systems generated by $F_{\text{min}}$, force trigger ($F_{\text{cnom}} = 250 \text{ pN}$), and force trigger ($F_{\text{cnom}} = 1000 \text{ pN}$) pulling routines. The overall probability of observing an unbinding event (blue) for lactose-G3 increases with increasing contact force. The probability of observing two or more (red), three or more (green), and four or more events (violet) also increase with increasing contact force. The probability of observing an unbinding event for mannose-G3 and lactose-KDPG aldolase also increases with increasing contact force. All blocked experiments contained 10 mM soluble α-methyl lactose.

To better understand the effect of compressive force on the formation of non-specific complexes we repeated the entire protocol replacing thiopentyl lactoside (2)
with thiopentyl mannoside (3), a ligand for which G3 has no known affinity (Figure 54B). As expected, few bound interactions were observed for both unblocked and blocked $F_{\text{min}}$ experiments $\rho_{\text{bind}} = 0.08$). Under compressive forces of 250 pN $\rho_{\text{bind}}$ increased to 0.41. This probability was diminished to $\rho_{\text{bind}} = 0.31$ during blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4), a 24% blocking efficiency. Under compressive forces of 1000 pN $\rho_{\text{bind}}$ increased to 0.47 and was again diminished by roughly 20% to $\rho_{\text{bind}} = 0.38$ upon blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4). The number of bound interactions per pull also increased with increasing compressive force. Given the lack of affinity of G3 for mannose, we attribute all observed interactions in configurations using mannose ligands to non-specific events.

Similar trends were observed for the second control system, lactose-KDPG aldolase (Figure 53C). As expected, virtually no bound interactions ($\rho_{\text{bind}} = 0.004$) were observed using the $F_{\text{cmin}}$ protocol. On the other hand, $\rho_{\text{bind}}$ increased to 0.07 and 0.42 using force triggers of $F_{\text{cnom}} = 250$ pN and 1000 pN, respectively. The probability using a force trigger of $F_{\text{cnom}} = 1000$ pN was not significantly diminished ($\rho_{\text{bind}} = 0.40$) upon blocking with $\alpha$-methyl lactose (10 mM in PBS pH 7.4); a 5% blocking efficiency. The number of bound interactions per pull also increased. Given the lack of affinity of KDPG aldolase for lactose, we attribute all observed interactions to non-specific processes.

### 3.2.6 Morphology of force vs. extension plots

A major goal of molecular recognition force microscopy is to obtain detailed information about the nature of the interacting molecular systems from force curve
morphologies, particularly in the identification, isolation and structural analysis of numerous ligand-receptor complexes.\textsuperscript{[163-164,198]} In order to gain insight into how contact forces affect force-extension curves, we carried out a systematic study using the three data collection routines previously described\textsuperscript{[195]} (F_{\text{min}} and force triggers F_{\text{cnom}} = 250 \text{ pN} and F_{\text{cnom}} = 1000 \text{ pN}) to probe the effect of compressive force on the morphology of force curves generated using the cognate binding system lactose – G3 and negative control molecular systems comprising lactose – KDPG aldolase and mannose – G3.

Figure 55 depicts typical force versus extension plots displaying rupture events for the lactose –G3, lactose – KDPG aldolase, and mannose – G3 systems. Evidence of binding can be seen in all retraction curves (black line). Force signatures generated with the lactose – G3 system using F_{\text{min}} were generally similar in morphology to those shown in Figure 55A. As described previously, single rupture events predominated, with \~73\% of all observed ruptures attributed to specific binding and can be described by an equilibrium expression. Although we concluded in the previous section that non-specific binding was present in \~77\% and \~94\% of all force curves that contained evidence of unbinding events using F_{\text{cnom}} = 250 \text{ pN} and 1000 \text{ pN}, respectively, the majority (>50\%) of these force curves are indistinguishable from those generated using the F_{\text{min}} protocol (Figure 55B and 55C). These results suggest that force curve morphology alone is insufficient to specific from non-specific rupture events.
Figure 55. Representative force distance curves for the lactose – G3, lactose – KDPG aldolase, and mannose – G3 systems generated by Fmin, force trigger (F_{cnom} = 250 pN) and force trigger (F_c = 1000 pN) pulling routines. Approach curves (green) and retraction curves (black) with unbinding events (blue circles) are depicted.

No specific bound complexes should arise during lactose – KDPG aldolase interaction. In the event, however, several force signatures for this system showed curves with morphologies similar or identical to those for specific lactose-G3 interactions. Although ruptures at short extensions (> 10 nm) were frequently observed when utilizing the F_{min} and F_{cnom} = 250 pN pulling routines (Figure 54D and 54E), higher compressive forces (F_{cnom} = 1000 pN) produced force curves identical to those of lactose – G3 (Figure 54F). Additionally several (presumably) non-specific mannose – G3 rupture events showed curve shapes identical to those arising from specific lactose – G3 force signatures (Figure 55G, 55H, and 55I). These results further suggest that force curve morphology alone is insufficient to determine the specific nature of a rupture. Thus, the mere presence of an unbinding event in the force curve does not implicitly arise from
the rupture of a specific bound interaction but may instead arise from non-specific adhesion.

### 3.2.7 Rupture force distributions

The distribution of rupture forces for the lactose-G3 system is unimodal with the most probable rupture force equal to 60±28 pN for all applied contact forces (Figure 55A), in good agreement with previously reported forces of 50±10 pN at equivalent retraction rates. The histogram profiles of Figure 56A are similar regardless of applied Fc in all respects except frequency. Thus, rupture force histogram profile alone is not indicative of specific binding. If conclusions concerning the specificity of the interacting species were made from the rupture force histogram alone, we would assume no difference between data collected using \( F_{\text{min}} \) and the force trigger (\( F_{\text{cnom}} = 1000 \)) other than \( p_{\text{bind}} \). However, there is clearly a difference in blocking efficiency and therefore the occurrence of specific interactions (Figure 56A). Thus, blocking experiments are essential for determining the specificity of the observed molecular interactions.

In contrast to the lactose – G3 system, the presumably non-specific interactions between mannose and G3 and between lactose and KDPGal aldolase produced a broader distribution of rupture forces (Figure 56B and 56C). The frequency of larger rupture forces increases significantly with increasing Fc.
Figure 56. Histogram of rupture forces observed for (A) lactose – G3, (B) mannose – G3 and (C) lactose – KDPG aldolase using force minimization (blue), and force triggers of 250 pN (red) and 1000 pN (green).

3.2.8 Rupture length distributions

The total extended length of a lactose-G3 molecular system containing a folded and functional CRD is ~60 nm (Figure 52). Interfacial G3 aggregation presumably adds to
the length of the system. Rupture lengths were plotted for all applied contact forces for the lactose – G3, mannose – G3, and lactose – KDPG aldolase systems (Figure 57). All ruptures at extensions less than 10 nm were considered non-specific adhesion.

Under minimal contact forces the rupture length histogram profile for the G3 – lactose interaction is unimodal with a distribution centroid near 50 nm. Shorter rupture length bins become more populated using force triggers of 250 pN and 1000 pN. Rupture length distributions for non-specific mannose – G3 and lactose – KDPG aldolase interactions decrease in frequency at longer lengths. The rupture length distribution for lactose – KDPG aldolase is shifted to shorter lengths, presumably due to the shorter molecular length of the interacting system (~16 nm) and the lack of an extendable collagen-like repeat.

Clearly, an increase in rupture frequency is not necessarily related to an increase in specific binding events. Again, the biophysical significance of ruptures cannot be fully understood on the basis of rupture length histograms alone. Blocking experiments remain essential for the interpretation of cantilever movement.
Figure 57. Histogram of rupture lengths observed for (A) lactose – G3, (B) mannose – G3 and (C) lactose – KDPG aldolase using force minimization (blue), and force triggers of 250 pN (red) and 1000 pN (green).
3.2.9 Effect of dwell time and linker length on binding probability

In addition to contact force, dwell time was investigated for the G3-lactose system at minimal contact forces ($F_{\text{min}}$). Figure 58 shows that increasing the contact time between tip and surface from 0 to 20 seconds increases the probability of observing an unbinding event from 0.2 to 0.9. The addition of blocking agent (10 mM β-methyl lactose) resulted in a 72% decrease in binding probability at a dwell time of 1 second (figure 58A). This blocking efficiency remained approximately the same at all dwell times, suggesting that only specific binding events increase with contact time. As an additional control, the non-specific KDPGal-lactose system was investigated at dwell times between 0 and 20 seconds (figure 58B). There was a 20% increase in observing a rupture between 0 and 20 seconds, however, the probability does not exceed 0.02; further suggesting that non-specific events do not increase with increasing contact times.

Figure 58 and 59 shows both a transient phase where $P_{\text{bind}}$ increases with time and also a steady phase where $P_{\text{bind}}$ reaches equilibrium. To further elucidate this behavior, we collected two additional time points in Figure 59 (t=3 and 7 seconds). The system appears to come to equilibrium after 1 second but then the binding probability again increase at 7 seconds. Overall, the results reveal an almost saturable relationship between dwell time and binding probability, providing insight into how extrinsic experimental parameters affect the interaction between immobilized moieties.
Figure 58. Histogram of binding probabilities at increasing dwell times. The probability of observing 2 or more events (red) 3 or more (green) and 4 or more (violet) also increase. Blocking experiments contained 10 mM soluble β-methyl lactose. A) G3-lactose B) KDPG-lactose control system.
In addition to measuring specific binding events between lactose and G3 as a function of contact time, we also altered the length of the PEG linker (x) between 2 and 12 oligoethylene units (x=2, 6, 12). As previously mentioned, the binding moieties should be attached to their corresponding surfaces with sufficient flexibility and mobility such that can freely interact with the complementary molecule to result in an observed binding event. Increasing the PEG spacer increases the total observed binding probability at each dwell time, suggesting that the longer tethers facilitate binding through increased conformational flexibility (Figure 59).

Figure 59. Binding probability observed for the G3-lactose system versus time with different PEG spacers. The total probability of observing an unbinding event increases with time for all linker lengths (blue). The probability of observing 2 or more events (red) and 3 or more also increases.

The dependence of rupture force on dwell time and linker length was also measured. The histogram of rupture forces exhibits a dominant peak at each dwell time and linker length corresponding to a most probable rupture force of 60 pN (Figure 60).
The frequency of rupture forces around 80 and 100 pN increases with contact time, suggesting that dwelling longer promotes the formation of multiple interactions or stronger bonds.

Figure 60. Binding probability observed for the G3-lactose system versus time with different PEG spacers. The total probability of observing an unbinding event increases with time for all linker lengths (blue). The probability of observing 2 or more events (red) and 3 or more also increases.
In conclusion, we have successfully demonstrated that contact force dramatically affects molecular recognition force microscopy experiments. For cognate binding partners, lactose and G3, minimal contact forces introduced minimal unblockable artifacts into data sets. Increased contact forces resulted in increased probabilities of binding concomitant with decreased blocking efficiencies, i.e. a greater number of unblockable artifacts resulting from greater contact forces. For molecular systems with no known affinity, mannose – G3 and lactose – KDPG aldolase, we demonstrated that increased contact forces resulted in non-specific ruptures that were similar or identical in morphology, rupture force, and rupture length to those of specific lactose-G3 interactions. Thus, the biophysical significance of rupture events cannot be fully understood on the basis of force curve shape, rupture force distribution, or rupture length distribution alone; blocking experiments remain essential for the interpretation of cantilever movement. Clearly, it is essential to design data collections routines that minimize contact forces to ensure that ruptures originate from specific, blockable interactions.

Furthermore, we demonstrate the effect of dwell time and PEG tether length on the observed binding probability between immobilized lactose and G3. Increasing dwell time results in an almost saturating binding probability; suggesting that longer contact times increases the frequency of observing a binding event. Longer, flexible tethers also result in an increase in binding probability between lactose and G3. The increase in conformational flexibility of the bound moieties likely facilitates their ability to bind.
3.3 A general and efficient cantilever functionalization technique for AFM molecular recognition studies

As was discussed in Chapter 3.2, AFM has emerged as a broadly applicable tool for the investigation of intermolecular interactions between ligands and receptors in both biological and abiological systems.\cite{164} Because interactions are probed between single species, ensemble averaging is absent and the reproducibility and interpretation of intermolecular force measurements is critically dependent on the stable and uniform adsorption of specifically oriented binding partners, on both the flat surface of a stage and the sharp tip of a cantilever. Although simple adsorption is frequently utilized, covalent immobilization is the only approach that meets the stringent requirements of stability and orientation.

Cantilever tips are most commonly constructed of silicon or silicon nitride: because of its mechanical properties, silicon nitride is generally preferred for molecular recognition studies.\cite{199} Surface modification of silicon nitride is most commonly achieved using the reaction of organic silanes with a native oxide layer.\cite{192} Although such monolayers form readily using either solution or vapor phase deposition, several inherent limitations, including polydisperse surface densities, the susceptibility of Si-O bond to hydrolysis and the formation of polymeric aggregates on surfaces, diminish their attractiveness for AFM studies.\cite{176b, 200} Immobilization techniques that facilitate attachment of a wide variety of organic and biological molecules, provide uniform surfaces, both with regard to the orientation of the immobilized species and the density.

\footnote{The following work was published in Biopolymers in 2012, vol. 97, pages 761-765. For additional experimental detail see experimental section 4.6}
of epitopes, and that withstand prolonged exposure to aqueous environments over a range of pH values would be of great value to the field of molecular recognition force spectroscopy.

Monolayers covalently bound to silicon or silicon nitride via Si-C bonds are considerably more stable than silane monolayers and have been shown withstand prolonged exposure to aqueous environments and prevent oxidation of the underlying silicon nitride.\textsuperscript{[201]} A common approach to the formation of such monolayers involves hydrosilylation, during which an unsaturated bond inserts into a silicon-hydride bond.\textsuperscript{[63, 70, 121, 199, 201b, 202]} Hydrosilylation of alkenes on silicon cantilevers has been reported for applications in high resolution imaging and sensing.\textsuperscript{[201a, 203]} Molecular recognition studies on soft or fragile samples, however, require a silicon nitride cantilever with a low spring constant so that the applied force is small.\textsuperscript{[204]} Here, we report the formation of stable, monodisperse, highly oriented monolayers highly suitable for force-based molecular recognition studies using silicon nitride cantilevers. The monolayers are formed through hydrosilylation of hydrogen-terminated silicon nitride AFM probes using a protected \(\alpha\)-amino-\(\omega\)-alkene; this functionalized hydrosilation substrate facilitates subsequent conjugation of biomolecules. The resulting monolayers are robust, bound to the underlying surface by a strong Si-C bond, completely uniform with regard to both epitope density and substrate orientation, and highly suitable for force microscopy studies.
3.3.1 Detection of lactose-G3 binding

To demonstrate the approach, we modified silicon nitride probes with oriented lactose in four sequential steps (Figure 56). Boc-terminated amines were immobilized on silicon nitride probes using a photochemical insertion of alkene 1 into H-terminated silicon nitride; deprotection under acidic conditions yielded an amino-terminated surface. The spacing of silicon atoms in the underlying substrate produces a disordered monolayer, and methylene surface area is presumably exposed to solvent. To prevent non-specific adsorption of biomolecules to this surface area, the amino-terminated substrate was reacted with heterobifunctional PEG linker (2). Finally, incubation of the surface with thiol (3) yielded a lactose-modified surface via conjugate addition to the pendant maleimide. The resulting probes were imaged using SEM to confirm that the tip morphology and radius of curvature remained unaffected by the protocol; details and images are included in the experimental section.
Figure 61. Top: Hydrosilylation and subsequent modification of silicon nitride AFM probes. Bottom: XPS spectra of Boc-modified and amino-terminated monolayers on silicon nitride.

XPS analysis of silicon nitride surfaces confirmed successful modification of silicon nitride with Boc-protected α, ω-amino alkenes. XPS spectra of the Boc-terminated substrate showed an increase in carbon compared to blank silicon nitride and also showed a C1s peak at 287 eV resulting from the carbamate carbonyl (Figure 61). XPS spectra of deprotected substrates lacked this peak and showed a diminished carbon signal that correlates with the calculated carbon concentration for completely
deprotected surface. Goniometry analysis in water of the t-BOC protected amine substrate ($\theta_{\text{adv}} = 75^\circ$, $\theta_{\text{rec}} = 65^\circ$) is reflective of the hydrophobic nature of the t-BOC protecting group. Water contact angle measurements of the deprotected amino-terminated substrate ($\theta_{\text{adv}} = 68^\circ$, $\theta_{\text{rec}} = 45^\circ$) are lower due to the decrease in hydrophobicity and protonation of the amino groups by TFA. Both the value of the contact angle and hysteresis are consistent with a disordered monolayer resulting from the Si-atomic spacing. Contact angle measurements are consistent with those previously reported.[202]

We have previously reported a method for patterning biological species on passivated silicon, and we utilized a similar strategy here to immobilize His$_6$-tagged galectin-3 (G3) on silicon (Figure 62).[121] The technique simultaneously protects silicon from degradation in biological environments and provides chelation sites for the uniform, highly oriented immobilization of proteins. H-terminated silicon was chlorinated with PCl$_5$ then alkylated with propenyl Grignard (4). The alkylated substrate was then reacted with diazirine (5) to yield an NHS modified monolayer (S4), which was sequentially reacted with lysine-N,N-diaceitic acid (6), then incubated with Ni$^{2+}$ and histidine tagged G3 providing the final surface (S6).
Figure 62. Modification of H-terminated silicon with G3 via alkylation and carbene addition.

The final molecular assemblies of Figures 61 and 62 were used for force spectroscopy experiments on a custom 3-axis AFM using a previously reported force minimization protocol.\(^{[195]}\) The surface-immobilized carbohydrate binding protein G3 binds immobilized lactose with an affinity of \(\sim 6400 \text{ M}^{-1}\). At the C-terminus, a 137 amino acid carbohydrate recognition domain (CRD), is oriented away from the surface and a disordered N-terminal 120 amino acid collagen-like-repeat (CLR) is anchored by an N-terminal His\(_6\) tag. When force is exerted on a bound G3-lactose complex the total estimated length, from tip to surface, of immobilized lactose bound to a properly folded CRD with fully extended CLR and linkers is \(\sim 60\) nm.

Our goal was to develop a general functionalization method for force spectroscopy that provides robust, monodisperse, highly oriented molecular systems that are stable to aqueous environments and do not degrade under mechanical strain.
To evaluate our surfaces against these requirements, 200 force/distance curves were generated at each of 20 different locations across the sample surface. All rupture forces >35 pN were considered resolved above noise. Unbinding events at extensions below 10 nm were considered non-specific adhesion. Our previously reported data collection routine was utilized: this approach minimizes contact forces and ensures that ruptures originate from specific, blockable interactions. Only force/distance curves with contact forces between 40 and 300 pN were considered.\textsuperscript{195}

Figure 63 depicts a representative force distance/curve for a bound G3-lactose interaction. The probability of observing a rupture event ($\rho_{\text{bind}} = \text{number of rupture-containing pulls/total pulls}$) over 200 pulls was 0.41. To confirm that the observed binding events represented lactose – G3 interaction, soluble β-methyl lactose (10 mM in PBS pH 7.4) was introduced into the AFM liquid cell to competitively inhibit interactions between surface-bound G3 and immobilized lactose. Upon introduction of this blocking agent, $\rho_{\text{bind}}$ decreased to 0.10. Previously we investigated the probability of binding between G3 and lactose immobilized through traditional silane-based methods. Here, we were able to achieve a higher binding probability ($\rho_{\text{bind}} = 0.41$ vs. 0.33), presumably reflective of more uniform surfaces, and similar blocking efficiency.
3.3.2 Detecting DNA hybridization

We extended this surface methodology to another biological system to show versatility and eventually the investigation of multivalent binding thermodynamics. DNA, as the primary information storage biomolecule, is involved in myriad biological processes and represents a relatively simple system that exhibits high affinity, specificity, and stability through its base pair’s hydrogen bond combinations. As such, we decided to investigate binding of immobilized ssDNAs using our novel competitive inhibition AFM method and the previously described immobilization technique.

The ssDNA molecular system was selected for MRFM study because it is fundamental to biology as the primary storage mechanism for genetic information, and
its interactions have been extensively investigated. The double stranded helix is a highly stable and specific complex mediated by hydrogen bond pairings between the nucleotide bases adenine and thymine, and guanine and cytosine and through base stacking interactions. Because the stability and affinity of dsDNA is dependent on both length and sequence, we were able to design a sequence that would be highly amenable to MRFM experimentation. The seven nucleotide sequence 5’-CAA AAA G-3’ and its complement 5’-CTT TTT G-3’ were selected because the double stranded complex exhibits a moderate binding affinity ($K_A = 1.8 \times 10^4 \text{ M}^{-1}$) that can be detected in a monovalent system yet improved through multivalency. Additionally, the short, seven-member sequence allows us to preclude self-association and more accurately neglect partially bound states, i.e. we can assume a binary binding profile. When force is exerted on the bound dsDNA complex the total estimated length, from tip to surface is estimated to be ~30 nm. For DNA blocking experiments, a non-thiolated 7-mer sequence (5’-CTT TTT G-3’) complementary to the surface-bound DNA was dissolved in 100 mM Na$_3$PO$_4$, 100 mM NaCl, pH 7.4.

Stable surface immobilization of DNA on silicon nitride cantilever tips (Bruker, $k_c = 120 \text{ pN/nm}$) and silicon <111> surfaces was achieved using covalent Si-C bond formation and subsequent linker chemistry to attach the oligomers. We followed the protocol outline in Figure 61 to immobilize the maleimide terminated PEG$_{24}$ linker. Covalent attachment of DNA to the maleimide terminated monolayers on both tips and surfaces was achieved through the addition of thiolated ssDNA. DNA with 1-hexanethiol conjugated to its 5’ end was purchased as a disulfide from Integrated DNA Technologies.
The sequence 5’-CAA AAA G-3’ and its complement 5’-CTT TTT G-3’ were immobilized to surfaces and tips, respectively. The disulfide DNA was reduced to the free thiol, prior to surface incubation, with tris (2-carboxyethyl) phosphine (TCEP) in DNA elution buffer (10 mM Tris, pH 8.0). In order to reduce the number of ligand-receptor interactions per approach-retract cycle observed with the hydrosilylation protocol, the monolayer surface density of DNA molecules was diluted. This was achieved by replacing a percentage of 25 µM thiolated DNA with 2-mercaptoethanol (BME). Reduction of both thiolated DNA and BME was performed prior to surface attachment using TCEP.

Figure 64. Schematic representation of the (A) surface and (B) tip modification.

The resulting molecular assemblies shown in Figure 64 were used for MRFM experiments using our previously reported force minimization protocol.[189, 195] Unblocked experiments resulted in binding probabilities consistently greater than 0.90. Introduction of DNA blocking agent at concentrations of 1.4x10^-2 M and 5.5x10^-2 M only
reduced $P_{\text{bind}}$ to 0.92 and 0.30, respectively. Examination of rupture force histograms revealed forces centered around 800 pN (Figure 65A). The inability to effectively block specific interactions at high concentrations of blocking solution combined with high rupture forces suggests the formation of multiple interactions between tip and surface per approach retract cycle.\textsuperscript{[164, 180a]} The hydrosilylation protocol, which relies on UV-mediated attachment of alkenes to H-terminated silicon, has been estimated to cap approximately 50% of the surface silicon atoms resulting in high surface density of functional species.\textsuperscript{[73]} Conventional silanization protocols, on the other hand, have estimated that vapor deposition of aminosilanes results in approximately $1.6 \times 10^3$ reactive amines/µm\textsuperscript{2}; 0.3% coverage on silicon oxide.\textsuperscript{[192]} Because the hydrosilylation protocol results in a higher density of reactive amines/µm\textsuperscript{2} compared to the silanization protocol, coupled with the formation of a more ordered and stable underlayer, the functionalization through hydrosilylation increases the fractional binding probability for immobilized molecular systems in MRFM. Achieving sufficiently high surface densities on both tips and surfaces is necessary to observed binding interactions in MRFM. However, in order to probe only single molecule interactions the number of functional moieties should be reduced by tuning the coverage through surface dilution.
In the final step of surface modification, thiolated DNA is conjugated to the maleimide terminated monolayer. Dilution of the surface immobilized DNA was achieved through mixed monolayer formation using 1% 2-mercaptoethanol (BME). The diluted system exhibited a reproducible maximum $P_{\text{bind}}$ of 0.55 that was blockable, with $1 \times 10^{-2}$ M soluble ligand down to 0.07; an 87% blocking efficiency (Figure 66). Rupture force histogram analysis revealed forces centered around 60 pN (Figure 65B). These forces are more indicative of interactions approaching the single molecule domain. We
calculated the monomeric rupture force for immobilized DNA complex to be 40 pN using a power spectrum method (see details in experimental section). This method was previously reported to determine the monovalent rupture force of lactose-G3.\textsuperscript{[172]}

![Figure 66. Typical rupture for observed for complementary DNA system and the corresponding binding probabilities with and without the addition of blocking solution.](image)

3.3.3 Conclusions

In summary, we have demonstrated a stable and efficient covalent immobilization of lactose and DNA to a silicon nitride AFM cantilevers using hydrosilylation chemistry. Our work provides a general and operationally simple method by which to covalently immobilize a wide variety of organic and biological molecules with complete control over both epitope density and orientation, and eliminates the need for optimization of each individual system. The technique utilizes robust Si-C bonds and, in conjunction with our previously reported technique for the formation of
stable, highly oriented monolayers of histidine tagged proteins on silicon, simplifies both the construction and interpretation of AFM-based molecular recognition studies. Furthermore, the application of the presented MRFM protocols will facilitate the investigation of the thermodynamic parameters of complex multivalent systems and surface-immobilized biological molecules. The presented monovalent DNA system is easily tunable to a multivalent system and is a promising way to investigate the multivalent binding thermodynamics of biomolecular complexes.
4. Experimental

4.1 Catalytic μCP on oxide-free silicon

4.1.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH₂O) were filtered through a 0.2 µm filter before use. SEM images were recorded on the FEI XL30 SEM-FEG microscope detecting secondary electrons at 6 cm working distance. GFP-patterned substrates were imaged using a Zeiss Axio Imager widefield fluorescence microscope with a mercury arc lamp (Zeiss HBO100). Images were taken using a green filter cube set and a 40x/0.75 DIC objective with 4 second exposure time. AFM images were obtained using contact mode lateral force microscopy on the Veeco D3100 microscope using Veeco DNP-S silicon nitride probes with a 0.32 N/m spring constant. Water contact angles were measured on the Rame-Hart NRL contact angle goniometer. XPS spectra were recorded on the Kratos Axis Ultra XPS spectrometer equipped with a mono-Al X-ray source. The plasmid pEGFP was purchased from BD Biosciences. Restriction enzymes, Antartic phosphatase and T4 Ligase were purchased from New England Biolabs. BL21 (DE3) singles, pET30b and Paint Pellet were obtained from Novagen. XL10 Gold cells were purchased from Stratagene. Qiaquick gel extraction kit and Miniprep kit were purchased from Qiagen. Dialysis tubing and Bradford Plus reagent was purchased from Pierce. Nickel chelating resin was manufactured by GE Healthcare. The lysis was performed by the Emulsiflex C-5 high pressure homogenizer. The protein was purified on the Amersham Bioscience AKTA FPLC.
4.1.2 Master Fabrication

Si/PMMA and Si/SiO₂ masters were prepared as previously described.[120b]

4.1.3 Polyurethane acrylate (PUA) monomer synthesis and stamp fabrication

PUA monomer was prepared according to a previously published protocol (Figure 67).[44] Isophorone diisocyanate (1) was reacted with 0.5 equivalents of PEG (2) for 3 hours in the presence of a urethane polymerization catalyst. PEG (2) was added dropwise slowly at 50°C. After 1 hr., the reaction temperature was increased to 65 °C and then to 70 °C for the remaining 1 hr. The resulting intermediate (3) was then reacted with an isomeric mixture of hydroxypropyl acrylate (4). Acrylate (4) was added slowly over 40 min at 70°C and the reaction proceeded for 3 hr. Target monomer (5) was diluted by 30% trimethylolpropane ethoxylate triacrylate to reduce the viscosity while continuously stirring for 20 min at 60 °C. The temperature was decreased to 50 °C and photoinitiators (1-Hydroxycyclohexyl phenyl ketone and 2-Hydroxy-2-methylpropiophenone) were added and the mixture was stirred for an additional 10 min. The resulting solution was polymerized between two glass microscope slides or between a glass slide and a Si/SiO2 master for 2 hr. under UV light (365 nm) at r.t.
4.1.4 Stamping Protocol

A corresponding polyurethane-acrylate stamp was placed on the top of the NHS-modified substrate at room temperature for a specific amount of time with no external load to hold them together. After the reaction the stamp and the substrate were separated. Following the reaction the substrates were rinsed with isopropanol and dried with filtered argon. The stamp was rinsed with EtOH, H₂O, EtOH, dried with filtered argon, and kept at room temperature before the next application.

4.1.5 NHS-modified diazirine synthesis

NHS-modified diazirine was prepared from (4-bromophenyl) methanol in 9 steps following the previously published protocol.²⁰⁶

4.1.6 GFP preparation and purification

pET30b-EGFP plasmid was prepared using standard DNA techniques.²⁰⁷ The final construct encodes EGFP with an in-frame N-terminal hexahistidine tag. EGFP-pET30b Plasmid DNA was transformed into BL21 (DE3) cells and a single colony was
used to start overnight cultures. One liter of TB and kanamycin were inoculated with the overnight culture and induced with 1 mM IPTG at OD$_{600}$=0.6. Cells incubated for an additional 3 hours. Harvested cells were lysed by multiple passages over the Emulsiflex between 15,000 and 20,000 psi. Following centrifugation, the supernatant containing His$_6$-GFP was loaded onto a pre-equilibrated column containing charged nickel chelating resin. The column was subjected to a wash in lysis buffer and a linear gradient of increasing imidazole. Fractions were analyzed by SDS-PAGE and those exhibiting EGFP were pooled. The EGFP was subjected to four rounds of dialysis, the first containing 5 mM EDTA, 50 mM sodium phosphate pH=7.6, and 300 mM NaCl and the remaining containing all components except for the EDTA. The protein concentration was quantified by an Edelhoch assay.

4.1.7 Si (111) functionalization

A silicon (111) chip (~1x1 cm) was washed with EtOH and dH$_2$O and blow-dried with filtered argon to remove dust particles. The substrate was then oxidized in Nano strip solution (cyantek Inc.) at 75°C for 15 min to remove all organic contaminants. The native oxide film was etched from the surface with 5% aq. HF at room temperature for 4 min yielding oxide-free polycrystalline surface displaying Si-H bonds. The silicon chip was transferred from the HF solution to a saturated phosphorus pentachloride solution in chlorobenzene containing small amount of benzoyl peroxide (0.1 % m/v) and heated in this solution for 1 hr. at 105°C. The chlorinated substrate was quickly washed with chlorobenzene, dried under a stream of argon, and immediately transferred into a 0.5 M
THF solution of 1-propenylmagnesium bromide. Subsequently, the surface was reacted in a sealed vial with the Grignard at 135°C for at least 24 hr. to produced stable close-packed propylene-terminated SAMs on oxide-free silicon. After the reaction, the surface was thoroughly rinsed with EtOH and dichloromethane (DCM) and dried first under a stream of argon and then on a hot plate at 75°C for 10 min. The propylene-functionalized chip was subsequently covered with ~ 50 µL of a 0.1 M solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate in CCl₄ and reacted under UV light (λ=254 nm, Intensity=4400 microwatts/cm² at 1.9 cm, lamp-surface distance=7.5 cm) for 1 hr. at room temperature. After the reaction, the NHS-functionalized surface was thoroughly washed with dichloromethane and isopropanol, dried under a stream of argon and used immediately.

4.1.8 Hydrolysis of NHS substrates with featureless and patterned catalytic stamps

Freshly prepared NHS-modified substrates were reacted with flat SO₃H modified polyurethane-acrylate stamps at room temperature for 5, 15 and 30 min. Following the reaction, the substrates were rinsed with isopropanol and dried with filtered argon. As a control experiment, an additional NHS modified substrate was reacted with an inactive PUA stamp which did not contain sulfonic acid moieties at room temperature for 30 min. To prepare a completely hydrolyzed reference substrate, we reacted an NHS-modified substrate with 1 M HCl solution at room temperature for 30 min. Following the reaction, the substrate was rinsed with dH₂O and IPA and dried under filtered argon.
Prepared substrates were analyzed by XPS for changes in carbon and fluorine concentrations.

In another set of experiments PUA stamp and HCL hydrolyzed substrates were reacted with a dichloromethane solution of pentadecafluorooctan-1-amine (20 mM) for 2 hr. at r.t. As a control experiment, a freshly prepared NHS-terminated substrate was reacted with the dichloromethane solution of pentadecafluorooctan-1-amine (20 mM) for 2 hr. at r.t. Following the reaction, the substrates were rinsed with 0.5 M aqueous HCl solution, dH$_2$O, and IPA. Dried under the stream of filtered argon and analyzed by XPS for changes in carbon and fluorine concentrations. To demonstrate that the proposed method obviates diffusive limitations of traditional µCP, we reacted a patterned catalytic stamp containing 125 nm lines with the NHS-modified silicon chips. The features were accurately transferred to the silicon surfaces, which would not be possible in the conventional µCP where the ink spreading is responsible for at least 50 nm enlargements of the feature edges.

**4.1.9 Functionalization of NHS-terminated monolayers**

NHS-modified substrates were further derivatized by reacting with mono Boc-protected ethylenediamine. The reaction was carried out in a 20 mM dichloromethane solution for 2 hr. at r.t. After reaction, the Boc-modified substrate was rinsed with DCM and EtOH. The Boc modified substrate was deprotected using 50% TFA in DCM for 1 hr. at r.t. The resulting surface was rinsed with DCM, EtOH and 10% potassium bicarbonate.
in water and dried under filtered argon. All surfaces were analyzed by XPS to determine elemental composition.

The NHS-patterned bi-functional substrate presenting an array of hydrolyzed 8 µm squares was submerged in Lysine-N,N-diacetic acid (20 mM) and Et₃N (100 mM) in DMF:H₂O (1:1) at room temperature for 1 hr. and then rinsed with dH₂O and EtOH. Theses substrates were subsequently incubated in a 50 mM NiSO₄ solution for 5 min at r.t. The chelated substrates were then rinsed excessively with water and binding buffer (20 mM NaP, 250 mM NaCl, 10 mM imidazole, pH 7.5) and submerged in a filtered GFP solution (~40 µM) for 1 hr. at 0°C. Substrates were immediately rinsed with binding buffer followed by PBS (pH 7.4). Substrates were kept hydrated in PBS at 0°C until they were ready for analysis.

4.2 Catalytic µCP on oxide-free germanium

4.2.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH₂O) were filtered through a 0.2 µm filter before use. SEM images were recorded on the FEI XL30 SEM-FEG microscope detecting secondary electrons at 15 mm working distance. XPS spectra were recorded on the Kratos Axis Ultra XPS spectrometer equipped with a mono-Al X-ray source. Germanium substrates (~ 1 cm x 1 cm) were manufactured by coating silicon wafers with a 100 Å titanium adhesion layer followed by a 230 Å germanium layer using an electron-beam metal evaporator (CHA Industries). Chemically modified germanium
substrates were analyzed for surface roughness using tapping mode atomic force microscopy on the Veeco D3100 using TESP silicon nitride probes with a 42 N/m spring constant. Patterned germanium substrates were visualized by contact mode lateral force microscopy on the Veeco D3100 microscope using ORC-8 silicon nitride probes with a 0.38 N/m spring constant.

4.2.2 Si/SiO\textsubscript{2} Master Fabrication

NR9-1500PY (Futurrex) was spun on a clean silicon chip bearing 1 µm of oxide at 3000 rpm for 40 s. The resulting substrate was baked on a digital hot plate at 155°C for 2 min to produce ~180 nm of the resist on Si. Photolithography (Karl Suss MA6/BA6) was performed using a photomask (Photo Sciences, Inc.) bearing 20 µm hexagonal rings with an exposure time of 10.5 s. After UV exposure, the substrate was baked on a digital hot plate at 105°C for 70 s, developed in RD6 (Futurrex) for 11 s and immediately rinsed with dH\textsubscript{2}O. The dried substrate was baked in an oven at 110°C for 5 min and descumed in oxygen plasma for 1 min at 100 watts and 6×10\textsuperscript{–1} mbar O\textsubscript{2} pressure (Emitech K-1050X plasma asher). The oxide layer was etched away through the opening in photoresist using reactive ion etching (Trion Technology Phantom II) for 22 min using CF\textsubscript{4} and O\textsubscript{2}. Any remaining oxide was removed using buffered oxide etch (BOE). The negative resist was removed with Nano strip (55°C, 2 min) producing a patterned silicon/SiO\textsubscript{2} master.

4.2.3 SAM formation

Germanium surfaces were rinsed briefly with dH\textsubscript{2}O, EtOH and IPA and dried under a stream of filtered argon. They were subsequently soaked at room temperature
in acetone for 25 min to remove any organic contaminants. Chlorine terminated surfaces were prepared by exposing the clean germanium surfaces to a 10% solution of HCl at room temperature for 15 min. The Cl-terminated surfaces were dried under argon and reacted with a Grignard solution (Octyl MgBr, 2M in THF) for 48 hr. at 130°C in sealed vials producing stable close-packed octyl-terminated SAMs on oxide-free Ge. After the reaction, the surface was thoroughly rinsed with EtOH and DCM and dried under a stream of argon. The octyl-functionalized chip was subsequently covered with ~ 50 µL of a 0.1 M solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate in CCl₄ and reacted under UV light (λ=254 nm) for 1.5 hr. at r.t. After the reaction, the NHS-functionalized surface was thoroughly washed with dichloromethane and isopropanol, dried under a stream of argon and used immediately.

4.2.4 Preparation of reactive stamps

Acidic polyurethane-acrylate stamps. Sulfonic acid modified and inactive stamps were prepared according a previously published protocol.[121]

4.2.4 Stamping Protocol

A corresponding polyurethane-acrylate stamp was placed on the top of the modified Ge substrate at room temperature for 2 min with no external load to hold them together. After the reaction the stamp and the substrate were separated. The substrate was rinsed with EtOH and dried with filtered argon. The stamp was rinsed with EtOH and dried with filtered argon, and kept at room temperature before the next application.
4.3 Multicomponent Patterning of Indium Tin Oxide

4.3.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied unless otherwise stated. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH$_2$O) were filtered through a 0.2 μm filter before use. SEM images were recorded on the FEI XL30 SEM-FEG microscope detecting secondary electrons at 15 mm working distance. XPS spectra were recorded on the Kratos Axis Ultra XPS spectrometer equipped with a mono-Al X-ray source. ITO substrates (~ 1 cm x 1 cm) were manufactured by coating silicon wafers with 2000 Å ITO using an RF dielectric sputter system (Kurt Lesker PVD 75). Chemically modified ITO substrates were analyzed for surface roughness using tapping mode atomic force microscopy on the Veeco D3100 using TESP silicon nitride probes with a 42 N/m spring constant.

4.3.2 Si/SiO$_2$ Master Fabrication

NR9-1500PY (Futurrex) was spun on a clean silicon chip bearing 1 μm of oxide at 3000 rpm for 40 s. The resulting substrate was baked on a digital hot plate at 155°C for 2 min to produce ~180 nm of the resist on Si. Photolithography (Karl Suss MA6/BA6) was performed using a photomask (Photo Sciences, Inc.) bearing 20 μm hexagonal rings or 8 μm hexagons. An exposure time of 10.5 s was used for the 20 μm hexagonal ring photomask and an exposure time of 12 s was used for the 8 μm hexagon photomask. After UV exposure, the substrate was baked on a digital hot plate at 105°C for 70 s, developed in RD6 (Futurrex) for 11 s and immediately rinsed with dH$_2$O. The dried substrate was baked in an oven at 110°C for 5 min and descummed in oxygen plasma for 1
min at 100 watts and 6×10\textsuperscript{-1} mbar O\textsubscript{2} pressure (Emitech K-1050X plasma asher). The oxide layer was etched away through the opening in photoresist using reactive ion etching (Trion Technology Phantom II) for 22 min using CF\textsubscript{4} and O\textsubscript{2}. Any remaining oxide was removed using buffered oxide etch (BOE). The negative resist was removed with Nano strip (55°C, 2 min) producing a patterned silicon/SiO\textsubscript{2} master

### 4.3.3 Monolayer Formation

**NHS-terminated SAM formation.** ITO surfaces were rinsed briefly with dH\textsubscript{2}O, EtOH and IPA and dried under a stream of filtered argon. They were subsequently cleaned in a plasma oxidizer (Emitech K-1050X, 5 min, 100 W) to remove any organic contaminants. Vapor phase silanization was accomplished using a protocol similar to that published by Riener et al. Octyl-terminated surfaces were made by placing ITO substrates in a glass bottle under high vacuum containing 2 mL vials of octylpropyltrimethoxysilanes (30 µL) and triethylamine (10 µL). After 16 hr. the surfaces were removed and rinsed thoroughly with DCM and EtOH and dried under a filtered stream of argon. Amino-terminated surfaces were formed by placing ITO substrates in a glass bottle containing 2 mL vials of aminopropyltrimethoxysilanes (30 µL) and triethylamine (10 µL). After 2 hr., the surfaces were removed and rinsed with dichloromethane and ethanol and dried under argon. The octyl-functionalized surface was subsequently covered with ~ 50 µL of a 0.1 M solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate in CCl\textsubscript{4} and reacted under UV light (λ=254 nm) for 1.5 hr. at room temperature\textsuperscript{121}. After the reaction, the NHS-functionalized
surface was thoroughly washed with DCM and IPA, dried under a stream of argon and used immediately.

**Carbene-terminated SAM Formation.** ITO surfaces were rinsed briefly with dH$_2$O, EtOH and IPA and dried under a stream of filtered argon. They were subsequently cleaned in a plasma oxidizer (Emitech K-1050X, 5 min, 100 W) to remove any organic contaminants. Amino-terminated surfaces were formed by placing ITO substrates in a glass bottle containing 2 mL vials of aminopropyltrimethoxysilanes (30 µL) and triethylamine (10 µL). After 2 hr., the surfaces were removed and rinsed with DCM and EtOH and dried under argon. The amino-functionalized surface was subsequently immersed in a 10 mM solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirine-3-yl)benzoate in CCl$_4$ for 2 hr. in the dark. After the reaction, the carbene terminated surface was thoroughly rinsed with dichloromethane and isopropanol, dried under a stream of argon, and reacted immediately with a 7 mM solution of C$_6$0 in benzene under UV light (λ=254 nm) for 1 hr. The C$_6$0 modified ITO substrate was rinsed with dichloromethane and ethanol and dried under argon.

### 4.3.4 Stamp Preparation

Sulfonic acid modified and inactive polyurethane-acrylate stamps were prepared according a previously published protocol.$^{[123]}$ Patterned and flat PUA stamps lacking the sulfonylic acid moiety were prepared according to a previously published protocol.$^{[121]}$ The PUA stamps were inked in either a 2 mM solution of azido-dPEG$_3$amine (Quanta
Biodesign, OH) in anhydrous dichloromethane or a 7 mM solution of C60 in benzene for 2 min at room temperature and dried under argon.

### 4.3.5 Stamping Protocol

For both catalytic and reactive printing applications, the appropriate polyurethane-acrylate stamp was placed on the top of the modified ITO substrate at room temperature for 2 min with no external load. After the reaction, the stamp and the substrate were separated. The substrate was rinsed with EtOH and dried with filtered argon. The stamp was rinsed with EtOH and dried with filtered argon, and kept at room temperature before the next application. To successfully pattern C60, the stamp/surface system was exposed to UV light (\(\lambda=254\) nm) for 2 min.

### 4.4 Tunable control over hole injection in OLEDs via organic monomolecular systems

#### 4.4.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied unless otherwise stated. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH\(_2\)O) were filtered through a 0.2 µm filter before use. SEM images were recorded on the FEI XL30 SEM-FEG microscope detecting secondary electrons at 15 mm working distance. XPS spectra were recorded on the Kratos Axis Ultra XPS spectrometer equipped with a mono-Al X-ray source. Work function was investigated via ultraviolet photoelectron spectroscopy (UPS) using a Kratos Axis Ultra DLD Ultraviolet Photoelectron Spectrometer. Dipole moment values were calculated using Hyper software package.
4.4.2 Primary Monolayer Formation

Silane Monolayers. ITO surfaces were sonicated in dH₂O and IPA for 5 min each and dried under a stream of filtered argon. They were subsequently cleaned in a plasma oxidizer (Emitech K-1050X, 5 min, 100 W) to remove any organic contaminants. Vapor phase silanization was accomplished using a protocol similar to that published by Riener et al. Octyl- and octadecyl- terminated surfaces were made by placing ITO substrates in a glass bottle under high vacuum containing 2 mL vials of silanes (30 µL) and triethylamine (10 µL). After 16 hr. the surfaces were removed and rinsed thoroughly with DCM and EtOH and dried under a filtered stream of argon. Control surface, C3, displaying fluorinated silanes was prepared in an identical manner using perfluorooctyltrimethoxysilanes. Amino-terminated control surface (C5) was formed by placing ITO substrates in a glass bottle containing 2 mL vials of aminopropyltrimethoxysilanes (30 µL) and triethylamine (10 µL). After 16 hr., the surfaces were removed and rinsed with DCM and EtOH and dried under argon yielding octyl-(P3) and octadecyl- (P1) functionalized surfaces.

Phosphonic acid Monolayers. ITO surfaces were sonicated in dH₂O and IPA for 5 min each and dried under a stream of filtered argon. The cleansed ITO surfaces were exposed to 10 mM octadecylphosphonic acid in IPA overnight for approximately 16 hr. The surfaces were removed, rinsed with IPA and dried under argon yielding surface P2. Control surface, C4, containing fluorinated phosphonic acid was prepared in an identical manner using octadecylphosphonic acid.
4.4.3 Secondary, reactive overlayer formation

*NHS-terminated SAM formation.* Primary monolayer surfaces, P1, P2 and P3 were subsequently covered with ~ 50 µL of a 0.1 M solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate in CCl$_4$ and reacted under UV light ($\lambda=254$ nm) for 1.5 hr. at room temperature$^{[121]}$. After the reaction, the NHS-functionalized surfaces (S1, S2, and S3) were thoroughly washed with DCM and IPA, dried under a stream of argon and used immediately.

4.4.4 Functionalization of the reactive overlayer

NHS-terminated surfaces (S1, S2, and S3) were exposed to either a solution of 10 mM 4-(aminomethyl)piperidine (F1 and F2) or 10 mM 1H, 1H-perfluorohexylamine in IPA (F3 and F4) for 30 min at room temperature. Following the reaction, the surfaces were rinsed with IPA and dried under filtered argon.

4.4.5 OLED fabrication

Modified ITO substrates were loaded into a custom-built vacuum chamber for thin film deposition. All the organic materials for thin film deposition were purified by entrainer gas sublimation before use. Cell fabrication was carried out in the vacuum chamber where the entire layer sequence: (1) a 50-nm-thick NPB layer as a hole transporting layer (HTL), (2) a 50-nm-thick Alq layer as a light-emitting layer (LEL), (3) a 10-nm-thick Bphen layer as an electron-transporting layer (ETL), (4) a 1-nm-thick LiF layer as an electron-injecting layer, and (5) a 200-nm-thick Al as a cathode. The deposition rate for organic layers was about 4 Å/s. The deposition rate for MoO$_3$, LiF
and Al was typically 2, 0.6 and 6 Å/s, respectively. The base pressure of the vacuum deposition system was about ~6 × 10^{-7} Torr. All thin film depositions were monitored by an Inficon IC5 controller. The cell active area was 0.1 cm², which was defined by the overlapping area of the ITO and Al electrodes. The electroluminescence was measured by PR-650 SpectraScan Colorimeter. For control sample C2, a 5-nm-thick MoO3 layer was the first organic thin film to be evaporated as a hole injecting layer.

4.4.6 Stamping Materials and Procedures

*Si/SiO₂ master fabrication.* NR9-1500PY (Futurrex) was spun on a clean silicon chip at 3000 rpm for 40 s. The resulting substrate was baked on a digital hot plate at 155°C for 2 min to produce 180 nm of the resist on Si. Photolithography (Karl Suss MA6/BA6) was performed using a photomask (Photo Sciences, Inc.) bearing 16 μm hexagons with an exposure time of 11.5 s. After UV exposure, the substrate was baked on a digital hot plate at 105°C for 70 s, developed in RD6 (Futurrex) for 11 s and immediately rinsed with dH₂O. The dried substrate was baked in an oven at 110°C for 5 min and descumed in oxygen plasma for 1 min at 100 watts and 6×10^{-1} mbar O₂ pressure (Emitech K-1050X plasma asher). The oxide layer was etched away through the opening in photoresist using reactive ion etching (Trion Technology Phantom II) for 22 min using CF₄ and O₂. Any remaining oxide was removed using buffered oxide etch (BOE). The negative resist was removed with Nano strip (55°C, 2 min) producing a patterned silicon/SiO₂ master.
**Preparation of Catalytic Stamps.** Acidic polyurethane-acrylate stamps. Sulfonic acid modified and inactive stamps were prepared according a previously published protocol.\[^{121, 145}\]

**Preparation of Reactive Stamps.** Polyurethane-acrylate stamps. Patterned and flat PUA stamps lacking the sulfonic acid moiety were prepared according to a previously published protocol.\[^{145}\] The PUA stamps were inked in a 10 mM solution of 4-(aminomethyl)piperidine in EtOH for 2 min at room temperature and dried under argon.

**Stamping protocol.** For both catalytic and reactive printing applications, the appropriate polyurethane-acrylate stamp was placed on the top of the modified ITO substrate at room temperature for 2 min with no external load. After the reaction, the stamp and the substrate were separated. The substrate was rinsed with ethanol and dried with filtered argon. The stamp was rinsed with EtOH and dried with filtered argon, and kept at room temperature before the next application. Following reactive printing with an inked stamp bearing 4-(aminomethyl)piperidine, substrates RA1 and RA2 were exposed to a solution of 10 mM 1H, 1H-perfluorohexylamine for 30 min at room temperature. The substrates (RB1 and RB2) were rinsed with IPA and dried under filtered argon.
4.5 *The effect of compressive force on the unbinding profiles of specific protein-ligand complexes*

4.5.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH₂O) were filtered through a 0.2 µm filter before use. Thin-layer chromatography was performed on Merc Silica Gel 60 F254 aluminum plates using phosphomolybdic acid stain for spot visualization. Column chromatography was performed on a CombiFlash Rf automated flash chromatography system (Teledyne ISCO) using RediSep Rf silica gel columns. ¹H and ¹³C NMR spectra were recorded on a Varian 300 MHz or Varian 500 MHz spectrometer. Oxygen plasma ashing was performed for 5 minutes at 100 watts and 6×10⁻¹ mbar O₂ pressure (Emitech K-1050X plasma asher). ESI mass spectrometry was performed on an Agilent Ion trap with electrospray ionization. UV irradiation was performed with a UVP mercury Pen Ray lamp 11sc with PS-1 power supply (4400 µC/cm²).

**Mercaptopentyl Lactose (2).** Synthesis of mercaptopentyl lactoside (2) was previously reported.¹⁷²

**NTA-maleimide linker (3).** Synthesis of N⁶-bis-carboxymethyl-N⁶-3-maleimido-propionyl lysine (3) was previously described.²⁰⁸
Pent-4-enyl-2,3,4,6-tetra-O-benzoyl-α-D-mannopyranoside (3). D-Mannopyranose, 1,2,3,4,6-pentabenoate (38.8 g, 55.5 mmol, 1 eq.) was dissolved in dichloromethane (200 mL) and cooled to 0°C under argon. To this was added 33%HBr/HOAc (82 mL, 1.4 mol, 25 eq) slowly via cannulation. After stirring at room temperature for 12 hr., the reaction was condensed on a rotavap and dichloromethane (200 mL) was added. The organic layer was washed with saturated NaHCO$_3$, dried over MgSO$_4$, and condensed to a foam. A dry flask containing a mixture of silver triflate (1.81 g, 7.1 mmol, 2 eq), 4-penten-1-ol (0.40 g, 4.6 mmol, 1.3 eq) and molecular sieves (4 Å) in dichloromethane was cooled to -30°C using dry ice in acetonitrile as a bath. To this was added a portion of the crude 1-bromo-2,3,4,6-tetra-O-benzoyl-mannopyranoside (2.33 g, 3.5 mmol, 1 eq) dissolved in dichloromethane (20 mL) via cannulation. After stirring for 4 hr. at -30°C, the mixture was filtered through celite and the resulting organic layer was washed with saturated NaHCO$_3$, and condensed to a yellow oil. Chromatography on a RediSep column (SiO$_2$, 40 g) using an automated column runner (Gradient: 0-70% ethyl
acetate in hexane) provided the desired product 3 (1.3g, 56% yield over both steps). Proton NMR was consistent with the reported spectrum.\[209\] MS (ESI) calculated for C39H36O10 [M + Na]+ 687.2 found 687.2.

**S-Acetyl-5-thiopentyl-2,3,4,6-tetra-O-benzoyl-a-D-mannopyranoside (4).** A portion of 3 (0.575g, 0.865mmol, 1eq.) was dissolved in 12 ml of dry THF. To this solution was added thiolacetic acid (1.31g, 17.3mmol, 20eq.) and AIBN (9mg, 0.0865mmol, 0.1eq). After UV irradiation for 3 hr. at r.t, the mixture was condensed to a yellow oil. Chromatography on a RediSep Column (SiO\(_2\), 40 g) using an automated column runner (Gradient: 0-50% ethyl acetate in hexane) provided the desired product 5 (0.3g, 47% yield). 1H NMR (300 MHz, CDCl3): \(\delta = 7.82-8.15\) (m, 8H), \(7.22-7.64\) (m, 12H), 6.12 (t, 1H, \(J = 10.0\) Hz), 5.92 (dd, 1H, \(J = 3.4, 6.7\) Hz), 5.70 (dd, 1H, \(J = 1.8, 1.4,\) Hz), 5.09 (d, \(J = 1.7\) Hz, 1H), 4.71 (dd, \(J = 2.5, 9.6\) Hz, 1H), 4.38-4.54 (m, 2H), 3.52-3.88 (m, 2H), 2.91 (t, \(J = 7.3\) Hz, 2H) 2.33 (s, 3H) 1.44-1.79 (m, 6H). MS (ESI) calculated for C\(_{41}\)H\(_{40}\)O\(_{11}\)S [M + Na]\(^+\) 763.2 found 763.1.

**5-Thiopentyl-a-D-mannopyranoside (5).** Compound 4 (0.30 g) was dissolved in a degassed solution of sodium methoxide (5ml, 0.2M in MeOH) and stirred under argon for 5 hr. at room temperature. The reaction mixture was neutralized to pH 7 using activated H\(^+\) Dowex resin, filtered, and condensed. The crude material was dissolved in ether, filtered, and condensed to give the pure desired product 3, as a clear oil (0.114 g, 61% yield). 1H NMR (500 MHz, CDCl3): \(\delta = 5.00-5.12\) (bd, \(J = 15.2\) Hz, 2H), 4.95 (bs, 1H), 4.82 (bs, 1H), 4.51 (bs, 1H), 3.88-4.01 (m, 3H), 3.84 (bs, 1H), 3.78 (d, \(J = 11.5\) Hz, 1H), 3.65 (dt, \(J = 6.6, 9.6\) Hz, 1H), 3.51 (d, \(J = 9.5\) Hz, 1H), 3.41 (dt, \(J = 6.12, 9.78\) Hz, 1H), 2.53 (q, \(J = 7.3\) Hz, 2H), 1.54-1.66 (m, 4H), 1.40-1.49 (m, 2H), 1.37 (t, \(J = 7.8\) Hz, 1H). 13C NMR
Expression and purification of murine His$_6$-tagged galectin-3

The His$_6$-tagged galectin-3 was expressed and purified as described by Snyder et al.$^{[172]}$. The gene for wild-type galectin-3 was obtained by PCR amplification from the plasmid prCBP35s (obtained from Dr. J. L. Wang) and digested with EcoRI and BamHI. The digested PCR product was ligated into a similarly prepared pET28b plasmid and transformed into BL21 (DE3) cells. A 5 mL portion of TB/Kanamycin (30 μg/mL) was inoculated with a single colony of BL21 (DE3) cells and incubated at 37°C overnight. Cells were grown until the optical density at 600nm ($\text{OD}_{600}$) reached 0.6-0.8 and expression was induced during 3 hr. with 125 mgL$^{-1}$ isopropyl thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, lysed by sonication, and the supernatant was applied to 10 mL of His Bind resin (Novagen, San Diego, CA). Galectin-3 was isolated according to manufacturer’s instructions and dialyzed against EDTA (1 mM) in sodium phosphate (50mM, pH 7.5) for 8 hr., followed by dialysis against sodium phosphate (50 mM, pH 7.5) for 24 hr. to remove imidazole and EDTA.

Expression and purification of His$_6$-tagged 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase

A plasmid containing the gene KDPG-pet36, which encodes His$_6$-tagged KDPG aldolase, was transformed into BL21 CodonPlus (DE3)-RIL cells. The transformation mixture was plated onto an LB/Kan (50 mg mL$^{-1}$) plate and incubated overnight at 37°C.
A single colony from the LB/Kan plate was introduced into 50 mL of TB/Kan (50 mg mL^{-1}). The culture was incubated at 37°C for 16 hr. and then 10 mL was transferred to 1 liter of TB/Kan (50 mg mL^{-1}) and shaken at 37°C until an OD_{600} of 0.8 was reached. Expression was induced by the addition of IPTG (230 mg L^{-1}, to 1 mM) to the cell culture. The induced culture was shaken at 37°C for an additional 4 hr. The cells were collected by centrifugation, lysed by sonication, and the supernatant was applied to 10 mL of His Bind resin (Novagen, San Diego, CA). KDPG aldolase was isolated according to manufacturer’s instructions and dialyzed into HEPES buffer (20 mM, pH 7.5) at 4°C.\(^{[210]}\)

**Functionalization of silicon nitride AFM tips**

AFM tips (NP, Veeco, nominal spring constant of 0.120 N/nm) were rinsed thoroughly in EtOH and dH\(_2\)O, dried under nitrogen, and cleaned in a plasma oxidizer (5 min, 100 W). Vapor phase aminosilanization was accomplished using a protocol similar to that published by Riener et al.\(^{[211]}\) Tips were placed in 50 mL glass bottles containing 2 mL vials of freshly distilled APTMS (30 µL) and triethylamine (10 µL) under an atmosphere of argon. After 2 hr. of incubation, the APTMS and triethylamine were removed. The bottle was flushed with argon and sealed for 2 days. Aminofunctionalized tips were rinsed with dichloromethane and immersed in a solution of NHS-PEG\(_{12}\)-Mal (Quanta Biodesign, 2 mM in DCM) with pyridine (1% w/v) for 1 hr., rinsed with ethanol and water, and dried under argon. The cantilevers were next submerged in an aqueous solution of mercaptopentyl lactoside (2) or mercaptopentyl mannoside (3) (2.25 mM) for 4 hr. at 37 °C, rinsed with water, and dried under argon.\(^{[172]}\)
Functionalization of silicon substrates

His$_6$-tagged galectin 3 was chelated to the surface using a protocol similar to that of Schmid et al.$^{[208]}$ Silicon wafers (prime grade <100>, Silicon Quest Int.) were rinsed with methanol and water and dried under nitrogen. The surfaces were immediately placed in a plasma oxidizer (5 min, 100 W) in order to generate surface hydroxyl groups on the native SiO$_2$ layer. Oxidized surfaces were silanized with mercaptopropyl trimethoxysilane (MPTMS) using the procedure described for aminosilanization of AFM tips. This process generated reactive thiols on the surface that were subsequently reacted during 1 hr. with 0.2 mg mL$^{-1}$ nitrilotriacetate (NTA)-maleimide linker (1) in sodium phosphate buffer (10 mM, pH 7.0). The surfaces were rinsed with water and then incubated for 5 min in NiSO$_4$ (50 mM). The surfaces were rinsed with water and bind buffer (50 mM imidazole, 20 mM Tris HCl, 0.5 M NaCl, pH 7.9) and then submerged in a solution of His$_6$-galectin-3 (50 µM) in sodium phosphate buffer (50 mM, pH 7.5) or His$_6$-KDPG aldolase (50 µM) in HEPES buffer (20 mM, pH 7.5) for 1 hr., then immediately rinsed with bind buffer followed by PBS (pH 7.4).

Unbinding experiments

Automated pulling experiments were carried out on a custom 3-axis AFM composed of a MultiMode head (Digital Instruments, Santa Barbara, CA) mounted on an xy- and z-positioning stages (Physik Instrumente, Auburn, MA).$^{[195]}$ For each experiment, 250 force curves were generated using an automated data collection routine. Sample was brought into contact with tip, held for a dwell time (DT) of 1 s, and then retracted at a velocity of 200 nm s$^{-1}$. For contact force minimization experiments, software
parameters were optimized to achieve contact forces (<100 pN) for each pull. Following
data collection, the photodetector was calibrated, and a cantilever spring constant was
determined using the thermal noise method.[212] The photodetector was calibrated prior
to data collection for force trigger experiments. The sample was then driven towards
the tip until a desired contact force was achieved (250 pN or 1000 pN). Unblocked
experiments were carried out in the presence of PBS, pH 7.4. Blocking experiments were
carried out in the presence α-methyl lactose (10 mM in PBS, pH 7.4); at this
concentration of ligand, 98% of available binding sites should be occupied by soluble
lactose, assuming an immobilized binding constant of 6400 M\(^{-1}\).[172]

4.6 A general and efficient cantilever functionalization technique for AFM
molecular recognition studies

4.6.1 Materials

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. Ethanol (EtOH), isopropanol (IPA) and deionized water (dH\(_2\)O) were filtered through a 0.2 µm filter before use. Thin-layer chromatography was performed on Merc Silica Gel 60 F254 aluminum plates using iodine vapors or CAM stain for spot visualization. Column chromatography was performed using Silicycle Silica-P Flash Silica Gel. \(^1\)H and \(^{13}\)C NMR spectra were recorded on a Varian 300 (300 MHz) spectrometer. XPS spectra were recorded on the Kratos Axis Ultra XPS spectrometer equipped with a mono-Al X-ray source. Silicon nitride substrates were manufactured by depositing 130 nm layer of silicon nitride on silicon chips using PECVD system (Advanced Vacuum Vision 310). Water contact angles were measured on the Rame-Hart NRL contact angle
goniometer. SEM images were recorded on the FEI XL30 SEM-FEG microscope detecting secondary electrons at 6 cm working distance. Optical micrographs were taken on the Nomarski type Zeiss Axiolmager microscope at 10 and 20X magnification. Automated pulling experiments were carried out using ORC8 cantilevers (Veeco Probes) and NP cantilevers (Bruker Probes) on a custom 3-axis AFM composed of a MultiMode head (Digital Instruments, Santa Barbara, CA) mounted on an xy- and z-positioning stages (Physik Instrumente, Auburn, MA). \textsuperscript{195}

4.6.2 SAM formation on silicon nitride substrates and silicon nitride AFM cantilevers

\textit{Boc-alkene synthesis}

Commercially available alkene (1) was prepared according to the previously published protocols.\textsuperscript{202}

\textit{Boc- and NH\textsubscript{2}-SAM formation on silicon and silicon nitride}

Boc-aminoalkene (1) was used to form Boc- and NH\textsubscript{2}-terminated SAMs on silicon nitride. As such, freshly prepared silicon nitride chips (~ 1x1 cm) were exposed to a 5% aq. HF solution for 5 min and dried under a stream of nitrogen to remove oxide layer. Subsequently, neat alkene (1) (10 µl) was applied to the surface of silicon nitride chips, which were immediately transferred into a nitrogen-filled glovebox. The samples were irradiated with UV light (UVP 11sc lamp, 4400 µC/cm\textsuperscript{2} at 2 cm distance) for 30 and 90 min at 2.5 cm and 5 cm distances. After the reaction, the substrates were removed from the glovebox, washed with dichloromethane, ethanol and water, and dried under a stream of filtered argon to give Boc-protected substrates. Amine-terminated
monolayers were prepared by deprotecting Boc-substrates in 50% TFA in DCM for 30 min and by rinsing the resulting surfaces DCM and EtOH.

Alkene (1) was then used to form Boc- and NH$_2$-SAMs on H-terminated silicon. As such, silicon chips (~1×1 cm, N/As, <1-1-1>, 0.002-0.004 ohm-cm) were exposed to 5% HF for 5 min, dried under a stream of nitrogen and reacted with alkene (1) following the protocol described above.

**XPS analysis of Boc-terminated SAMs on silicon and silicon nitride**

Following SAM formation, functionalized surfaces were analyzed by XPS to compare relative carbon concentration in silicon and silicon nitride substrates and to determine optimal reaction conditions. Additionally, modified substrates were analyzed by goniometry to provide further comparison between the densities of SAMs on silicon and silicon nitride and to confer a degree of SAM ordering.

Table 5 shows that the UV reactions on both materials yielded SAMs with almost identical densities. In both cases, the substrates exposed to UV light for 90 min at 2.5 cm (samples A2, B2) had elevated carbon concentration, suggesting potential polymerization of alkene 1 on the surface at these conditions. Samples A1, A3, A4, B1, B3, B4 demonstrated very similar C1s/Si2p values, suggesting that complete SAM formation on either silicon or silicon nitride can be achieved after a 30 min UV exposure at 2.5 cm. Goniometry measurements again confirmed formation of identical monolayers on silicon and silicon nitride and were consistent with Boc-and NH$_2$-terminated SAMs on silicon, previously reported in the literature.$^{[202]}$
Table 5. XPS analysis and contact angle measurements of Boc-terminated SAMs on Si and Si₃N₄

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrate</th>
<th>UV lamp distance (cm)</th>
<th>Reaction time (min)</th>
<th>C1s/Si2p XPS ratio</th>
<th>Water contact angle (Rec/Adv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Si</td>
<td>2.5</td>
<td>30</td>
<td>0.547</td>
<td>78/68</td>
</tr>
<tr>
<td>A2</td>
<td>Si</td>
<td>2.5</td>
<td>90</td>
<td>0.747</td>
<td>79/66</td>
</tr>
<tr>
<td>A3</td>
<td>Si</td>
<td>5</td>
<td>30</td>
<td>0.365</td>
<td>72/63</td>
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Boc- and NH₂-SAM formation on silicon nitride AFM tips

Based on the XPS results, the silicon nitride AFM cantilevers were functionalized with Boc- and NH₂-terminated SAMs using the abovementioned procedure and 30 min 2.5 cm UV exposure conditions. To ensure the integrity of AFM tips in this functionalization protocol, we exposed four AFM cantilevers to a 5% HF solution for 1 – 2 min and analyzed them by SEM (Figure 69). As it can be seen from the SEM images, the silicon nitride tips remained their morphology even after a 2 min HF exposure guaranteeing the consistency of the curvature radios between individual silicon nitride probes.
Figure 69. SEM images of the HF treated tips: (A) no HF, tip height 4.83 µm, tip base 4.87 µm; (B) 30 sec HF exposure, tip height 4.87 µm, tip base 4.67 µm; (C) 60 sec HF exposure, tip height 4.83 µm, tip base 4.78 µm; (D) 120 sec HF exposure, tip height 5.02 µm, tip base 4.87 µm.

Likewise, we analyzed the integrity of the AFM cantilevers following exposure to alkene (1) under UV light. Figure 70 shows that the silicon nitride cantilevers did not bend upon UV exposure, ensuring their consistency in the laser focusing step.
4.6.3 Functionalization of silicon with the G3-protein

**NHS-diazirine synthesis**

2, 5-Dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate (5) was prepared from (4-bromophenyl)methanol in 9 steps following the previously published protocol.[206]

**G3 purification**

The His$_6$-tagged galectin-3 was expressed and purified as previously described.[172]

**G3 immobilization on silicon substrates**

A silicon (111) chip (~1×1 cm) was washed with EtOH and dH$_2$O and dried with filtered argon. The substrate was then oxidized in Nano strip solution (cyantek Inc.) at 75°C for 15 min to remove all organic contaminants. The native oxide film was etched from the surface with 5% aq. HF at room temperature for 4 min. yielding oxide-free polycrystalline surface displaying Si-H bonds. The silicon chip was transferred from the HF solution to a saturated phosphorus pentachloride (PCl$_5$) solution in chlorobenzene.
containing small amount of benzoyl peroxide (0.1 % m/v) and heated in this solution for 1 hr. at 105°C. The chlorinated substrate was quickly washed with chlorobenzene, dried under a stream of argon, and immediately transferred into a 0.5 M THF solution of 1-propenylmagnesium bromide. Subsequently, the surface was reacted in a sealed vial with the Grignard at 135°C for at least 24 hr. to produced stable close-packed propylene-terminated SAMs on oxide-free silicon. After the reaction, the surface was thoroughly rinsed with ethanol and dichloromethane and dried first under a stream of argon and then on a hot plate at 75°C for 10 min. The propylene-functionalized chip was subsequently covered with ~ 50 µL of a 0.1 M solution of 2,5-dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate in CCl₄ and reacted under UV light for 1 hr. at room temperature. After the reaction, the NHS-functionalized surface was thoroughly washed with dichloromethane and isopropanol, dried under a stream of argon and used immediately.

The NHS-functionalized substrate was submerged in lysine-N,N-diacetic acid (20 mM) and Et₃N (100 mM) in DMF:H₂O (1:1) at room temperature for 1 hr. and then rinsed with dH₂O and EtOH. The sample was subsequently incubated in a 50 mM NiSO₄ solution for 5 min at room temperature. The chelated substrate was then rinsed excessively with water and binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and submerged in a filtered G3 solution (~30 µM) for 1 hr. at 0°C. The G3-modified surface was immediately rinsed with binding buffer followed by PBS (pH 7.4). All functionalized substrates were kept hydrated in PBS at 0°C until they were ready for analysis.
4.6.4 Functionalization of silicon and silicon nitride with ssDNA

**Surface and Tip Functionalization**

Surfaces rinsed with dH$_2$O, cleaned in Nano strip for 30 min at 75°C, rinsed with dH$_2$O, and etched in a 5% HF solution for 5 min at r.t. Similarly, the tips were rinsed with dH$_2$O, cleaned in Nano strip for 30 s at 75°C, rinsed with dH$_2$O, and etched in 5% HF for 1 min at r.t. Both surfaces and tips dried with nitrogen and covered with (~10 µl) neat Boc-protected amino-PEG-alkene. UV-mediated (254 nm) conjugation of alkene was carried out under nitrogen atmosphere for 2 hr. at 2.5 cm for surfaces and for 30 min at 5 cm for tips. Surfaces and tips rinsed with DCM and EtOH, dried under argon, and submerged in trifluoroacetic acid (TFA) (50% in dichloromethane) for 1 hr. at r.t to deprotect the Boc-amine monolayer. Amine terminated surfaces were then exposed to a 2 mM solution of NHS-PEG$_{24}$-Maleimide (Quanta BioDesign) in DCM with 1% Et$_3$N for 2.5 hr. at r.t and under argon. Disulfide terminated DNA was diluted to 25 µM using elution buffer and reduced to the corresponding thiol using one equivalent of TCEP. Following disulfide reduction for 15 min at r.t, BME was added to the solution such that it was 1% of the total DNA concentration. Tips and surfaces were allowed to react overnight (~16 hr.) with the thiolated DNA solution in a humid chamber. The tips and surfaces were rinsed with filtered elution buffer and filtered water, dried with filtered argon and stored in an inert atmosphere until they were ready to use. XPS analysis was performed to verify successful immobilization of DNA. Results from the preparation of the PEG modified surface from native silicon was consistent with previously reported spectra.\textsuperscript{129b, 189} XPS analysis revealed an increase in the N 1s signal after immobilization
of thiolated DNA (Figure 71). The N1s/Si2p ratio increases from 0.07 to 0.09 from surface 3 to 4 in Figure 71, consistent with successful insertion of thiolated DNA to the maleimide terminated surface.
Figure 71. Functionalization of silicon with thiolated ssDNA and corresponding XPS spectra.
**AFM experimental parameters**

All experiments were conducted using the previously reported force minimization program. Dwell time and approach/retract velocities are kept constant at 1 s and 200 nm/s. Ten pulls were collected at 5 x-positions along 5 y-coordinates for a total of 250 pulls covering an area of 25 nm$^2$. All experiments were performed in the presence of a buffer (100 mM Na$_3$PO$_4$, 100 mM NaCl, pH 7.4) to effectively submerge the cantilever tip.

The fundamental rupture force was calculated using a previously reported method using a power spectrum. A histogram bin spacing of 20. This value is based on the resolution afforded by the cantilever. A polynomial fit was applied to the histogram. The order of the polynomial (n=7) was selected to capture the best fit of the dominant peak. From this fit, an autocorrelation function was derived and a Fourier transform was computed to identify the fundamental force present in the data (Figure 72).
Figure 72. (A) Histogram of forces, (B) autocorrelation function, (C) Power Spectra for the rupture of immobilized complementary DNA.
References


Biographical Sketch

*Carleen M. Bowers*

### EDUCATION

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<td>10/2012</td>
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<tr>
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<tr>
<td>University of Virginia, Charlottesville, VA</td>
<td>B.S. in Chemistry, Concentration in Biochemistry</td>
<td>05/2007</td>
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### RESEARCH EXPERIENCE

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<td>Duke University, Durham, NC</td>
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<td>08/2007 – present</td>
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<tr>
<td>University of Virginia, Charlottesville, VA</td>
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<td>12/2004 – 05/2007</td>
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### AWARDS and FELLOWSHIPS

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TEACHING EXPERIENCE

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<td>Teaching Assistant. Org. Chemistry 151 and 152 Labs (3 semesters)</td>
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MEMBERSHIPS

Graduate Women in Science, Rho Tau Chapter

Phi Lambda Upsilon

PUBLICATIONS


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<td><strong>Carleen Morris</strong>, “Chemical Reactions at Surfaces for Hybrid Material Fabrication and Molecular Recognition.” Structural Biology and Biophysics Seminar, Durham, NC, <strong>January 2011</strong></td>
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<td><strong>Carleen Morris</strong>, “Probing Multivalency in Ligand Binding Using Atomic Force Microscopy.” Structural Biology and Biophysics Seminar, Durham, NC, <strong>March 2009</strong></td>
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