New Approaches to Studying Non-Covalent Molecular Interactions in Nano-Confined Environments

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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

2010
ABSTRACT

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Abstract

The goal of this work is to develop novel molecular systems, functionalization techniques, and data collection routines with which to study the binding of immobilized binding partners. Our ultimate goal is the routine evaluation of thermodynamic parameters for immobilized systems through interpretation of the variation of the binary probability of binding as a function of soluble ligand concentration. The development of both data collection routines that minimize non-specific binding and functionalization techniques that produce stable ordered molecular systems on surfaces are of paramount importance towards achievement of this goal. Methodologies developed here will be applied to investigating the thermodynamics of multivalent systems.

In the first part of this work, the effect of contact force on molecular recognition force microscopy experiments was investigated. Increased contact forces (>250 pN) resulted in increased probabilities of binding and decreased blocking efficiencies for the cognate ligand-receptor pair lactose-G3. Increased contact force applied to two control systems with no known affinity, mannose-G3 and lactose-KDPG aldolase resulted in non-specific ruptures that were indistinguishable from those of specific lactose-G3 interactions. Thus, it is essential to design data collections routines that minimize contact forces to ensure that ruptures originate from specific, blockable interactions.

In the second part of this work we report the first example of the preparation of stable self assembled monolayers through hydrosilylation of a protected aminoalkene onto hydrogen-terminated silicon nitride AFM probes and subsequent conjugation with
biomolecules for force microscopy studies. Our technique can be used as a general attachment technique for other molecular systems.

In the third part of this work we develop novel molecular systems for tethering oriented vancomycin and its cognate binding partner L-Lys-D-Ala-D-Ala to surfaces and AFM tips. Unbinding experiments demonstrated that traditional methods for forming low surface density amine layers (silanization with APTMS and etherification with ethanolamine) provided molecular constructs which displayed probabilities of binding that were too low and showed overall variability too high to use for probabilistic evaluation of thermodynamics parameters. Instability and heat–induced polymerization of APTMS layers on tips and surfaces also prohibited their utility. Formation of Alkyl SAMs on silicon provides a more reliable, stable molecular system anchored by Si-C bonds that facilitates attachment of vancomycin and is capable of withstanding prolonged exposure to heated organic and aqueous environments. It follows that covalent immobilization of KDADA to silicon nitride AFM tips via Si-C bonds using hydrosilylation chemistry will be similarly advantageous. These methods offer great promise for probabilistic evaluation of thermodynamic parameters characterizing immobilized binding partners and will permit unambiguous determination of the role of multivalency in ligand binding, using an experimental configuration in which intermolecular binding and aggregation are precluded.
Dedicated to Natalie and Jackson.
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List of Abbreviations

AFM – atomic force spectroscopy
AIBN - azobisisobutyronitrile
APTMS – aminopropyltrimethoxy silane
BBSA – biotinylated bovine serum albumin
BDE – bond dissociation energy
Boc – tert-butoxycarbonyl
CLR – collagen-like repeat
CRD – carbohydrate recognition domain
DBU - 1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC – dicyclohexylcarbodiimide
DCM - dichloromethane
DMF – dimethylformamide
DMSO – dimethylsulfoxide
ESI – electrospray ionization
$F_c$ – contact force
$F_{nom}$ – nominal contact force
$F_{min}$ – force minimization protocol
$F_{rup}$ – rupture force
G3 – galectin-3
GlcNAc - N-acetyl glucosamine
HBTU – 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
His$_6$ - hexahistidine
HPLC – high pressure liquid chromatography
ITC – isothermal titration microcalorimetry
KDADA - L-Lys-D-Ala-D-Ala
KDPG - 2-keto-3-deoxy-6-phosphogluconate
MeOH – methanol
MPTMS – mercaptopropyltrimethoxy silane
MRFM – molecular recognition force microscopy
MurNAc - N-acetyl muramic acid
NHS – N-hydroxy-succinimide
NMR – nuclear magnetic resonance
NTA – nitrilotriacetic acid
OEG – oligoethylene glycol
OtBu – tert-butyl ester
PEG – polyethylene glycol
\( \rho_{\text{bind}} \) - the probability of observing a rupture event
\( \rho_{\text{multiple}} \) - probability of observing two or more rupture events
SAM – self-assembled monolayer
Si\(_3\)N\(_4\) – silicon nitride
TCEP – triscarboxyethyl phosphine
TFA – trifluoroacetic acid
THF – tetrahydrofuran
XPS – X-ray photoelectron spectroscopy
\( Z_{\text{con}} \) – Z contact position
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1. Introduction

1.1 Overview

Non-covalent complexation in both an inter- and intramolecular sense is involved in virtually all biological processes.\textsuperscript{1-3} Life itself proceeds through a series of highly interconnected molecular recognition events that control vital processes including, but not limited to, thoughts, movements, respiration, and reproduction. Our ability to fight and eradicate pathogenic diseases without attacking our own tissues and organs depends on the elegant organization and exquisite control of self vs non-self multivalent molecular recognition events at the surfaces of properly functioning immune cells. Molecular recognition events are also the basis for many biosensing technologies,\textsuperscript{4, 5} and considerable effort has been devoted to understanding the underlying thermodynamics characterizing non-covalent recognition events both in solution and at surfaces.

Because of its ability to spatially manipulate and measure forces between individual molecules, the atomic force microscope (AFM) is well suited for the consideration of non-covalent interactions. AFM is a cantilever-based technique widely applied to the study of molecular association, both in aqueous and non-aqueous environments. A major goal of our lab is the evaluation of thermodynamic parameters characterizing specific non-covalent interactions between immobilized molecules using AFM-based techniques. The foci within this work are (i) the development of AFM data collection routines that isolate and characterize individual bound interactions, (ii) the...
development of methodologies for oriented, stable covalent immobilization of binding partners, and (iii) understanding the physical and thermodynamic consequences of binding partner orientation. Such methodologies 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.

Although AFM has been successfully used to study force driven dissociation, there are several challenges associated with using AFM to determine equilibrium constants. First, because forced dissociation is far from equilibrium, rupture forces do not provide direct insight into equilibrium thermodynamic parameters. Second, the shape of unbinding profiles, the probability of binding during an approach-retract cycle, and degree of non-specific adhesion are highly dependent the data collection protocol used. In order to measure rupture forces, binding partners are immobilized to macroscopic surfaces which are driven together and then separated. Achieving physical proximity of receptor and ligand is clearly essential for binding. On the other hand compressive strain may cause protein denaturation, desorption, and/or tip fouling. Third, the physical consequences of immobilization can significantly affect the affinity of a pair of immobilized partners. The chemistry of immobilization and site of tether attachment may preclude partners from orienting properly for binding and may produce systems that are unstable to experimental conditions. Moreover, if numerous partners are immobilized in a heterogeneous manner, no single binding constant will accurately describe the affinities of the ensemble. It is therefore imperative that an appropriate set of experimental protocols be developed to overcome these limitations.
In our lab, we overcome the first challenge by relying on a force signature to differentiate bound versus null complex during an approach-retraction cycle. A binding constant for the immobilized complex can be determined, not from rupture forces, but from variations in the probability of bound complex formation as a function of soluble ligand concentration. This technique is described in detail in section 1.6.2.6.

In this work we describe ongoing efforts to overcome the second and third challenges described above, i.e. efforts to develop general methodologies for oriented, stable covalent immobilization of binding partners and to develop AFM data collection routines that facilitate the evaluation of thermodynamic parameters. Specifically, we investigate the effect of contact force 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 vs. extension plots. We also report the first example of the preparation of stable self assembled monolayers through hydrosilylation of a protected aminoalkene on hydrogen-terminated silicon nitride AFM probes and subsequent conjugation with biomolecules for force microscopy studies. Lastly, we investigate the effect of immobilization strategy and geometry on unbinding experiments.

1.2 Principles of AFM: imaging and force measurements

Since Binnig, Quate, and Gerber first described the atomic force microscope (AFM) as a cantilever-based tool for measuring “ultrasmall forces” on particles as small as atoms,7, 8 AFM has provided researchers extraordinary opportunities to image, spatially manipulate, and measure forces between atoms and molecules. The
capabilities of the AFM have been expanded far beyond the device originally described by Binnig for measuring interatomic forces ranging from ionic bonds ($10^{-7}$ N) to van der Waals forces ($10^{-11}$ N).

![AFM applications.](image)

**Figure 1:** AFM applications. (A) Imaging surface features. (B) Unfolding a biopolymer. (C) Molecular recognition between tip-immobilized ligand and surface-immobilized receptor.

AFM has been widely used as a tool for imaging surfaces.\textsuperscript{7-10} In a typical AFM imaging protocol, a sharp tip at the end of a flexible cantilever is raster scanned across a surface in a series of parallel lines. Repulsions between surface features and tip cause cantilever deflections which are detected and quantified (Figure 1A). Reconstruction of these deflection patterns provides three dimensional images of surface structures with sub nanometer resolution. Over the years, significant improvements in resolution have been achieved through advancement in instrumentation, cantilever fabrication and acquisition protocols.\textsuperscript{10} Perhaps the most spectacular example of sub-nanometer imaging resolution was the observation of individual atoms and bonds within a single pentacene molecule.\textsuperscript{11} The ability to operate in aqueous solutions allows for the direct imaging of biological systems including the conformational structure of single
immobilized proteins,\textsuperscript{12} light induced damage to individual DNA strands,\textsuperscript{13} and bound protein-protein interactions.\textsuperscript{14}

A second important application of AFM utilizes the device to study the effect of force on the stability of polymers and folded proteins. Advances in instrument design, cantilever fabrication, and surface preparation techniques have enabled spatial manipulation of immobilized molecules with sub-nanometer accuracy.\textsuperscript{15-17} Such experiments typically involve anchoring one end of an individual protein or polymer, through physisorption or chemisorption, to a cantilever tip and the other end to a surface (Figure 1B). Retraction of the surface from the cantilever applies force to the protein or polymer, causing unfolding and eventual detachment from the surface. AFM has been used to probe the forces required to reversibly unfold proteins\textsuperscript{18} and even to break covalent bonds in polymers.\textsuperscript{19, 20}

A third modern biophysical application of force microscopy is the investigation of force driven dissociation of non-covalently bound complexes. Robust techniques for stable molecular immobilization have extended the capabilities of AFM beyond the study of interatomic forces. Researchers can now measure the forces exerted by individual species through non-covalent interactions.\textsuperscript{1, 15-17, 21-26} Additionally, it is possible to alter the orientation and lifetime of non-covalent bonds through the direct application of force to molecular complexes. In a typical experiment, each element of a ligand-receptor pair is immobilized at either a cantilever tip or sample (Figure 1C). The sample is brought into contact with the tip, allowing complex formation between immobilized molecules. Retraction of the sample applies force to the assembly causing
cantilever deflection and, ultimately, force-driven dissociation (rupture) of the complex. A plot of force vs. extension provides a curve from which both rupture force and length can be determined.

AFM-based investigations of the forced dissociation of specific bound complexes, often called molecular recognition force microscopy (MRFM), has provided insight into the nature and dynamics of non-covalent complexation, including those between biotin and avidin, complementary DNA strands, antibodies and antigens, and proteins and carbohydrates. MRFM techniques have also produced experimental methods for force-based biosensing, for the recognition of specific biomolecules on the surface of live cells and for imaging the distribution of individual receptors adsorbed to solid surfaces through spatial mapping of recognition events.

The investigation of intermolecular interactions using AFM-based techniques is not trivial. 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 that include, but are not limited to, the compressive force applied by the data collection routine, the specific biomolecules used, the nature and geometry of immobilization, and the composition of the solution in which the interactions are studied. Here we describe efforts to understand how these variables affect the specific interactions between galectin-3 and carbohydrate ligands, and between vancomycin and its cognate binding partner L-Lys-D-Ala-D-Ala and how force microscopy can best be utilized to study solution phase molecular recognition.
1.3 Thermodynamics characterizing non-covalent association

1.3.1 Equilibrium in solution and at surfaces

The ultimate goal or our program is to develop MRFM for the routine evaluation of thermodynamic parameters for immobilized binding partners. The achievement of this goal is non-trivial, for several reasons. An immobilized complex under force exists in a state far removed from equilibrium.\(^57-59\) Moreover, forced ligand-receptor unbinding is an irreversible process. By contrast, solution phase equilibria between freely diffusing molecules is a microscopically reversible process for which measured phenomena can be related to thermodynamic state parameters.\(^59\)

A non-covalent solution phase association can be represented as

\[ A + B \rightleftharpoons AB \]  \hspace{1cm} (1)

where \(A\) is a monovalent chemical species with affinity for monovalent species \(B\). Over sufficient time and constant pressure and temperature, the system will achieve equilibrium, at which point the net rate of formation of unbound species is equal to the rate of formation of bound complex (\(AB\)). The association constant \((K_a)\) for the system can be described as the ratio of the activity of bound complex to the product of the activities of unbound species \(a_A\) and \(a_B\).

\[ K_a = \frac{a_{AB}}{a_A a_B} \]  \hspace{1cm} (2)

Activity is a unitless value that approaches numeric equivalence with the concentration of a solute, typically expressed as molarity (moles / L), as the concentration of that solute approaches zero.\(^60\) Activity deviates from concentration
due to non-idealities caused by phenomena such as solvation and solute-solute interactions. Activity \(a_i\) and concentration \(C_i\) are proportionally related through an activity coefficient \(\gamma_i\),

\[
a_i = \gamma_i C_i
\]  

Activity coefficients take on positive integer values and approach unity as the concentration of solute approaches infinite dilution. Typically it is assumed that activity and concentration coalesce at concentrations less than \(10^{-3}\) molar. This assumption facilitates the calculation of association constants based on concentrations of species in solution; however the validity of this assumption for complex biomolecules is difficult to verify and deviations from ideality often significantly affect the behavior of dissolved solutes.

Although no studies have been reported that directly probe activity coefficients for immobilized molecules, tethering and immobilization may cause the activity of biomolecules to deviate significantly from ideality. Tethered molecules can no longer freely sample all space within the surrounding solvent, but instead remain constrained near a surface restricted to volumes defined by the effective radius of the molecule and its tether. Molecular crowding experienced by tightly packed monolayers may act to exclude solvent altering the solvation sphere around a molecule. Crowding may also prohibit a partner from binding or at least alter the geometry of the interaction.

Surfaces may also develop charge, depending on the composition of the bulk material comprising the surface. Charge localization at surfaces can strongly perturb
immobilized biomolecules through electrostatic interactions. Giessler and coworkers studied the effect of repulsive and attractive Coulombic interactions on the pKa of immobilized diacids.\textsuperscript{61} Their work demonstrated that pKa values for surface-immobilized maleic acids were shifted toward values higher than solution phase values. pKa values were further affected by alterations in the hydrogen bonding capacities caused by the ionic strength of the surrounding solution. Although these pKa shifts do not provide direct quantitative insight into the effect of tethering on activity, we can infer, because molecular recognition events rely heavily on electrostatic interactions and hydrogen bonding, that activities of binding partners will be similarly perturbed by both surface charge and solution ionic strength.

Because binding constants are related to activities, and not concentrations, it is reasonable to imagine that immobilization might alter the nature of association between immobilized partners relative to solution phase binding. As immobilization affects affinity, so too is free energy affected based on the relationship between association constants and free energy ($\Delta G$),

$$\Delta G^\circ = -RT \ln K_a$$

(4)

where $R$ is the gas constant and $T$ is temperature in Kelvin.
Figure 2: Representation of equilibrium between immobilized partners.

AFM can be used to drive a tip-immobilized ligand into close proximity with a sample immobilized receptor. If the tip-sample distance remains less than the combined effective length of the tethers, then the system can equilibrate between bound and unbound states (Figure 2). Our efforts to develop a set of experimental protocols for probing equilibria by AFM are crucial for understanding association between these immobilized partners. Because both ligand and receptor are immobilized, a cantilever-based method for probing affinity using competitive equilibria establishes AFM as a powerful tool for determining binding constants which reflect the physical consequences of tethering both partners.
1.3.2 Multivalent ligand-receptor binding

1.3.2.1 Principles of multivalency

Force microscopy can be used to probe multivalent ligand-receptor interactions and the nature of additivity in ligand binding in a manner that precludes intramolecular binding and aggregation. Multivalency is utilized in myriad instances by nature to overcome weak binding of individual ligand-receptor pairs by clustering multiple receptors into high affinity multivalent systems.\textsuperscript{2, 3, 62, 63} Characterized by the concurrent formation of multiple associations between a multivalent ligand and a multivalent receptor, multivalent binding forms strong overall interactions from distributions of individually weak ones. Here, the valency of a molecule is defined as the number of separate associations that can be formed with another molecule through ligand-receptor interactions.

A broad array of biological processes, including antibody-antigen recognition, viral and bacterial binding to host cells, and other cell-cell interactions compensate for weak monovalent affinities by using enhancements gained through multivalency. A key example of such behavior includes, T-cell, platelet, and tumor cell binding to epithelial cells. Another example is the anchoring of influenza particles to bronchial epithelial cells, which occurs via interaction between densely packed hemagglutinin, a carbohydrate binding protein on the viral surface, and sialic acid moieties arrayed on the host cell surface.\textsuperscript{3}

Understanding the molecular basis of multivalency is fundamentally important for probing thermodynamic parameters that characterize the magnitude of multivalent
binding enhancements.\textsuperscript{64-66} Only by understanding these parameters can researchers hope to design multivalent molecules that specifically influence biological processes through multivalent interactions. In this way, progress can be made toward the lofty goal of designing multivalent therapeutics for treating diseases in ways that are fundamentally different from and more effective than monovalent drugs.\textsuperscript{67}

Multivalent interactions have unique properties that are distinct from monovalent interactions in a number of ways. First, multivalent associations predominantly display additive enhancements over analogous monovalent binding on a per ligand basis. Second, literature reviews have shown remarkable variations in the magnitude of these enhancements depending on the assay used. Although a relationship does exist between the assay and the magnitude of enhancement, there is no apparent general relationship between the valency of interaction e.g. mono-, bi-, or trivalent, and the magnitude of enhancement.\textsuperscript{2} Furthermore, the same multivalent interactions measured by different assays often display different affinities.\textsuperscript{68}

1.3.2.2 Thermodynamics of multivalent binding

The simplest formulation of multivalency involves a conceptual bivalent ligand associating with a bivalent receptor (Figure 3). Because thermodynamic parameters are state quantities, we can describe the overall binding process as the sum of the parameters describing each step. In stepwise binding, chelation begins with the initial association of a single ligand and receptor. Provided the second ligand can orient properly for binding, the second binding event follows in an intramolecular sense.
Figure 3: (A) Monovalent ligand-receptor association. (B) Intermolecular association of bivalent partners to form a chelate complex.

Thermodynamic parameters characterizing multivalent association can be parsed using the formalism of Jencks for additivity in ligand binding.\textsuperscript{69} For the bivalent case, any thermodynamic parameter characterizing association (\(\Delta J^o_{\text{bi}}\)) can be expressed as the sum of that parameter for each monovalent interaction (\(\Delta J^o_{\text{mono}}\)) plus a parameter encompassing the energetic consequence of physically tethering monovalent ligands and receptors together (\(\Delta J^o_{\text{int}}\)).

\[
\Delta J^o_{\text{bi}} = 2\Delta J^o_{\text{mono}} + \Delta J^o_{\text{int}}
\]  \hspace{1cm} (5)

Where “mono” refers to that parameter for the analogous monovalent association, and “int” refers to the interaction parameter, which accounts for the physical consequences of linkage.
When considering the overall change in free energy for chelate binding, the interaction parameter ($\Delta G_{\text{int}}$) can be expressed as a combination of the enthalpy ($\Delta H_{\text{int}}$) less the product of temperature ($T$) and entropy of interaction ($\Delta S_{\text{int}}$).

$$\Delta G_{\text{int}} = \Delta H_{\text{int}} - T\Delta S_{\text{int}} \quad (6)$$

The entropy of interaction can be further divided into terms describing translational, rotational, and conformational motion plus the entropy of solvation, i.e.

$$\Delta S_{\text{int}} = \Delta S_{\text{trans}} + \Delta S_{\text{rot}} + \Delta S_{\text{conf}} + \Delta S_{\text{solv}} \quad (7)$$

Enhancements in bivalent affinity occur when $|\Delta G_{\text{bi}}| > 2|\Delta G_{\text{mono}}|$, i.e. when $\Delta G_{\text{int}}$ is negative (favorable). This behavior is most commonly rationalized in entropic terms. The translational and rotational entropy ($S_{\text{trans}}$ and $S_{\text{rot}}$) of a molecule arise from its freedom to translate and rotate in space. During an initial association event between bivalent ligand and bivalent receptor a total of three translational and three rotational degrees of freedom are lost. Because the magnitude of $S_{\text{trans}}$ and $S_{\text{rot}}$ are related logarithmically to the mass and the dimensions of the molecule, respectively, tethering two molecules does not significantly alter their solution phase translational and rotational entropy. Therefore the magnitude of $\Delta S_{\text{trans}} + \Delta S_{\text{rot}}$ for monovalent binding is essentially equivalent to that for the association between bivalent partners, and a net entropic savings is realized during the subsequent intramolecular binding event that leads to a chelate complex, compared to monovalent binding.

Although this translational and rotational entropic savings must contribute to the thermodynamics of bivalent binding, other contributions, both entropic and enthalpic, must be considered. First, the entropic savings can be partially or completely
offset by conformational penalties incurred as conformational degrees of freedom are lost in the linker domain. This tradeoff between favorable translational and rotational entropy and unfavorable conformational entropy may sum to an overall unfavorable $\Delta_{\text{int}}$.

Enthalpic effects may also contribute to or dominate the underlying thermodynamics of bivalent associations. For a bivalent system composed of identical ligands and receptors, the enthalpic term for each individual interaction is presumed to be identical to the monovalent value. Enthalpic interaction contributions arising from tethering ligands can either diminish or enhance binding. $\Delta H_{\text{int}}$ certainly depends on the nature of the linker; specifically its length, flexibility, and ability to interact with ligand and receptor surface. Chelate binding may induce strain in the linker, causing unfavorable enthalpic changes. Furthermore, gauche and eclipsing interactions may develop as a result of restricting linker motion which will have deleterious effects on binding. Unfavorable interactions may also arise from contact between the linker and ligand or receptor surface. Conversely, favorable hydrogen bonding or hydrophobic interactions between linker and surface may stabilize the chelate complex and enhance binding.

The importance of enthalpic effects was demonstrated in previous work from our lab. The binding of a series of mono-, bi-, tri-, and tetravalent carboxylate ligands to Ca(II) was studied by ITC. Enhancements in affinity coincident with increasing valency were shown to be enthalpic in origin. These enhancements were attributed to the relief of repulsive ion-ion interactions between tethered carboxylate ions in the unbound
ligands during ligand binding. The contribution of enthalpic effects, therefore, should not be ignored.

1.3.3.2 Intermolecular binding, aggregation, and precipitation

Aggregation states are also important to consider during interpretation of binding data. Although intramolecular binding is frequently assumed, solution phase binding between a multivalent ligand and a multivalent receptor can also occur in an intermolecular fashion (Figure 4). Intermolecular binding can lead to cross-linked networks of multivalent partners, forming polydisperse aggregates of varying solubility. Following an initial binding event, the probability of intra- vs. intermolecular binding depends on the thermodynamics of the various competing reactions as well as a range of factors including, but not limited to, concentration, temperature, ionic strength, and pH. Although a competitive equilibrium may be established between intra- and intermolecular binding, the diminished solubility of cross-linked complexes can kinetically trap aggregates through an effectively irreversible precipitation.
Figure 4: Modes of bivalent binding in solution. Initial association can be followed by intra- or intermolecular binding. Subsequent intermolecular binding can form insoluble aggregates.

The potential for intermolecular binding, aggregation, and precipitation complicate the evaluation of multivalent binding affinities. Thermodynamic parameters consistent with aggregation rather than enhanced ligand-receptor affinity are well documented.\textsuperscript{65, 68} Furthermore, the formation of crystal structures comprising cross linked concanavalin A with dendritic ligands demonstrate the importance of insoluble aggregate formation.\textsuperscript{65} Competitive aggregation, therefore, significantly limits our ability to probe equilibrium thermodynamics of intramolecular multivalent association.\textsuperscript{2, 70, 71}

Commonly used solution phase assays including ultraviolet and fluorescence titration do not prohibit aggregation states, nor do they provide detailed structural information about the bound complex. Other assays suffer additional limitations. Hemmaglutination inhibition assay (HIA) evaluates the ability of a ligand to inhibit
aggregation caused by the hemagglutination reaction. Enzyme-linked lectin assay (ELLA) evaluates the ability of soluble ligand to inhibit the binding of a receptor to an immobilized ligand. Both HIA and ELLA report on processes only tangentially related to reversible equilibrium and provide IC$_{50}$ values, which are not necessarily equateable to binding constants. Isothermal titration microcalorimetry (ITC), although a powerful technique that provides reliable binding constants based on heat evolved during ligand binding, cannot distinguish between inter- and intramolecular modes of binding. Without detailed structural information about the products of complexation reactions, it remains difficult to directly relate observed phenomena, e.g. absorption or emission of light or evolution of heat, from solution phase assays to binding constants that describe reversible equilibrium of intramolecular multivalent association. Clearly an experimental method that unambiguously precludes aggregation is required to properly consider the nature of additivity in ligand binding.
Recently, we reported a technique for using AFM to determine affinities of tip-immobilized ligands to surface-immobilized receptors from threshold force measurements. A binding constant for the immobilized complex was determined from the variation of probability of bound complex formation as a function of soluble ligand concentration.\(^6\) Because binding partners are immobilized to surfaces (Figure 5), aggregation is prevented. This technique provides a means of probing multivalent affinity with a new level of detail. Furthermore, it provides a framework for the development of portable bio-sensing devices.
1.4 An AFM-based approach

1.4.1 Instrument design

Measurement of the displacement of a flexible cantilever in response to interactions between tip- and surface-immobilized molecules provides the basis for molecular recognition force microscopy. Figure 6 shows a basic AFM design, consisting of a sample, often comprised of crystalline silicon, glass, or mica, affixed to a piezo scanner positioned below the tip at the end of a flexible cantilever. A laser, positioned above and focused onto the free end of the cantilever, is reflected onto a position-sensitive photodiode detector. The cantilever is held in place by a fluid cell (not displayed) capable of maintaining a liquid environment at the tip-surface interface. Precise nano- and micro-scale movement of the sample, relative to the tip, in the X, Y, and Z direction is accomplished through the application of voltage to the piezo scanner.

The AFM measures forces that develop as the piezo scanner is driven through multiple approach-retract cycles in the Z-direction. During an approach-retract cycle the sample is first brought into contact with the tip and then retracted at a known velocity. Repulsive and attractive forces cause the cantilever to deflect from its equilibrium position, in turn causing movement of the laser across the photodiode detector. Laser movement is proportional to cantilever deflection which is in turn proportional to force. With accurate calibrations of photodetector sensitivity and cantilever spring constant, the forces generated by a non-covalently bound molecular complex between tip and surface can be monitored throughout the course of forced unbinding.
Figure 6: Schematic of select components of the AFM apparatus.

1.4.2 Force curves

Probing molecular recognition using AFM requires interpreting the biophysical significance of the movement of a flexible cantilever in response to interactions between immobilized binding partners. Such interpretation is accomplished by generating and analyzing force curves, or plots of force exerted on the cantilever as a function of position as the sample is driven through multiple approach-retract cycles in the Z-direction. In a typical experiment, one element of a ligand-receptor pair is immobilized to a cantilever tip and the other to a solid sample. At a given \((X,Y)\) position, the sample is driven toward the tip, in the Z-direction, until cantilever
deflection is observed\textsuperscript{29,72,73} or until tip-sample distance is sufficiently close to allow for ligand-receptor complexation.\textsuperscript{74} If complexation occurs, retraction of the sample applies force to the immobilized complex causing cantilever deflection and, ultimately, force-driven dissociation (rupture) of the complex.

Cantilever deflection, in turn, causes laser movement across the photodiode detector. Raw voltage signal from the photodiode is converted to force through two conversions. First the sensitivity of the photodiode detector is determined and used to convert voltage to deflection distance ($x$) in nanometers. Second deflection is converted to force ($F$) by assuming that the cantilever acts as a spring with linear elasticity using Hooke’s law ($F = k_c x$), where $k_c$ is the cantilever spring constant with units of pN nm$^{-1}$. A plot of force vs. Z-stage extension for a single approach-retract cycle provides a force curve from which both rupture force and length can be determined.

Inspection of a force curve provides insight into the dynamics of bond rupture under load. Figure 7 displays a representation of a force curve indicating tip-sample contact during approach and a single rupture event during retraction. During approach (A to C) the Z stage begins from a position that is separated from the sample (A). With no external load, the cantilever remains oscillating at an equilibrium position (1). As the sample is driven towards the tip, contact is made (B) and the cantilever begins to deflect upward (2). Maximum force is applied when the Z-stage is fully extended (C). It is important to note that contact forces act not only on the cantilever, but also on molecules compressed at the tip-sample interface. Compressive forces acting on these immobilized molecules may eventually cause denaturation and/or desorption.\textsuperscript{74}
Retraction of the Z stage results in a rapid decrease in contact force (C to D) as cantilever bending decreases. The cantilever returns briefly to its equilibrium position (3) before tip separation from the surface begins. At point D, the retraction curve inflects toward horizontal, signifying tip-sample separation.

Figure 7: Representation of a force vs. extension plot with evidence of a rupture event.

Hysteresis in the retraction curve arises from the extension of molecular tethers as load is applied to a bound complex causing downward cantilever deflection (4). A non-linear increase in force (D to E) followed by a sharp linear decrease (E to F) is
generally interpreted as evidence of an unbinding (rupture) event. Although the shape of the force curve resulting from tether extension (E to F) may vary, the convex form depicted here is often observed during extension of flexible oligo(ethylene glycol) tethers. Rupture forces ($F_{rup}$) are determined from the overall magnitude of vertical deflection that evinced rupture. The overall magnitude of deflection is defined as the vertical difference between the apex (E) and the midpoint of noise at the base of each rupture event (F).

Rupture length is defined as the difference in molecular extension between inflection point (D) and the apex (E) of the unbinding event. Following the forced rupture of all non-covalent interactions between tip and surface, the cantilever returns to equilibrium position (1). The probability of observing a rupture ($\rho_{bind} =$ number of rupture-containing pulls / total pulls) can be accurately determined using several hundred force curves generated with a single tip-sample system.

1.4.3 Physical proximity of receptor and ligand

Achieving physical proximity of receptor and ligand is essential for binding. Thus the approach phase of an approach-retraction cycle is a critical aspect of data collection. Bond formation is enabled by positioning tip and sample at a separation distance less than the combined length of tethered ligand and receptor (Figure 8A). As shown above, a typical method for ensuring close proximity involves driving sample and tip into contact, i.e. the point at which cantilever deflection begins. Contact forces can also deform and potentially degrade molecules at the tip-sample interface (Figure 8B).
Recent studies have suggested that high contact forces cause tip wear\textsuperscript{76}, binding partner desorption, protein denaturation\textsuperscript{77,78} and tip fouling,\textsuperscript{74, 79} events that alter or obscure the behavior of molecules under investigation. Although the extent to which MRFM experiments are affected by such processes depends on the stability of the individual molecular system, it seems reasonable that increased contacts force will have increasingly deleterious effects on even the most robust systems.

![Diagram](image)

**Figure 8:** Representation of potential results of tip-sample proximity (not to scale). (A) Tip-sample distance on the order of the total length of the molecular system. (B) High contact forces act on the cantilever and molecules at the tip-sample interface.

The most common method for controlled approach involves force-based triggering of sample movement. In this approach a sample is driven toward the tip until contact force crosses a threshold value, which triggers retraction of the sample. Typically force triggers on the order of several hundred piconewtons are employed.\textsuperscript{29, 73} Several groups, however, recently described data collection protocols that apply nanonewton scale force triggers to binding partners anchored by covalent or ionic bonds.\textsuperscript{80-83} Because nanonewton forces are sufficient to rupture even covalent bonds,\textsuperscript{19, 78}
it is likely that binding partners are denatured or degraded by repeated application of such high contact force.

Tip-sample proximity can significantly affect the distribution of rupture forces and lengths, as well as the probability of binding for a given molecular system. Probability of binding depends on both ligand-receptor proximity and on association rate. If close proximity is never achieved, then specific binding, i.e. complexation between ligand and receptor of interest, cannot occur. If binding partners are brought into close proximity without tip-sample contact (8A), then rupture forces reflect specific ligand-receptor bond strength, however, because molecular extension is a prerequisite for binding, rupture lengths will not accurately reflect the overall length of the molecular system. If tip-sample contact is made, then rupture forces and lengths may reflect specific bound interactions or may reflect some other non-specific binding mechanism, i.e. binding between species other than the ligand and receptor of interest. Protein denaturation and desorption can result in a diminished number of specific bound interactions per pull. However, if non-specific binding is indistinguishable from specific binding, then binding probabilities may be erroneously high. A systematic study of the effect of compressive force on binding probability, blocking efficiency, rupture force, and rupture length is described in Chapter 2.

1.4.4 Immobilization of molecules to tip and surface

In order to diminish the lifetime of a non-covalently bound complex by the application of force, it is essential to immobilize each binding partner to the surface of a
macroscopic object that can be physically manipulated and detected. For cantilever-based MRFM, the cantilever serves as macroscopic force transducer, while the tip serves an anchor point for the immobilization of a binding partner. Cantilever tips are typically fabricated from silicon (Si) or silicon nitride (Si$_3$N$_4$). Nanoscale radii of curvature of commercially available tips range from 2 nm to 60 nm facilitating nanoscale contact over areas of $\sim$12 to $\sim$11x10$^3$ nm$^2$. The binding partner is typically immobilized to a solid support such as a polymeric bead,$^{30, 31, 34}$ amorphous glass,$^6$ freshly cleaved mica,$^{34, 38}$ or crystalline silicon$^{84}$ which is in turn affixed to a piezo scanner and physically manipulated in the X, Y, and Z directions.

1.4.5 The physical consequences of tethering

Our approach to affinity measurement using competitive equilibria establishes AFM as a powerful tool for evaluating binding in a configuration that reflects the physical consequences of tethering both mono- and multivalent partners. As discussed previously, we intuitively expect tethering and immobilization to cause the activity of biomolecules to deviate from ideality. Immobilization may denature proteins or preclude partners from orienting properly for binding. Furthermore, tethered molecules can no longer freely translate throughout the surrounding solvent, but remain constrained near a surface and are capable of sampling volumes defined by the effective length of the tether.$^{85}$ Tethering a molecule to a macroscopic surface thus significantly limits both translational and rotational entropy. It follows then that the magnitude of $\Delta S_{\text{trans}} + \Delta S_{\text{rot}}$ for interactions between immobilized partners may be
significantly different than that of the corresponding interaction between soluble partners. Analogous to chelate binding described earlier, net rotational and translational entropic savings can result from the association of immobilized partners. These entropic savings may be partially or completely offset by conformational penalties incurred through limiting linker mobility. Because activity and entropy directly affect affinity, it is conceivable that immobilization may have a significant impact on affinity.

For these reasons it is imperative that molecular design and immobilization strategies be chosen judiciously. Efficient protocols for the rapid, robust, and uniform adsorption of oriented binding partners to surfaces and tips will facilitate accurate and reproducible data collection for studying thermodynamics using MRFM. Such strategies are described below.

1.4.6 General criteria for immobilization

Several factors must be considered when choosing an immobilization strategy. Generally, the immobilized molecules must not desorb from the tip-surface interface under experimental conditions and must maintain their ability to bind. We have previously compiled a number of criteria for effective immobilization. First, because heterogeneities in surface orientation produce heterogeneities in the affinities of the immobilized molecules, it is necessary to immobilize binding partners with a specific, defined orientation. Second, immobilization should form stable molecular systems that remain intact during functionalization reactions, in aqueous environments during binding studies, and under the force required to rupture the complex of interest. Third,
the immobilized molecules at the tip-surface interface must retain their ability to form a bound complex. Fourth, non-specific binding must be minimized. Finally, the surface density of immobilized molecules must be sufficient to observe binding within nanoscale tip-sample contact areas.

1.4.6.1 Heterogeneity vs homogeneity

Immobilization strategies can be broadly grouped into three classes; non-specific physisorption (Figure 9A), semi-specific adsorption to one of several reactive moieties, often lysine or cysteine residues (9B) on protein or ligand surface, and site-specific adsorption to a single, orthogonally reactive moiety on the protein or ligand surface, often at the C- or N-terminus (9C). Although easier to accomplish experimentally, non-specific and semi-specific methods have inherent limitations for AFM binding studies. The random or semi-random nature of adsorption may result in binding site occlusion, prohibiting a significant fraction of immobilized partners from binding. Moreover, if numerous partners are immobilized in a heterogeneous manner, no single binding constant will accurately describe the affinities of the ensemble.

The magnitudes of association constants have been shown to be highly dependent on immobilization strategy. Vijayendran and Leckband reported a range of association constants for immobilized antibody-antigen interactions. Antibodies, immobilized to glass surfaces via non-specific, semi-specific, and site-specific adsorption protocols, displayed affinities for soluble antigen ranging from $6.1 \times 10^6$ to $7.1 \times 10^7$. All affinities were 1-2 orders of magnitude lower than the analogous solution-phase affinities.
Figure 9: Immobilization strategies for receptors (top) and antibodies (bottom). (A) Random physisorption. (B) Semi-specific covalent adsorption. (C) Site-specific, oriented covalent adsorption.

Vijayendran and Leckband suggested that non-specific adsorption strategies introduced heterogeneities in orientation and binding site occlusion that were responsible for inconsistencies in affinity. They demonstrated that homogeneous, site-specifically adsorbed proteins exhibited more uniform binding and kinetic constants. In a separate study, homogenously-oriented antibody fragments were shown to provide more reproducible data for AFM-based binding experiments than configurations using randomly oriented antibodies. It is therefore imperative that experimental protocols are chosen to provide oriented site-specific adsorption of both binding partners.

1.4.6.2 Stability of immobilized partners

Immobilization protocols generally require multi-step procedures involving molecular conjugation in both aqueous and organic environments. In many cases, a reactive monolayer is first deposited on tip and sample. Subsequent conjugation of heterobifunctional tethers and binding partners is carried out at room temperature or under elevated temperatures and pressures. The resulting systems are exposed to
atmospheric gasses and to aqueous buffers during storage and data collection, and immobilization protocols should form stable molecular systems that facilitate attachment of various organic and biological molecules. These systems must tolerate prolonged exposure to air as well as organic and aqueous environments over a range of temperatures.

A key requirement for AFM binding studies is that the immobilized partners do not desorb under the force required to rupture the complex of interest. If this criterion is not met, then there is no way to assign rupture data to complex unbinding. Because the strengths of specific and non-specific interactions are similar in magnitude, random physisorption does not typically meet this requirement. Conversely, forces required to rupture specific non-covalent interactions are typically weak (on the order of $10^1$-$10^2$ pN) compared to covalent bonds ($10^3$-$10^4$ pN). Thus, stable adsorption is best achieved through covalent attachment. While modern synthetic conjugation methods are readily applied to covalent immobilization of most small molecules as well as to proteins containing single cysteine residues, engineering orthogonal reactive moieties into biomolecules with multiple cysteine or lysine residues poses significant difficulties.
Figure 10: Site directed nickel-nitrilotriacetate (Ni-NTA) immobilization of recombinant C- or N- terminal hexahistidine (his6) tagged proteins.

Figure 10 shows the use of site directed nickel-nitrilotriacetate (Ni-NTA) chelation of recombinant C- or N- terminal hexahistidine (his6) tagged proteins for oriented immobilization. NTA-Ni-his6 complexation is routinely used for affinity purification and does not generally diminish receptor-ligand affinity. Furthermore, this technique has previously been utilized to anchor his6 tagged protein to NTA terminated monolayers for AFM binding studies.\textsuperscript{25,91-94} Although the forces required to rupture NTA-Ni complexes are small (on the order of several hundred piconewtons),\textsuperscript{25} compared to covalent bonds, the stability of the system is often sufficient for probing weaker (e.g. protein-carbohydrate) non-covalent interactions.

1.4.6.3 Regiochemistry of tethering and tether flexibility.

Molecules immobilized at the tip-surface interface must retain the ability to form a bound complex. The method of immobilization must not denature the molecule or
occlude binding sites and must allow the adsorbed molecules to orient properly for binding. Tethers should also be of sufficient length to facilitate the resolution of rupture events from tip-surface adhesion in force vs. extension plots. This condition is frequently achieved by using long flexible tethers that allow partners to orient freely within a given hemispherical volume, defined by the effective length of the linker-partner conjugate.21,25

The dependence of affinity on linker length is well documented.40,41 Antibodies tend to display higher antigen capture efficiencies when immobilized via longer poly(ethylene glycol) tethers as compared to either short tethers40,41 or random physisorption to a surface.95,96 This trend is frequently attributed to greater availability of the antigen binding site due to the conformational mobility of longer tethers.

In order to ensure that immobilized molecules retain the ability to bind, attention must be paid to the site and method of tether attachment. Tether attachment should neither sterically occlude key contacts between ligand and receptor nor otherwise inhibit binding through interactions between tether and protein surface. Additionally, immobilization protocols should neither denature nor degrade protein, ligand, or the underlying reactive monolayer.

Oligomers of ethylene glycol are well suited to function as flexible tethers for the conjugation of binding partners to surfaces. These moderately hydrophilic polymers have a high degree of conformational flexibility in aqueous media. Reactive functionality that facilitates conjugation can be introduced at one end of the oligo(ethylene glycol) (OEG) polymer through well-established desymmetrization
Conjugation of OEG chains to biomolecules is a well established technique for the development of biocompatible therapeutic antibodies with nonimmunogenic and nonantigenic properties. The antibody-OEG conjugates maintain their ability to bind therapeutic targets while evading immune response from the host organism. Based on these attributes, heterobifunctional poly(ethylene glycol) tethers have been described as the “ideal spacer” for MRFM studies.

1.4.6.4 Non-specific binding

A key aim of MRFM studies is the characterization of specific bound complexes between a tip-immobilized ligand and a sample-immobilized receptor. Non-specific adsorption of protein or ligand to other elements of the tip-sample interface, including, but not limited to, molecular tethers, underlying monolayers, or unprotected elements of the underlying solid may obscure specific rupture events and significantly complicate data analysis. Non-specific binding indistinguishable from specific events complicates the interpretation of experimental results, including rupture forces, lengths, and binding probabilities.

Oligomers of ethylene glycol also function as protective layers that confer non-fouling properties to surfaces. Surfaces presenting well ordered oligo(ethylene glycol) monolayers resist non-specific adsorption of proteins from solution. Although the amount of protein adsorption has been shown to vary monotonically with the length of the OEG monolayer, SAMs presenting hexa(ethylene glycol) groups have been shown to effectively protect surfaces from protein deposition. Furthermore, mixed monolayers comprising protective OEG chains interspersed with reactive OEG
chains have been shown to facilitate specific binding while maintaining non-fouling properties.\textsuperscript{101, 102} In order to minimize non-specific binding, both underlying monolayer and extendible tether must not display affinity for either ligand or receptor. A body of work has shown that the non-adhesive properties displayed by hexa(ethylene glycol) groups are also displayed by longer (≥10 nm) tethers typically used for MRFM.\textsuperscript{21, 29, 103, 104} Oligo(ethylene glycol) polymers are therefore useful both as non-adhesive tethers and as underlying protective monolayers.

1.4.6.5 Surface density

Precise control of adsorption chemistry is necessary to facilitate the production of surfaces with tailorable uniform densities of reactive groups. Isolated clusters of reactive groups may hinder reproducibility of rupture forces and binding probabilities. Immobilization protocols should produce stable molecular systems that facilitate facile conjugation and uniform distribution of binding partners on both the sample and tip.

1.4.7 Examples of tip and surface immobilization

1.4.7.1 Random Physisorption

Pioneering examples of molecular recognition force microscopy described the force-driven dissociation of non-covalently bound complexes comprising biotin and streptavidin. Immobilization was achieved by means of non-specific physisorption of biotinylated bovine serum albumin (BBSA) to surfaces. In the first example (Figure 11A), Florin, Moy, and Gaub described the formation of a BBSA adhesion layer on silicon nitride cantilevers.\textsuperscript{30, 31} Streptavidin, a tetrameric protein with four biotin binding sites,
was specifically immobilized to biotin moieties on BBSA. Binding experiments were performed using tip-immobilized streptavidin and a biotinylated polymer bead. In the second example, Lee, Kidwell, and Colton described the formation of a BBSA adhesion layer both on mica surfaces and on glass beads glued to cantilevers (Figure 11B).\textsuperscript{34} Streptavidin was immobilized to BBSA-modified mica surfaces and binding between tip and surface was probed.

![Diagram](image)

**Figure 11:** Examples of early methods for tip and sample prep using non-specifically bound biotinylated BBSA as an adhesion layer for streptavidin.

Although successful for the first AFM-based binding experiments, these methods have several important drawbacks. A fundamental assumption was that non-specific adsorption of BBSA to mica, glass, and silicon nitride occurred “spontaneously and
Although this condition may hold under zero force conditions, BBSA desorption under the force required to rupture streptavidin-biotin bonds is quite probable. Furthermore, the adhesive nature of BSA does not necessarily facilitate the minimization of non-specific interactions. Also, the non-specific nature of BBSA adhesion undoubtedly resulted in heterogeneities in the orientation of surfaced bound biotin. A significant amount of reactive sites were likely improperly oriented or completely prohibited from binding.

1.4.7.2 Oriented covalent adsorption

Whereas pioneering MRFM studies relied on random non-specific adsorption of binding partners, subsequent experiments probed the advantages of specific, oriented covalent adsorption using flexible non-fouling tethers. Strategies for the covalent modification of AFM tips and samples were developed to produce widely spaced functionality that facilitates binding between single molecular pairs. As previously described, cantilevers are commonly fabricated from silicon or silicon nitride. Conventionally, these materials are either 1) coated with a thin layer of gold which supports monolayers of alkanethiols, or 2) covalently modified using ethanolamine or organic silanes that react with the amorphous native silicon oxide layer at the surface-air interface.

These methods alleviate many of the drawbacks of random physisorption. Covalently immobilized layers are more resistant to force driven desorption. Furthermore, reactive layers can be conjugated to extendible OEG tethers that minimize non-specific interactions and can be covalently linked to binding partners.
Nonetheless, these methods have several important drawbacks that limit the precise control of adsorption chemistry and the stability of the adsorbed layers.

Figure 12: Covalent immobilization strategies: Silanization and subsequent coupling of tethered ligands to AFM tips and chemisorption of thiolated antibodies to gold.

Ros and coworkers described a method for probing fluorescein-antibody binding (Figure 12A). Flat gold surfaces were first protected with a monolayer of mercaptoethanesulfonate. Anti-fluorescein antibodies with C-terminal cysteine residues were anchored by Au-S bonds formed by displacement of protective sulfonate thiols. Although widely used for AFM, the stability of chemisorbed thiol layers on gold depends heavily on the density of monolayer packing. While straight-chain alkanethiols form densely packed, stable semi-crystalline monolayers, minor structural differences greatly affect stability. Thiols bearing functionality, including reactive amines, acids, or biomolecules often form diffuse layers susceptible to desorption by oxidation or displacement by other thiols. Although mixed monolayers can be used
to space functional groups within a well-ordered protective layer,\textsuperscript{108} reactive thiols do not necessarily distribute evenly, but rather form phase separated domains.\textsuperscript{106} Furthermore, Au-S bonds are less stable than many other bonds including covalent C-C bonds and ionic NaCl bonds (Table 1).

**Table 2: Homolytic bond dissociation energies**

<table>
<thead>
<tr>
<th>Bond</th>
<th>Dissociation Energy (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au-S</td>
<td>224.7</td>
</tr>
<tr>
<td>Na-Cl</td>
<td>242.6</td>
</tr>
<tr>
<td>Si-C</td>
<td>326.8</td>
</tr>
<tr>
<td>Si-O</td>
<td>498.4</td>
</tr>
<tr>
<td>C-C</td>
<td>607</td>
</tr>
<tr>
<td>C-N</td>
<td>945.3</td>
</tr>
</tbody>
</table>

Fluorescein antigens were tethered to silicon nitride tips by Si-O-Si bonds (Figure 12B). Tips were first oxidized in acidic peroxide solution. Silanization with aminopropyltrimethoxy silane (APTMS) provided a layer of reactive amines. Coupling of fluorescein-poly(ethylene) glycol-NHS ester provided a means of antigen immobilization that alleviated many of the problems of non-specific methods, however, significant drawbacks remained. Silanization with APTMS forms polydisperse aggregates with variable degrees of cross linking, and precise control of layer thickness and uniformity is limited. Furthermore, reactivity of amines within these multilayers is highly variable.
making precise control of the surface density of ligands difficult. Although thermodynamically stable, Si-O bonds remain susceptible to hydrolysis.

**Figure 13: Ethanolamine etherification of tips and mica surfaces and subsequent conjugation of reactive tethers and binding partners.**

Hinterdorfer and coworkers have described a method for probing polyclonal antibody-antigen binding.38 This study conclusively demonstrated the utility of covalent immobilization of binding partners through the use of long extensible tethers. Anti-HSA (human serum albumin) and HSA, covalently attached to tip (Figure 13A) and surface (Figure 13B), respectively, by 8nm long heterobifunctional poly (ethylene glycol) tethers, retained their capacity to bind throughout numerous approach-retract cycles in a single lateral position. Unbinding events, occurred at molecular lengths up to 30 nm, and were well resolved due to extension of the flexible tethers. The underlying monolayer was formed by etherification of the oxide layer on silicon nitride cantilevers and mica surfaces with ethanolamine.
This method also has several important drawbacks. Irreproducible oxide distribution precludes precise control of subsequent adsorption chemistry. Also, ethanolamine layers are anchored by Si-O bonds, which are susceptible to hydrolysis in aqueous environments. Therefore the need still exists for new reliable protocols for the tailorable, stable covalent functionalization of silicon nitride and silicon using thermodynamically stable bonds, such as Si-C or N-C bonds that are also stable to hydrolytic scission.

1.5 Thermodynamics of single molecule interactions.

1.5.1 Overview

Our goal is to use the AFM to probe thermodynamic parameters of specific non-covalent interactions between single immobilized molecules. Many fundamental properties of matter including the compressibility of gasses and the viscosities and surface tensions of liquids can be rationalized by the strength and direction of forces acting between molecules. The consideration of forces acting on biomolecules gives insight into the dynamics of biological processes that are driven by force-sensitive intermolecular interactions. Myriad examples exist of enzymes using strain to destabilize substrates towards high energy transition states thereby speeding the conversion of substrate to product. Supramolecular springs and ratchets serve as mechanochemical engines of motility for cells by exerting forces through the arrangement protein subunits into filaments and bundles that extend and collapse in response to molecular signals.\textsuperscript{109} Motor proteins such as kinesins transport
comparatively massive cargos throughout cells. Energy derived from ATP hydrolysis is used to drive kinesin movement stepwise along microtubule filaments generating enough force to move vesicles and organelles. In all cases, forces generated by molecular recognition events dynamically affect reaction rates. Molecular recognition and molecular strain provide specificity, directionality, and control in biological systems.

1.5.2 Force removes systems from equilibrium

Force can be used, much like temperature and pressure, as a variable to affect the progression of chemical reactions. The application of force can drive reversible processes to favor reactants or products. AFM can be used to control the force on a molecular complex, thereby changing its thermodynamic stability, increasing or decreasing free energy differences between bound and unbound states, and altering rates of dissociation. Pulling on a bound complex forces it from a state of equilibrium and lowers kinetic barriers to dissociation. By exerting directional force, reactions are driven to completion along direction-specific reaction coordinates. Reactions under force transiently sample high energy transition states allowed by the vector and speed of pulling.

Because the process of forced unbinding is carried out far from equilibrium, consideration of thermodynamic parameters for the force-driven dissociation of individual complexes is not straightforward. In the absence of force, a single ligand-receptor complex is allowed to freely sample energetic states around the global minimum of a potential well. Given enough time, the bond may be thermally induced to
cross the activation energy barrier for unbinding, escape the potential well, and dissociate under zero force. The application of force removes the system from equilibrium by tilting and deforming the energy well, lowering the kinetic activation energy barriers for dissociation.¹¹¹⁻¹¹³ Once the bond between ligand and receptor is broken by the action of force, each binding partner essentially experiences infinite dilution as tip and sample are separated.⁶⁰ Thus, force driven dissociation is effectively an irreversible process, because experimental conditions preclude microscopic reversibility.

1.5.3 No direct relationship between forces and free energy

Rupture forces obtained from force curves are indirect measurements of the force required to dissociate a bound complex. Bond rupture under AFM conditions is a stochastic, non-deterministic process for which transitions to future (unbound) states of the system are described by probability distributions. Repeated applications of force to the same bound complex at the same loading rate and duration will result in a distribution of rupture forces. Bond strength under load corresponds to the force that most frequently produces a rupture.⁶⁰

Investigations into the dependence of rupture force on solution phase affinity for various ligand-receptor complexes have been reported. Although no direct correlation between rupture force and solution phase free energy of interaction is apparent, a linear relationship between unbinding forces and enthalpy of interaction has been reported. ³¹, ¹¹⁴ Moy and coworkers suggested that the proportionality between
enthalpy change and rupture force implies a negligible entropic contribution to forced ligand-receptor dissociation. Any entropic changes that occur after the point that applied force exceeds rupture force may influence the free energy of the system, but are immaterial to the rupture force.31 Chilkoti and coworkers substantiated these findings and further demonstrated that rupture forces are more closely related to the activation enthalpy for unbinding and not equilibrium enthalpy. Their work supported the theory that force measurements do not directly probe entropic contributions to equilibrium and are therefore not directly linked to overall free energies of interaction.114

1.5.4 Relating near-equilibrium bond strength to kinetic and thermodynamic parameters

As the loading rate changes, so does the distribution of rupture forces.112 Rupture forces for bound complexes under dynamic loading rates have been used to probe kinetic barriers to unbinding. Rupture forces increase linearly with the logarithm of the loading rate. Extrapolation of this trend to zero force provides an approach to determine kinetic off-rates and the energy required to reach the transition state from a complex at equilibrium. In practice, however, this dependence deviates from linearity at near equilibrium (zero-force) conditions, thereby rendering linear extrapolations inaccurate.111 At high loading rates, chemical bonds sample energy states that are far from equilibrium transition state energies. At slower, near-equilibrium, loading rates, rupture forces asymptotically approach a minimum plateau since the bound complex samples energy states that approach those of the equilibrium transition state.
Rupture forces for a given molecular interaction also depend on tether stiffness and on the stiffness of the surfaces to which they are anchored. The dependence of rupture force on tether stiffness was originally demonstrated by Evans.\textsuperscript{111} Friddle and coworkers recently noted an important dependence of rupture force, not only on tether stiffness, but also on cantilever flexibility.\textsuperscript{115} The overall shape of the potential energy landscape describing forced unbinding was shown to depend on both ligand-receptor interaction energy and cantilever flexibility. At near-equilibrium conditions, changes in cantilever stiffness changed the shape of the potential energy surface, thus changing the magnitude of unbinding force. This observation was proposed to account for up to 10-fold variation of rupture forces reported by different groups for the same interactions at equivalent loading rate.\textsuperscript{116}

Friddle further demonstrated that at sufficiently low loading rates rupture forces become independent of loading rate and vary, instead, as the square root of the product of cantilever stiffness and the equilibrium free energy change between the bound and unbound states.\textsuperscript{115} This equality allows for the estimation of free energy differences by measuring rupture forces using cantilevers of varying stiffness. Although a relatively straightforward method for probing thermodynamic values characterizing immobilized partners, its direct applicability to biomolecular receptor-ligand interactions has yet to be demonstrated.

Furthermore, the method is predicated on the assumption that dissociation reactions are driven to completion along direction-specific reaction coordinates defined by the angle of pulling. Ke and coworkers demonstrated the significance of pulling
geometry induced errors in single molecule rupture force measurements. Given the importance of the “pulling angle effect” (Figure 14), the assumption of a single unbinding pathway may be significantly in error.

![Figure 14](image)

**Figure 14** Potential variations in pulling geometry. (A) Vertical alignment of ligand and receptor. (B) Lateral offset results in a complex that is extended at an angle $\theta$ from the vertical.

1.5.5. Unzipping of an RNA helix using Jarzynski relationship

Collin and coworkers used Crook’s fluctuation theorem to describe the work fluctuations associated with the forward and reverse changes in a system as it is driven from equilibrium by an external force. A consequence of Crook’s theorem is the Jarzynski equality, which relates the equilibrium free energy difference between two states of a system to the work required to switch the system between states. This relationship has been used to estimate thermodynamic parameters for reversible folding and unfolding of RNA hairpins stretched by optical tweezers. Although valid for microscopically reversible processes, this approach is limited as a method for probing the thermodynamics of molecular association.
1.6 Avoiding the limitations of force-based experiments

1.6.1 Thermodynamics from kinetic parameters \((k_{on} \text{ and } k_{off})\)

Hinterdorfer and coworkers described an AFM-based method for determination of a binding constant for polyclonal antibody-antigen binding through the estimation of the kinetics of association and dissociation.\(^3\) The main difference between this and previous approaches is that thermodynamic parameters were based entirely on measurements of time, distance, and probability without invoking rupture forces collected under non-equilibrium conditions to describe properties of a system at equilibrium. In this study, approach-retraction curves were generated by oscillating the tip in the Z-direction while passing laterally over a single antigen. The effective length of the tethered molecular assembly was determined by plotting the probability of binding as a function of lateral position. A rate constant for the association of immobilized antibody to immobilized antigen \((k_{a,ii})\) was then estimated from the normalized probability of binding during the time tip-sample distance was less than the overall effective length of the molecular assembly. Binding interactions were then blocked by the addition of soluble antigen. A rate constant for the dissociation of soluble antigen from immobilized antibody \((k_{d,is})\) was then estimated from the recovery time for binding to return after rinsing with buffer. The main limitation of this approach is that it reports an equilibrium constant based on the ratio of rate constants for the association of immobilized antigen and the dissociation of soluble antigen \((k_{a,ii} / k_{d,is})\), rather than the affinity between two immobilized binding partners.
1.6.2 Competitive binding

Our approach to the evaluation of surface and solution phase thermodynamic parameters relies on use of a force signature, derived from a force vs. extension plot, to differentiate bound complex from no complex during an approach-retraction cycle. A binding constant for the immobilized ligand-receptor complex is determined from the variation of binary probability of bound complex formation as a function of soluble ligand concentration. The main advantage of this approach is that thermodynamic parameters are based entirely on probability of binding without invoking rupture forces collected under non-equilibrium conditions to describe properties of a system at equilibrium. This methodology was first demonstrated using an immobilization system of the carbohydrate binding protein galectin-3 (G3) and lactose.\textsuperscript{6}

Figure 15: Competitive equilibrium probed in the presence of soluble ligand. Immobilized protein (P\textsubscript{i}) binds free ligand (L\textsubscript{f}) with an association constant K\textsubscript{if}. P\textsubscript{i} binds immobilized ligand (L\textsubscript{i}) with an association constant K\textsubscript{ii}.
Competitive equilibrium was established upon addition of soluble ligand to the system (Figure 15). Binding of immobilized protein (Pi) to free ligand (Lf) formed bound complex (PiLf) with an association constant described by $K_{if}$. Binding of immobilized protein (Pi) to immobilized ligand (Li) formed bound complex (PiLi) with an association constant described by $K_{ii}$.

**Figure 16:** (A) In the absence of soluble ligand, surface sites remain unblocked. A force curve indicates binding. (B) Sufficiently high concentrations of soluble ligand will block all available surface sites. No specific binding events are evident.
In the absence of soluble ligand, the fractional occupancy of sample-immobilized receptors was zero; all binding sites were unblocked and complexation was possible between immobilized partners (Figure 16A). As soluble ligand was titrated into the system, binding sites become populated and the probability of observing a rupture event decreased as fractional occupancy increased. At sufficiently high concentrations of soluble ligand, surface sites became saturated and the probability of observing a specific binding event was at a minimum. A binding isotherm was fit to an expression derived from the binding polynomial for the competing equilibrium. An association constant (K_{ii}) for immobilized receptor-ligand binding was determined. Because both ligand and receptor were covalently tethered to surfaces, the binding constant reflected the physical consequences of tethering both partners. Furthermore, aggregation states could not contribute to the observed phenomena of binding. The approach is a powerful new method for probing both monovalent and multivalent binding at a new level of detail.
2. The effect of contact force on molecular recognition force microscopy

2.1 Overview

Non-covalent associative forces dramatically affect virtually all biological processes\(^2,3,65,67\) and are the basis for many biosensing technologies.\(^4,5\) Because of its ability to spatially manipulate and measure forces between individual molecules, the atomic force microscope (AFM) is well suited for the consideration of non-covalent interactions. Our goal is to develop AFM methodologies that facilitate evaluation of thermodynamic parameters characterizing specific non-covalent interactions between single immobilized molecules. Such methodologies will allow us to better understand the behavior of interacting species, both at surfaces and in solution.

Molecular recognition force microscopy (MRFM)\(^27-29\) is a cantilever-based technique that has been widely applied to the study of protein unfolding\(^18\) and biomolecular association, both in aqueous and non-aqueous environments. Non-covalent rupture forces between biotin-avidin,\(^30-33\) complementary DNA strands,\(^35-37\) antibody-antigen,\(^38\) and protein-carbohydrate\(^6,43\) 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,\(^6,18,30,31,35,36,38,43\) the nature and geometry of immobilization,\(^40\) and composition of the solution in which molecular interactions take place.\(^41,56\)
Our approach to the evaluation of surface and solution phase thermodynamic parameters relies on use of a force signature, derived from a force vs. extension plot, to differentiate bound complex from no complex during an approach-retraction cycle. Recently, we reported a threshold force-based AFM sensing technique using the carbohydrate binding protein galectin-3 (G3) and tip-immobilized lactose. A binding constant for the immobilized lactose-G3 complex was determined from the variation of binary probability of bound complex formation as a function of soluble ligand concentration.6

The formation of non-specific complexes complicates force curve analysis, masking the true fractional probability of specific complex formation. In order to better understand the extent to which experimental design affects the behavior of interacting species immobilized on force microscope tips and sample surfaces, we carried out a systematic evaluation of the effect of contact forces on MRFM force signatures. The results of this investigation provide fundamental insights into the proper design and accurate interpretation of MRFM experiments.

Because cantilever behavior is interpreted in terms of specific non-covalent interactions, a robust, reproducible methodology is needed to isolate these interactions and resolve them from other non-specific tip-sample interactions. The forces required to rupture specific non-covalent interactions are typically i) weak (on the order of $10^1$-$10^2$ pN) compared to covalent interactions ($10^3$-$10^4$ pN),19 ii) operative over the same length scales as non-specific interactions and iii) similar in magnitude to non-specific adhesion forces.
In a typical experiment, each element of a ligand-receptor pair is immobilized to a cantilever tip or sample.\textsuperscript{21, 25} Most frequently, 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 vs. extension provides a curve from which the rupture force and length can be determined.

Achieving physical proximity of receptor and ligand is essential for binding. On the other hand compressive strain is believed to cause both protein denaturation\textsuperscript{77, 78} and tip fouling.\textsuperscript{74, 79} Efforts have been made to avoid these complications by using a method of ‘compression-free’ force spectroscopy, in which close proximity is achieved without tip-sample contact.\textsuperscript{74} Additionally, Vander Wal and coworkers found that contact forces ranging from 100 pN to 10 nN caused significant increases in the most probable rupture force of a DNA duplex.\textsuperscript{119} To date however, no systematic study of the effect of compressive force on binding probability, blocking efficiency, rupture force, and rupture length has been conducted.

Here we investigate the effect of applied contact force 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 vs. extension plots. The cognate ligand-receptor pair lactose-G3 along with two control systems with no known affinity, mannose-G3 and lactose-2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, were studied.
2.2 Molecular assemblies for binding studies

2.2.1 Immobilization of saccharides to AFM tips

A goal of MRFM is to study the force driven dissociation of specific non-covalent ligand-receptor complexes between tip and surface-immobilized binding partners. Modification of silicon nitride AFM tips for molecular recognition binding studies is typically achieved by covalent linkage one binding partner to an extendible tether, which is, in turn, covalently linked to the tip surface.\textsuperscript{21, 29, 40-42, 52, 72, 73, 103, 104} The relative thermodynamic stability of covalent over non-covalent bonds is sufficient to ensure against ligand/receptor desorption from the tip during force driven dissociation of non-covalent complexes. In order to minimize non-specific binding, the extendible tether must not display affinity for either ligand or receptor. Tethers must also have sufficient conformational flexibility to properly orient the system for binding and should be sufficiently long, when fully extended, to facilitate the resolution of rupture events from tip-surface adhesion in force vs. extension plots. Heterobifunctional poly(ethylene glycol) tethers have been shown to be suitable for MRFM studies.\textsuperscript{75}

For this study we required a method to covalently conjugate saccharides to long (>5nm), flexible, non-adhesive tethers which are covalently bound to the surface of the tip. The tether should be covalently linked to a region of each ligand that will not inhibit its ability to form a bound complex. With this requirement in mind, we endeavored to prepare derivatives of lactose and mannose with a thiol terminated alkyl chain at the reducing end of each saccharide; such thiols would facilitate conjugation through reactive maleimide moieties on surface immobilized poly(ethylene glycol) chains. Such
a molecular assembly should achieve ligand immobilization with sufficient conformational flexibility to properly orient the system for binding and should not interfere with ligand-receptor interaction.

Figure 17 describes the molecular assemblies used for AFM tip modification for this investigation. Plasma oxidized silicon nitride AFM tips were aminosilanized with aminopropyltrimethoxy silane (APTMS) and the resulting amine layer was coupled to a 10 nm heterobifunctional poly(ethylene glycol) tether comprised of maleimide-dPEG$_{24}$-NHS ester (NHS-PEG$_{24}$-Mal, Quanta Biosdesign). Conjugate addition of thiopentyl saccharide derivatives 1 and 2 provided immobilized mannose and lactose. Lactose forms a specific complex with galectin-3. Mannose, a molecule for which galectin-3 has no known affinity, was used as a negative control.
Figure 17: Molecular assembly covalently linked to a silicon nitride AFM tip. Thiopentyl mannose (1) and thiopentyl lactose (2) are conjugated to an aminosilanized silicon nitride tip through a heterobifunctional poly(ethylene glycol) linker (NHS-PEG24-Mal).

2.2.2 Immobilization of proteins to Si <100> surfaces

We reasoned that adsorption of proteins to silicon for binding studies required a covalently anchored system to which proteins could be conjugated in a site-specific, homogeneous manner. Furthermore an effective anchoring strategy should orient all proteins in a manner that will not diminish protein-ligand affinity. We reasoned that site directed nickel-nitrilotriacetate (Ni-NTA) chelation of recombinant C- or N- terminal hexahistidine (his6) tagged proteins could be used to achieve this goal. This method of
chelative immobilization of proteins has previously been utilized to anchor his₆ tagged protein to NTA terminated monolayers for AFM binding studies.²⁵, ⁹¹⁻⁹⁴ We reasoned that by minimizing heterogeneity in protein orientation, heterogeneity in affinity might also be minimized.⁸⁶

Figures 18 and 19 depict the molecular assemblies used to modify silicon <100> surfaces for this investigation. The carbohydrate binding protein G3 was selected because it displays binding that is specific to disaccharides of the β-galactoside form and not monosaccharides. Whereas calorimetric methods have been used to demonstrate a solution phase affinity of his₆-galectin-3 for β-methyl lactose on the order of 18,000 M⁻¹, the same study reported a 56% decrease in affinity for the monosaccharidic components of lactose, β-methylglucose and β-methylgalactose.⁶³ We infer from this drop in affinity and from the dearth of evidence to the contrary, that G3 will exhibit little affinity for β-methylmannose. Surface-immobilized G3 binds immobilized lactose with an affinity of ~6400 M⁻¹.⁶ KDPG aldolase has no known affinity for either lactose or mannose and was used as a negative control. N-terminal his₆-galectin-3 (Figure 18) and C-terminal his₆-KDPG aldolase (Figure 19) were immobilized via his₆-Ni²⁺-NTA coordination to a covalently anchored linker 3 on mercaptosilanized silicon <100>.

Galectin-3 is a 35 kDa chimeric lectin which contains at its C-terminus a 137 amino acid carbohydrate recognition domain (CRD) that is oriented away from the surface (Figure 18). A disordered N-terminal 120 amino acid collagen-like-repeat (CLR) that is both flexible and extendable is connected to the CRD and anchors the construct to the surface by an N-terminal his₆ tag. In its crystalline form the G3 CRD is ~4.5 nm at
its widest point. In fully extended form, the G3 CLR is \( \sim 44 \) nm in length. When force is applied to a bound lactose-G3 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 \( \sim 63 \) nm. Interfacial G3 aggregation and tip fouling can potentially cause unbinding events at extensions beyond the total estimated length of the molecular assembly.

Hexa-histidine tagged KDPG aldolase, a protein with no measurable affinity for hemiacetal forms of glucose, galactose, or lactose, was chosen as a negative control. KDPG aldolase binds the open chain form of 2-keto-3-deoxy-6-phosphogluconate and related straight-chain aldehydes.\(^{120}\) Active KDPG aldolase exists in solution as a trimeric assembly of identical subunits 3.9 nm in length\(^{121}\). The total estimated length, from tip to sample, of the monomeric KDPG aldolase immobilized construct is 16.4 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 18: His$_6$-galectin-3 is immobilized via N-terminal-his$_6$-Ni$^{2+}$ coordination (shown in red) to a covalently anchored NTA linker (3) on mercaptosilanized silicon <100>. A crystal structure for the G3 C-terminal CRD is shown (adapted from PDB file 2NMO). The disordered N-terminal G3 CLR is represented (blue line).
Figure 19: His$_6$-KDPG aldolase is immobilized via NTA -Ni$^{2+}$-his$_6$ coordination (shown in red) to a covalently anchored NTA linker (3) on mercaptosilanized silicon <100>. A crystal structure for the KDPG aldolase monomer is shown (adapted from PDB file 1WA3).
2.3 Materials for surface functionalization

2.3.1 Synthesis of mercaptopentyl saccharide ligands

We chose to covalently immobilize saccharide ligands to AFM tips through the conjugate addition of thiolated saccharides to surface immobilized maleimide groups. Thiopentyl mannoside 1 was synthesized from perbenzoylated mannose 4 in four steps (Figure 20). Bromination of mannose pentabenzoate under acidic conditions provided the glycosyl bromide 5 which was converted glycoside 6 by silver triflate promoted glycosylation with 4-penten-1-ol. Radical addition of thiolacetic acid across the terminal olefin provided thioacetate 7, which was fully deprotected under basic conditions to provide thiopentyl mannoside 1. All synthetic transformations in this scheme were performed by Carleen Morris.

![Synthesis of 5-thiopentyl-α-D-mannopyranoside (1).](image)

Figure 20: Synthesis of 5-thiopentyl-α-D-mannopyranoside (1).

Thiopentyl lactoside 2 was synthesized from precursor 8 (Figure 21). Radical addition of thiolacetic acid across terminal alkene 8 (a generous gift from James Parise...
Jr. Ph.D.) in the presence of uv light provided the thioacetate 9. Methanolysis provided the thiopentyl lactoside 2.

![Chemical structure diagram]

**Figure 21: Synthesis of 5-thiopentyl-β-lactoside (2).**

### 2.3.2 Synthesis of maleoyl NTA-lysine

We chose to covalently immobilize the nickel chelating NTA functionality through conjugate addition of NTA maleimide 3 to thiolated silicon <100> surfaces. Synthesis of maleoyl NTA-lysine 3 was achieved in four steps (Figure 22). Cbz-NTA lysine 10 was esterified via carbodiimide coupling in the presence of catalytic dimethylaminopyridine to form the tris(trimethylsilyl)ester 11. Hydrogenolysis over palladium on carbon provided the primary amine 12. Carbodiimide coupling to N-maleoyl-β-alanine in the presence of hydroxybenzotriazole provided the amide 13. Deprotection in TFA/DCM (1:1) yielded the desired maleoyl-NTA-lysine 3.
Figure 22: Synthesis of Nα-bis-carboxymethyl-Nε-3-maleimidopropionyl lysine (3).

2.3.3 Immobilization of lactose and mannose on silicon nitride tips

Aminofunctionalization of silicon nitride AFM tips was accomplished using the method of Ebner et al.\textsuperscript{103} Briefly, plasma-cleaned tips were subject to vapor phase deposition of 3-aminopropyltrimethoxy silane (APTMS). The resulting amine-terminated surface was conjugated to a 10 nm heterobifunctional (oligoethylene)glycol linker (Mal-PEG24-NHS ester, Quanta Biosedign). Thiopentyl lactoside 2 and thiopentyl mannoside 1 were covalently bound to the surface by conjugate addition to the reactive maleimide functionality following a previously described procedure.\textsuperscript{6} Thiopentyl mannoside 1, a monosaccharide for which galectin-3 has no known affinity, was used as a negative control.

2.3.4 Immobilization of galectin-3 and KDPG aldolase

Vapor deposition of freshly distilled 3-mercaptopropyltrimethoxy silane (MPTMS) onto plasma-oxidized silicon <100> samples provided thiol-terminated
surfaces. Conjugate addition of maleimide 3 provided sites for Ni\(^{2+}\)-mediated immobilization of his\(_6\)-G3 or his\(_6\)-KDPG aldolase via his\(_6\)-Ni\(^{2+}\)-NTA chelation. The terminal his\(_6\) tag, routinely used for adsorption of biomolecules for AFM studies\(^{25,92}\) and affinity purification of galectin-3 and KDPG aldolase, does not significantly affect solution phase affinities of either biomolecule.

![AFM Image](image)

**Figure 23:** Tapping mode AFM image of a 5\(\mu m^2\) area of Si <100> following mercaptosilanization and conjugation to NTA maleimide (3). A feature depth profile is shown for the entire area.

Tapping mode AFM images of mercaptosilanized Si <100> before and after immobilization of his\(_6\)-G3 his\(_6\) were acquired using unmodified versions of the same cantilevers used for binding experiments. As is evident from Figure 23, mercaptosilanization of plasma cleaned Si <100> followed by conjugation of maleimide (3) produced a uniform surface with average feature depth of 1.5 nm. This depth correlates well with the expected length of the molecular assembly (~1.8 nm). No visible patterns or features with depths greater than 2 nm were evident in the scanned area.
Adsorption of G3 via his$_6$-Ni$^{2+}$-NTA chelation produced the pattern of features shown in Figure 24. Scan area A1 was imaged in a high resolution first pass of the scanning probe from left to right across a 1 $\mu$m$^2$ area of the surface. Feature depths ranged from 2-8 nm with an average feature depth of 5.8 nm.

A second lower resolution image was obtained by scanning a 5 $\mu$m$^2$ area that contained area A1 at its center. This doubly imaged area, A2, contained features comparable in depth to the original A1 image, although the surface density of the remaining features was diminished. As seen in Figure 24, much of the original material had been shifted into a ridge-like formation in area B at the boundary of A2. Average feature depths in area B range from 10 to 15 nm, consistent with the accumulation of material in that area.

We conclude that the initial high resolution first pass of the scanning probe across area A1 caused desorption of material which was then pushed across the surface by the tip to accumulate at the edge of the scan area. This phenomenon was dubbed the snow plow effect. Material outside of the initial first scan area remained unmodified. Surface features in this newly imaged area C were similar to, but slightly deeper than, those in areas A1 and A2.
Figure 24: Tapping mode AFM image of a 1µm² area of Si <100> after immobilization of his6-G3 via his6-Ni²⁺-NTA chelation (A1). A second image obtained by scanning a 5 µm² area that contained A1 at its center (A2). The snow plow effect (B): accumulation of material at the edge of area A2. Material outside of area A2 remained unplowed (C). Feature depth profiles are shown for each area.
2.4 Unbinding experiments

2.4.1 Automated data collection routines

Our goal was to gain insight into the effect of compressive forces on the formation of both specific and non-specific complexes produced during molecular recognition force microscopy experiments. This goal was accomplished by investigating the effect of contact forces on force vs. extension plots generated during approach-retraction pulling cycles. In a typical MRFM experiment, the sample is driven toward the tip until a pre-determined contact force (force trigger), often several hundred piconewtons \(^{29, 73, 103, 104}\) and occasionally several nanonewtons \(^{78, 124, 125}\) is reached, triggering retraction of the sample. Low force triggers (<100 pN) often cause premature retraction of the sample and are not typically relied upon to generate sub-100 pN contact forces. \(^{126}\) Here we considered complex formation using three pulling routines: a custom force minimization routine \((F_{\text{min}})\), which can be optimized to reliably achieve sub 100 pN contact forces, and force trigger routines with nominal contact force \((F_{\text{cnom}})\) triggers of 250 pN and 1000 pN.

Minimal contact forces were achieved by optimizing the \(F_{\text{min}}\) pulling routine for the lactose-G3, lactose-KDPG aldolase, and mannose-G3 systems. This custom pulling routine uses data recorded during the first approach-retract cycle to alter the position of the sample surface so that minimal contact force is exerted on the tip throughout the next approach-retract cycle while achieving tip-sample proximity so that binding can occur (Figure 25). This process is repeated for a predetermined number of cycles (250 for each experiment described here).
Auto F\textsubscript{min} Pulling Protocol
1. Manually find the surface to set initial Z contact position (Z\textsubscript{con1})
2. Initiate approach-retract pulling cycle (Z\textsubscript{con1} = fully extended Z position)
3. If signal crosses Contact Threshold 1, then adjust Z\textsubscript{con2} < Z\textsubscript{con1} for next pull
4. If signal crosses Contact Threshold 2, then adjust Z\textsubscript{con2} << Z\textsubscript{con1} for next pull
5. If signal crosses Rupture Threshold, then Z\textsubscript{con2} = Z\textsubscript{con1} for next pull
6. If signal crosses no threshold, then Z\textsubscript{con2} > Z\textsubscript{con1} for next pull

Figure 25: F\textsubscript{min} automated pulling protocol (code provided by Monica Rivera). Z\textsubscript{con} adjustment magnitudes and all threshold values can be optimized.

Initially, the surface was manually brought into close proximity to the tip. This was accomplished by driving the sample towards the tip in 5 nm increments until the first sign of contact, reproducible tip deflection, was observed. An initial Z contact position (Z\textsubscript{con1}) was chosen from the Z extension value that first produced tip deflection. Additional user defined variables were chosen and applied to all experiments: approach and retraction velocities (200 nm s\textsuperscript{-1}), approach and retraction distances (300 nm), dwell time (1 sec), X and Y start positions, X iterations (5), Y iterations (5), and Z iterations (10). Using these inputs, the first approach-retract pulling cycle was initiated and the sample on the Z-stage was extended to Z\textsubscript{con1}, held at this position for a dwell time of 1 sec, and retracted. A plot of vertical tip deflection vs. Z extension was automatically
generated. Tip-sample contact was inferred if the vertical tip deflection crossed the contact threshold line (Figure 25A). Cantilever movement was interpreted as an adhesion/rupture event if the vertical deflection crossed the rupture threshold line. If the surface was not driven into close proximity to the tip, then neither contact nor rupture threshold lines were crossed (Figure 25B) and the sample was driven closer on the subsequent approach-retract cycle. The Z contact point for the next pull ($Z_{con2}$) was chosen based on a series of rules outlined in Figure 25. All threshold values and $Z_{con}$ adjustment magnitudes were optimized so that minimal average contact forces ($\leq 100$ pN) were achieved for each set of approach-retract pulling cycles. (Rivera et al., ASME 2009)

To achieve higher contact forces a second routine, dubbed force trigger, was utilized. The sample was driven towards the surface until a nominal contact force ($F_{cnom}$ = 250 pN or 1000 pN) was reached, triggering a pause in sample movement for 1 sec followed by retraction of the sample at a rate of 200 nm s$^{-1}$. For a typical experiment, 250 force curves were generated at 25 different locations on the sample surface.

2.4.2 Data analysis

Force vs extension plots were analyzed according to a predefined set of rules applied uniformly across all data sets. Rupture forces ($F_{rup}$) were determined by applying Hooke’s law of elasticity ($F_{rup}=kx$), where $k$ is the cantilever spring constant and $x$ is the overall magnitude of vertical deflection.
Figure 26 shows a typical force curve for lactose-G3 with evidence of a rupture event. Both approach (green line) and retraction (black line) curves are displayed. During approach, the Z stage is driven towards the sample and applies maximum contact force at its apex of movement (A). Retraction of the Z stage results in a vertical decrease in contact force (A to B) as cantilever bending reduces. At point B, the tip begins to separate from the surface and the retraction curve angles toward horizontal. This inflection point (B) was defined as the point of zero molecular extension.

A non-linear increase in force (B to C) followed by a sharp linear decrease (C to D), exemplified in Figure 26, was considered evidence of an unbinding (rupture) event. Analyses of these sharp linear decreases in vertical deflection provided rupture forces and rupture lengths for all unbinding events.

Rupture forces \( (F_{rup}) \) were determined by applying Hooke’s law of elasticity \( (F_{rup}=kx) \), where \( k \) is the cantilever spring constant and \( x \) is the overall magnitude of vertical deflection that evinced rupture. The overall magnitude of deflection was defined as the vertical difference between the apex (C) and the midpoint of noise at the base of each rupture event (D). All rupture forces >35 pN were considered to be resolved above noise. Rupture length was defined as the molecular extension at the apex (C) of the unbinding event. Following the forced rupture of all non-covalent interactions between tip and surface, the cantilever oscillates about its equilibrium position (E).

2.4.3 Analysis of contact forces

Using the $F_{\text{min}}$ routine, an average contact force of 84 pN (standard deviation = 16 pN) was obtained for the lactose-G3 system (Figure 27). Slightly higher contact forces were observed for the lactose-KDPG aldolase system (average = 116 pN, standard deviation = 69 pN). An average contact force of less than 100 pN for the mannose-G3 system (average = 76 pN, standard deviation = 38 pN) was measured for the data set containing 250 approach-retract cycles.

Figure 27: Histograms of observed contact forces for the lactose-G3 system, lactose-KDPG aldolase, and mannose-G3 systems using the $F_{\text{min}}$ data collection routine.
The force trigger data collection routine typically resulted in broader contact force distributions than \( F_{\text{min}} \). Furthermore, due to variable delays in computer processing speed, average applied forces tended to be larger than the nominal force trigger (Figure 28). The smallest variations were observed in experiments performed with the lactose-G3 and mannose-G3 systems. For these two systems, greater than 90% of all observed contact forces fell within a 250 pN range (200 – 450 pN) for experiments using \( F_{\text{nom}} = 250 \) pN. Also, for these two systems, nearly 90% (88%) of all observed contact forces fell within a 250 pN range (1100 – 1350 pN) for experiments using \( F_{\text{nom}} = 1000 \) pN. All data from these experiments were used in subsequent analyses.

![Figure 28](image)

*Figure 28: Histograms of observed contact forces for the lactose-G3 system, lactose-KDPG aldolase, and mannose-G3 systems using nominal force triggers of 250 pN and 1000 pN.*

The largest contact force variations were observed for the lactose-KDPG aldolase system; these variations, however, did not significantly affect the probability of observing a bound complex. For the lactose-KDPG aldolase system, 85% of all observed
contact forces fell within a 250 pN range (200 – 450 pN) for the experiment using $F_{\text{nom}} = 250$ pN. The probability of observing a rupture event ($\rho_{\text{bind}} = \text{number of rupture-containing pulls/total pulls}$) following 200 – 450 pN of applied force ($\rho_{\text{bind}} = 0.10$) was similar to the probability of observing a rupture event for the entire data set collected using $F_{\text{nom}} = 250$ pN experiment ($\rho_{\text{bind}} = 0.07$). All data from this experiment was used in subsequent analyses.

In experiments using the lactose-KDPG aldolase system, 54% of all observed contact forces fell within a 250 pN range (1100 – 1350 pN) for the experiment using $F_{\text{nom}} = 1000$ pN. The probability of observing a rupture event following 1100 – 1350 pN of applied force ($\rho_{\text{bind}} = 0.57$) was again similar to the probability of observing a rupture event for the entire $F_{\text{nom}} = 1000$ pN dataset ($\rho_{\text{bind}} = 0.42$). All data from this experiment were used in subsequent analyses.

2.4.4 The probability of observing a rupture

2.4.4.1 Key objectives

Our primary goal in MRFM is the evaluation of solution phase thermodynamic parameters for immobilized binding partners. Our approach determines a binding constant for immobilized partners based on the variation of the probability of binding as a function of soluble ligand concentration. In order to accomplish this goal, we require clear differentiation between the presence and absence of specific bound complexation during multiple approach-retract cycles. Because non-specific complexation can mask the true fractional probability of specific complex formation, it is essential to distinguish specific complexation from non-specific binding.
Of the three systems studied here, only lactose-G3 forms a specific complex. A fundamental assumption is that the binding sites of immobilized G3 can be occupied by soluble ligand thereby preventing the formation of specific bound interactions with lactose-modified tips, an assumption supported by previous studies.\textsuperscript{6, 38} We therefore anticipated that the addition of soluble β-methyl lactose would diminish the probability of observing a specific rupture events for this system. Additionally, all unbinding events observed after saturation of immobilized G3 with β-methyl lactose were interpreted as non-specific adhesion.

Because there should be no affinity between mannose-G3 or lactose-KDPG aldolase, no specific bound interactions were expected for either of these systems. Furthermore, the addition of soluble β-methyl lactose should have no effect on the probability of observing a rupture for these systems.

We investigated the relationship between the magnitude of applied contact force using the $F_{\text{min}}$ and force trigger ($F_{\text{cnom}} = 250$ pN and $F_{\text{cnom}} = 1000$ pN) data collection routines and the probability of observing both specific and non-specific rupture events for the lactose-G3, lactose-KDPG aldolase, and mannose-G3 systems. Approach-retract cycles were performed at 25 positions across each surface (10 cycles per position). Experiments were performed in PBS (pH 7.4) and then repeated with a new tip and surface in the presence of β-methyl lactose (10 mM in PBS pH 7.4).

2.4.4.2 Variations in probability and blocking efficiency

As evident from Figure 29, the probability of observing a rupture ($\rho_{\text{bind}} = \text{number of rupture-containing pulls / total pulls}$) increased with increasing contact force, as did
the probability of observing two or more ruptures ($\rho_{\text{multiple}} = \text{number of pulls containing two or more ruptures/total pulls}$) for lactose-G3 interactions in PBS (pH 7.4) (Figure 29A). Under the minimal compressive force applied in the $F_{\text{min}}$ routine, single rupture events predominated, with a total $\rho_{\text{bind}}$ of 0.33 and $\rho_{\text{multiple}}$ of 0.02. After blocking with β-methyl lactose (10 mM in PBS pH 7.4), $\rho_{\text{bind}}$ decreased to 0.09, representing a 73% blocking efficiency ($\Delta \rho_{\text{bind}} / \rho_{\text{bind,unblocked}}$). Based on our previously measured immobilized lactose-G3 affinity,6 98% of surface sites should be blocked at 10 mM soluble lactose. Because we assume that all unbinding events observed under saturating β-methyl lactose conditions were non-specific in nature, we conclude that non-specific binding occurred in ~27% of all force curves that showed evidence of unbinding events in the unblocked experiment.

Use of the force trigger protocol to apply significant contact forces ($F_{\text{nom}} = 250$ pN) resulted in an increase in both the total probability of binding ($\rho_{\text{bind}} = 0.57$) and the probability of observing two or more ruptures ($\rho_{\text{multiple}} = 0.28$) with the unblocked system. The total probability of binding was diminished to $\rho_{\text{bind}} = 0.44$ upon blocking with β-methyl lactose (10 mM in PBS pH 7.4), representing a 23% blocking efficiency. Because we assume that all unbinding events observed after saturation of immobilized G3 with β-methyl lactose were non-specific in nature, we conclude that non-specific binding was responsible for ~77% of all unbinding events observed in the unblocked experiment.

Under the maximum compressive force used for this study, ($F_{\text{nom}} = 1000$ pN) a significant increase in the total probability of binding ($\rho_{\text{bind}} = 0.66$) was observed for the
unblocked system. Alternatively, the probability of observing two or more ruptures 
\( \rho_{\text{multiple}} = 0.32 \) remained constant relative to \( \rho_{\text{bind}} \) compared to the \( F_{\text{cnom}} = 250 \) pN experiment. The total probability was diminished to \( \rho_{\text{bind}} = 0.62 \) upon blocking with \( \beta \)-methyl lactose (10 mM in PBS pH 7.4); a 6% blocking efficiency. Because we assume that all unbinding events observed after saturation of immobilized G3 with \( \beta \)-methyl lactose were non-specific in nature, we conclude that non-specific binding was responsible for \( \sim 94\% \) of all unbinding events observed in the unblocked experiment.

It is evident from these experiments that the application of increasing compressive force leads to significant (up to 2×) increases in binding probability along with concomitant drops in blocking efficiency. Mechanistic details accounting for these trends are unclear from these experiments. Nevertheless, we rationalized that increased binding probabilities may result from greater surface area at the tip-surface interface caused by greater tip implantation. If this scenario leads to a higher incidence of specific bound interactions, we expect that they would remain blockable, however, they do not. A possible explanation for decreased blocking efficiency is compression induced soluble ligand extrusion of \( \beta \)-methyl lactose from a properly folded G3 CRD. We exclude this possibility because compressive extrusion of soluble ligand would undoubtedly cause denaturation of the G3 CRD rendering it unable to specifically bind both soluble and tethered lactose. We, therefore, exclude the possibility that increases in binding probability along with concomitant drops in blocking efficiency result from a higher incidence of specific binding. It is more likely that higher contact forces result in
protein denaturation, tip fouling, and concomitant formation of multiple non-specific complexes.

The probability of observing multiple ruptures per pull ($\rho_{\text{multiple}}$) is also minimized through the use of the $F_{\text{min}}$ routine, but increases dramatically to $\sim50\%$ of all observed ruptures when using $F_{\text{cnom}} = 250$ pN and 1000 pN. This phenomenon results from increased tip implantation from higher contact force, which presumably produces larger contact areas and, potentially, a higher number of simultaneous specific bound interactions. Conversely, a higher degree of molecular compression may result in protein denaturation, tip fouling, and concomitant formation of multiple non-specific complexes.

In order to further investigate the effect of compressive forces on tip integrity, a freshly prepared lactose modified tip and G3 modified sample were subjected to 50 approach-retraction pulling cycles using the force trigger $F_{\text{cnom}} = 1000$ pN. The tip was then moved several micrometers laterally, and the $F_{\text{min}}$ pulling protocol was initiated providing 250 force curves ($\rho_{\text{bind}} = 0.09$). This probability of binding was significantly lower than a typical $F_{\text{min}}$ experiment and comparable to fully blocked $F_{\text{min}}$ experiments suggesting that repeated application of compressive forces on the order of 1000 pN renders a lactose-functionalized tip unable to form specific complexes with G3.
Figure 29: 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), the probability of observing 2 or more events (red) 3 or more events (green) and 4 or more events (violet) are shown. Blocked experiments contained 10 mM soluble $\beta$-methyl lactose.
To better understand the effect of compressive force on the formation of non-specific complexes we repeated the study, but replaced thiopentyl lactoside 2 with thiopentyl mannoside 1, a ligand for which G3 has no known affinity. As evident in Figure 29B, few bound interactions were observed for both unblocked and blocked $F_{\text{min}}$ experiments ($\rho_{\text{bind}} = 0.08$) and ($\rho_{\text{multiple}} = 0.01$). Under compressive forces of 250 pN $\rho_{\text{bind}}$ increased to 0.41 and $\rho_{\text{multiple}}$ increased to 0.15. This probability was diminished to $\rho_{\text{bind}} = 0.31$ upon blocking with $\beta$-methyl lactose (10 mM in PBS pH 7.4), representing a 24% blocking efficiency. Under nominal compressive forces of 1000 pN $\rho_{\text{bind}}$ increased to 0.47 and $\rho_{\text{multiple}}$ remained 0.15. The overall probability was diminished to $\rho_{\text{bind}} = 0.38$ upon blocking with $\beta$-methyl lactose (10 mM in PBS pH 7.4); a 19% blocking efficiency.

The lack of G3 affinity for mannose implies that all mannose-G3 ruptures resulted from non-specific binding. Nevertheless, the addition of $\beta$-methyl lactose unexpectedly blocked 24% and 19% of ruptures observed using force trigger ($F_{\text{com}} = 250$ pN), and force trigger ($F_{\text{com}} = 1000$ pN), respectively. A possible explanation for this reduction in non-specific binding is that complexation of soluble lactose increased the thermodynamic stability of G3. Ligand binding has been shown to increase protein stability in theory$^{127}$ and in vitro.$^{128,129}$ A stabilized lactose-G3 complex may resist force-induced denaturation thereby reducing somewhat the formation of non-specific complexes.

As expected, the KDPG aldolase-lactose system (Figure 29C) showed a small number of bound interactions ($\rho_{\text{bind}} = 0.004$ and $\rho_{\text{multiple}} = 0$) using the $F_{\text{min}}$ protocol. On the other hand, $\rho_{\text{bind}}$ increased to 0.07 and $\rho_{\text{multiple}}$ increased to 0.02 using a force trigger
Further increases in ruptures were observed ($\rho_{\text{bind}} = 0.42$ and $\rho_{\text{multiple}} = 0.22$) using a force trigger of $F_{\text{nom}} = 1000$ pN. The probability of binding was not significantly diminished ($\rho_{\text{bind}} = 0.40$) using a force trigger of $F_c = 1000$ pN upon blocking with β-methyl lactose (10 mM in PBS pH 7.4). Given the lack of affinity of KDPG aldolase for lactose, we attribute all observed interactions to non-specific processes. Furthermore, the low degree of blocking is in accord with the fact that soluble β-methyl lactose has little ability to prevent KDPG aldolase denaturation under compressive forces.

### 2.4.5 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. Additionally, it is critical to ensure that the observation of discontinuity in a force curve in fact arises from the rupture of a specific bound interaction and not from non-specific adhesion. In order to gain insight into how contact forces affect observed force-distance curves, we carried out a systematic study described in section 2.4.4. Briefly, three data collection routines ($F_{\text{min}}$ and force triggers $F_{\text{nom}} = 250$ pN and $F_{\text{nom}} = 1000$ pN) were used 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. Approach-retract cycles were performed at 25 positions across each surface (10 cycles
Here we consider the morphology of force curves that displayed unbinding events.

Figure 30 depicts typical force vs. 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). Each retraction curve shows one or two non-linear increases in force followed by sharp linear decreases in force (blue dots) back to baseline position, indicative of rupture of non-covalent interactions (either specific or non-specific) between tip-immobilized and sample-immobilized molecules as the sample is retracted from the tip. Following the force-driven rupture of all non-covalent interactions between tip and surface the cantilever returns to its equilibrium position.

Figure 30: Force vs. extension plots depicting ruptures for the lactose-G3, lactose-KDPG aldolase, and mannose-G3 systems generated by $F_{min}$, force trigger ($F_{nom} = 250$ pN) and force trigger ($F_{nom} = 1000$ pN) pulling routines. Approach curves (green) and retraction curves (black) with unbinding events (blue circles) are depicted.
Force signatures generated with the lactose-G3 system using \( F_{\text{min}} \) were generally similar in morphology to those shown in Figure 30A. As discussed in the previous section, single rupture events predominated with \(~73%\) of all observed ruptures attributed to specific binding. 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 \( F_{\text{nom}} = 250 \) pN and 1000 pN, respectively, the majority (>50%) of these force curves are indistinguishable from those generated using the \( F_{\text{min}} \) protocol (Figure 30B and 30C). These results suggest that force curve morphology alone is insufficient to determine the specific nature of a rupture.

No specific bound complexes were expected for lactose-KDPG aldolase interaction. As it was, several force signatures for this system were similar or identical in morphology to those of specific lactose-G3 interactions. Although ruptures at shorter extensions were frequently observed when utilizing the \( F_{\text{min}} \) and \( F_{\text{nom}} = 250 \) pN pulling routines (Figure 30D and 30E), higher compressive forces (\( F_{\text{nom}} = 1000 \) pN) resulted in force curves that were identical to those of lactose-G3 (Figure 30F). Additionally several presumably non-specific mannose-G3 rupture events were identical to those observed from specific lactose-G3 force signatures (Figure 30G, 30H, and 30I). These results further suggest that force curve morphology alone is insufficient to determine the specific nature of a rupture. Thus, the 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.
Occasionally force curve morphologies diverged from the single and double ruptures previously discussed. Evident in Figure 31, these ruptures displayed complex unbinding profiles and unbinding well beyond the total estimated length of each system (63 nm for lactose-G3 and mannose-G3, 16.4 nm for lactose-KDPG aldolase). The probability of observing such events was less than 0.02 for all $F_{\text{min}}$ experiments, but increased significantly to 0.10 in force trigger experiments, confirming that greater tip implantation results in larger contact area and, potentially, a higher number of simultaneous specific bound interactions. Conversely, a higher degree of molecular compression may result in protein denaturation, tip fouling, and concomitant formation of multiple non-specific complexes.$^{74,77,79}$

**Figure 31:** Force vs. extension plots displaying evidence of multiple ruptures.
Unbinding events at extensions less than 10 nm were generally similar in morphology to those displayed in Figure 32, and differed qualitatively from those at extensions greater than 10 nm (Figure 30 A-C). The probability of observing these events was similar for unblocked (0.02) and blocked (0.05) lactose-G3 experiments using the F_\text{min} protocol. For these reasons unbinding events at extensions less than 10 nm were considered indistinguishable from non-specific adhesion, e.g. surface-surface, linker-surface, or linker-linker interactions, and were not considered in subsequent analyses.

![Figure 32: Unbinding events at extensions less than 10 nm.](image)

### 2.4.6 Rupture force distributions

The distribution of rupture forces for the G3-lactose system is unimodal with the most probable rupture force = 60±28 pN for all applied contact forces (Figure 33A), in good agreement with previously reported forces of 50±10 pN using the same retraction rate.\(^6\) Histogram profiles in Figure 33A are similar regardless of applied F_c in all respects except frequency. Thus, rupture force histogram profile alone is not indicative of specific binding. If conclusions were drawn on the basis of this histogram alone, we would assume no difference between data collected using F_\text{min} and the force trigger.
(\(F_{\text{cnom}} = 1000\) pN) other than \(p_{\text{bind}}\), although there is clearly a difference in blocking efficiency (Figure 29A). We conclude that blocking experiments are an essential component of an understanding of the biophysical significance of cantilever movement.

In contrast to G3-lactose interactions, non-specific interactions for G3-mannose and KDPG aldolase-lactose resulted in a broader distribution of rupture forces (Figure 33B and 33C). The frequency of observing larger rupture forces increases significantly with increasing contact force.
Figure 33: Histogram of rupture forces observed for (A) G3-lactose, (B) G3-mannose, and (C) KDPG aldolase-lactose using force minimization (blue), and force triggers of 250 pN (red) and 1000 pN (green).
2.4.7 Rupture length distributions

The total extended length of a G3-lactose molecular system containing a folded and functional CRD is ~63 nm. Removal of the collagen-like repeat using collagenase has no effect on the binding behavior of the carbohydrate recognition domain, and we thus assume that specific G3-lactose affinity is unaffected by extension of the CLR. We also assume that specific G3-lactose binding is not possible if the CRD is denatured or partially unfolded. Therefore, while force signatures at extensions between 10 and 70 nm potentially originate from the rupture of specific complexes, events at extensions >70 nm must originate from non-specific association. Regardless of extension, all unbinding events (up to eight per pulling cycle) were included in subsequent data analyses. Rupture lengths beyond ~63 nm may indicate protein unfolding, assuming partially unfolded protein retains at least some affinity.

A histogram of rupture lengths was plotted for all applied contact forces for the lactose-G3, mannose-G3, and lactose-KDPG aldolase systems (Figure 34). All ruptures at extensions less than 10 nm were considered indistinguishable from non-specific adhesion.

Unbinding events occurring beyond the length of the fully unfolded system imply compression-induced processes that extend the overall length of the interacting system. It is conceivable that high contact forces result in a “snow plow effect” (discussed previously, Figure 24) as the cantilever tip is driven into and rastors across the surface. A possible result of this process is ablation of protein from the surface and tip fouling, transfer of G3 or KDPG aldolase aggregates,\textsuperscript{130} from sample to tip.
For lactose-G3 under minimal contact forces the rupture length histogram profile is unimodal with the centroid of the distribution at ~50 nm. Shorter rupture length bins become more populated using force triggers of 250 pn and 1000 pN. Rupture length distributions for non-specific G3-mannose and KDPG aldolase-lactose interactions decrease in frequency at longer lengths. The rupture length distribution for KDPG aldolase-lactose is shifted to the left due to the shorter molecular length of the system (16.4 nm) and the lack of an extendable collagen-like repeat on KDPG aldolase.

The increase in frequency of rupture events 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. Rather, blocking experiments remain essential for the interpretation of cantilever movement.
Figure 34: Histogram of rupture lengths observed for (A) G3-lactose, (B) G3-mannose and (C) KDPG aldolase-lactose using force minimization (blue), and force triggers of 250 pN (red) and 1000 pN (green).
2.5 Conclusions

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 resulted from greater contact forces. Thus, it is essential to design data collections routines that minimize contact forces to ensure that ruptures originate from specific, blockable interactions.

Our goal here was to interpret cantilever movement in terms of specific non-covalent interaction between immobilized biomolecules. 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.
3. A general and efficient functionalization technique for AFM molecular recognition studies

3.1 Overview

Our primary goal is the routine evaluation of thermodynamic parameters for immobilized binding partners through the interpretation of the variation of binary probability of binding as a function of soluble ligand concentration. The development of methodologies that produce stable ordered molecular systems on surfaces will facilitate the achievement of this goal. Determination of binding probabilities by observing the rupture of piconewton-scale attractive forces between ligands and receptors requires efficient protocols for the rapid, robust, and uniform adsorption of oriented binding partners to surfaces and tips at the end of flexible AFM cantilevers.\textsuperscript{131-136}

Traditionally, techniques for the chemical modification of AFM tips have been designed to produce widely spaced functionality in order to ensure binding between single pairs of molecular species.\textsuperscript{24, 25, 29, 104} The success of our approach, however, relies on maximizing the probability of forming specific non-covalent interactions in a reproducible manner. Additionally, the minimization of non-specific interactions that might obscure or be indistinguishable from specific binding is essential to ensure accurate statistical evaluations of bound interactions. We rationalize that by optimization of these experimental parameters we can achieve accurate and reproducible dynamic range between binding probabilities of blocked and unblocked systems. To this end, we sought methods for the formation of protective monolayers that would facilitate the production of surfaces with tailorable densities of reactive
groups on both tip and sample. These methodologies form stable Si-C bonds to both silicon and silicon nitride (Si$_3$N$_4$) surfaces and, when optimized, ensure that binding partners are distributed uniformly across both the sample and tip, orient partners properly for binding, and produce surfaces that are stable to experimental conditions.

The most commonly used cantilever materials are silicon and silicon nitride. Because of its flexibility, high fracture toughness, and resistance to wear$^{137}$, silicon nitride is the preferred material for AFM-based molecular recognition studies.$^{21, 38, 138}$ Paradoxically the same chemical inertness that renders silicon nitride attractive for other applications, e. g., as a refractory ceramic for engine turbine fabrication, also impedes the adsorption of biomolecules to its surface. Exposure to air results in the oxidative formation of a trilayer comprising a layer of silicon oxynitride (Si$_2$N$_2$O) between Si$_3$N$_4$ and amorphous silicon oxide (SiO$_2$) at the air interface.$^{137, 139, 140}$ A similar oxidative process creates amorphous SiO$_2$ at the surface of crystalline silicon (Figure 35). Covalent modification of silicon and silicon nitride is conventionally achieved using ethanolamine or organic silanes that react with this silicon oxide layer at the surface-air interface.$^{21, 42, 72, 73, 103, 105}$

![Figure 35: Native oxide layers on Si$_3$N$_4$ and crystalline Si.](image-url)
Despite widespread use, these methods have several important drawbacks. The amorphous nature of the oxide layer implies that the ratio of Si-O-Si to Si-OH functionalities at the surface is highly variable.\textsuperscript{141, 142} This irreproducible oxide distribution precludes precise control of subsequent adsorption chemistry. Moreover, the susceptibility of the underlying crystalline surface to further oxidation leads to instability of the adsorbed layers.\textsuperscript{141, 142}

Although ethanolamine and silane layers can be easily formed on oxides using solution or vapor phase deposition, there are inherent deficiencies in these chemistries that limit their utility for our AFM studies. Such deficiencies include difficulty in controlling surface density, susceptibility of Si-O bond to hydrolysis in aqueous environments and the formation of polymeric aggregates.\textsuperscript{21, 143, 144}

So called “etherification”, or dehydration of SiOH groups with ethanolamine, produces low surface density amino functionalization and highly diffuse reactive groups anchored by single Si-O-C bonds.\textsuperscript{21, 38} The lack of cross links and the hydrolytic susceptibility of the Si-O-C bond result in poor monolayer stability. Mitigation of these instabilities can be achieved through the use of silanes with multiple reactive groups capable of cross linking in the presence of water, e.g. APTMS.

Cross-linked silanes have been used to create structurally characterized\textsuperscript{145-149} reactive amine layers for AFM binding studies\textsuperscript{103, 150, 151}. Despite their utility in single molecule studies, significant limitations have been reported. Heterogeneous water mediated polymerization during deposition\textsuperscript{141, 142, 147, 152} results in polydisperse aggregates with variable degrees of cross linking.\textsuperscript{145} Similar polymeric multilayers have
been observed on oxides of silicon and Si₃N₄. Amines may be occluded under or within these polymeric structures at surfaces (Figure 36). Furthermore, hydrogen bonding of these buried amines with surface oxides has been shown to render them unreactive towards subsequent coupling reactions with phenylsulfonyl chloride and NHS-esters.

![Figure 36: Depiction of possible microscopic chemical structure of aminosilane layer on amorphous silicon oxide adapted from proposed structures. Vertical polymerization creates polymeric aggregates and multilayers. Hydrogen bonding renders amines unreactive towards subsequent functionalization.](image)

Various methods of deposition using vapor- and solution-phase silanes combined with post-deposition heat curing mitigate but do not completely resolve these problems. Aggregates and unreactive amines can be reduced but not eliminated by vapor deposition and precise humidity control, but instability during subsequent functionalization and in aqueous environments during binding studies provide a powerful motivation for further development of novel surface functionalization.

We required a reliable, stable molecular system for AFM studies that would facilitate attachment of various organic and biological molecules and that can withstand prolonged exposure to organic and aqueous environments. Monolayers directly bound
to silicon or Si₃N₄ via Si-C bonds are more stable than silane and ethanolamine monolayers capable of withstanding harsh reactive environments. A common approach to the formation of such monolayers on polycrystalline silicon is hydrosilylation, which involves the addition of silicon hydride (Si-H) across a terminal carbon-carbon multiple bond. Conjugation of biomolecules to reactive monolayers has been demonstrated. Additionally, protective oligo (ethyleneglycol) monolayers with tailorable densities of reactive groups have been shown to support the conjugation of biotin and subsequent specific binding of avidin while minimizing non-specific protein adsorption.

Although successful application of hydrosilylation has been demonstrated on Si₃N₄ surfaces and on silicon AFM probes, to date no group has applied this method to Si₃N₄ cantilevers. Here we report the first example of the preparation of stable self assembled monolayers through hydrosilylation of a protected aminoalkene onto hydrogen-terminated silicon nitride AFM probes and subsequent conjugation with biomolecules for force microscopy studies.

3.2 Comparison of immobilization strategies using XPS

We first set out to compare the composition and structure of aminosilane monolayers on oxidized silicon and Si₃N₄ to that of monolayers formed through hydrosilylation of a protected aminoalkene on hydrogen-terminated silicon and Si₃N₄. Silicon nitride substrates were prepared by depositing a 130 nm layer of silicon nitride on a silicon wafer using plasma enhanced chemical vapor deposition (PECVD system,
Advanced Vacuum Vision 310). Vapor phase aminofunctionalization of silicon and Si$_3$N$_4$ wafers was accomplished using a method optimized by Ebner et al.$^{103}$ for use on Si$_3$N$_4$ AFM tips. Briefly, the amorphous silicon oxide surface of oxygen plasma- or nanostrip-oxidized wafers was subject to vapor phase deposition of APTMS in the presence of triethylamine vapor for two hours in a sealed chamber under argon gas. Room temperature “curing” in an argon atmosphere for two days followed by rinsing in dichloromethane provided the aminosilanized surfaces.

XPS analysis of oxidized Si$_3$N$_4$ before and after APTMS deposition (Figure 37) confirmed the formation of a silane layer with significant hydrogen bonding of amines to surface oxides. The C1s signal of nanostrip oxidized Si$_3$N$_4$ can be attributed to carbon impurities trapped during PEVCD deposition.$^{137}$ The C1s/Si2p signal ratio following APTMS deposition (0.978) is more than triple that reported for a well ordered uniform monolayer comprised of a three carbon chain (see Figure 42, surface S3 below) on silicon$^{170}$ (~0.290) and is suggestive of polymeric multilayers at the surface. Although partially obscured by the nitrogen signal from the underlying surface, the N1s signal of aminosilane modified Si$_3$N$_4$ contains a shoulder at ~400 eV consistent with hydrogen bonding of buried amines to surface oxides$^{146,147,155}$ while the nanostrip cleaned surface shows a N1s peak consistent only with oxidized Si$_3$N$_4$.$^{153}$
Figure 37: Aminosilanization of oxide layer on silicon nitride with APTMS and XPS spectra of surfaces before and after aminosilanization.

Silane aggregation and multilayer formation with concomitant hydrogen bonding of amines to surface oxides is further confirmed by XPS analysis of APTMS deposition on oxidized silicon <100> (Figure 38). Amorphous silicon oxide is clearly present both before and after aminosilane deposition. The C1s signal of the aminosilane modified surface contains a shoulder at 286 eV indicative of C-N bonding. The C1s/Si2p signal ratio of the aminosilane modified surface (0.662) is double that reported for a well ordered uniform monolayer comprised of a three carbon chain (see Figure 42, surface...
S3 below) on silicon\(^{170}\) (~0.290) and is suggestive of polymeric multilayers at the surface implying a multilayer composed of silane aggregates. The N1s signal of the aminosilane modified surface is increased relative to that of the oxidized surface and clearly shows two peaks consistent with previously reported free amines (~398-400 eV) and unreactive hydrogen-bonded amines (~400-402 eV) formed during aminosilane depositon.\(^{146, 147, 155}\)

Figure 38: Aminosilanization of oxide layer on silicon with APTMS and XPS spectra of surfaces before and after aminosilanization.
Hydrosilylation of protected Boc-amino SAMS on silicon <111> and silicon nitride wafers was achieved using a protocol optimized for use on AFM tips by Carleen Morris and Alexander Shestopalov Ph.D. (Figure 39). Briefly, surfaces were oxidized in nanostrip at 75 °C for two minutes and then submerged in HF (5% aq.) for two minutes to remove native oxides and form hydrogen terminated surfaces. The wafers were rinsed with water, methanol, coated with neat alkene 14 and placed in a nitrogen glove box. Hydrosilylation was initiated with uv light at a distance of 5 cm for 30 min.

Figure 39: Preparation of Boc protected alkyl SAMS on silicon nitride and silicon <111> wafers.

XPS analysis of the resulting Boc-protected alkane modified silicon <111> and silicon nitride surfaces confirmed the formation of SAMS anchored by stable Si-C bonds (Figure 40). The C1s signals from both substrates displayed peaks indicative of C-N and C=O bonding from the carbamate moiety in 14. The C1s/Si2p signals (0.560 and 0.366)
are less than that expected (1.55) for a well ordered uniform monolayer with equivalent number of carbons on hydrogen terminated silicon (extrapolated from the reported value for a three carbon chain on silicon\textsuperscript{170}: $0.29 \times 16/3 = 1.55$). Although the steric bulk of the Boc-protected amine functionality precludes the formation of ordered crystalline SAMS\textsuperscript{171}, it has been demonstrated that, following deprotection, the resulting amine spacing enhances reactivity towards further conjugation relative to closely packed amine SAMS.\textsuperscript{172-174} The protective capacity of the Boc-protected monolayers on silicon $<111>$ is demonstrated by the small residual silicon oxide peak (Figure 40) relative to that of native silicon oxide (Figure 38).

In conclusion we have demonstrated methodology for the formation of Boc protected alkyl SAMS on passivated silicon nitride. This methodology can be utilized for the functionalization of silicon nitride AFM tips and should ensure that binding partners are distributed uniformly across the surface and that monolayer desorption caused by hydrolysis or air oxidation of the underlying surface will be minimized.
3.3 Molecular assemblies for binding studies

Here we demonstrate the preparation of stable self assembled monolayers through hydrosilylation of a protected aminoalkene onto hydrogen-terminated silicon nitride AFM tips and subsequent conjugation with biomolecules for force microscopy studies using the cognate ligand-receptor pair lactose-G3. The surface-immobilized
carbohydrate binding protein (G3) binds immobilized lactose with an affinity of ~6400 M\(^{-1}\). We investigate the probability of observing a rupture event, the normalized number of blockable rupture events per pull, and rupture force and length distribution in force vs. extension plots using surface prepared via our novel method and compare to results obtained using APTMS-based silanization.

### 3.3.1 Hydrosilylation of silicon nitride AFM tips

Our approach takes advantage of hydrosilylation chemistry to modify silicon nitride probes with lactose molecules in five consecutive steps (Figure 41). Boc-terminated amines were immobilized on silicon nitride probes using UV activated reaction between H-terminated silicon nitride and alkene 14. The Boc-terminated monolayer was deprotected under acidic conditions yielding an amino-terminated surface. To prevent non-specific adsorption of biomolecules, the amino-terminated surface was reacted with heterobifunctional PEG linker 15. Finally, incubation in thiol 2 yielded a lactose modified surface.

XPS analysis confirmed successful formation of Boc-protected monolayers on both silicon nitride wafers and cantilevers prepared using the same conditions. XPS spectra of Boc-modified SAMs showed an increase in carbon signal compared to silicon nitride and contained an additional C1s peak at 287 eV corresponding to the carbonyl moiety of alkene 14 (Figure 41). XPS spectra of deprotected substrates lacked the C1s peak at 287 eV consistent with a decrease in carbon signal that correlates with that calculated for the completely deprotected substrate. Water contact angle values were
also consistent with those previously reported for Boc-protected and amino-terminated substrates.\textsuperscript{142}

\begin{itemize}
  \item[i.] Nanostrip 2 min, 70°C, then HF (5% soln), r.t., 2 min
  \item[ii.] UV light, N\textsubscript{2} atmosphere, r.t., 30 min
  \item[iii.] TFA:DCM (1:1 v/v), r.t., 30 min
  \item[iv.] Mal-dPEG\textsubscript{12}-NHS ester 15 (3mM DCM), Et\textsubscript{3}N (10mM DCM) r.t., 2 h
  \item[v.] 2.25 mM H\textsubscript{2}O\textsubscript{2}, 4h, 37°C
\end{itemize}

\textbf{Figure 41:} Top: Hydrosilylation and subsequent modification of silicon nitride AFM probes. Bottom: XPS spectra of Boc-modified and amino-terminated monolayers on silicon nitride.
3.3.2 Immobilization of G3 on silicon <111> surfaces

We previously reported a novel method for patterning biological molecules on passivated silicon. Here, we utilized a similar strategy to immobilize histidine-tagged galectin-3 (G3) on silicon (Figure 42). The technique simultaneously protects silicon from degradation in biological environments and provides a chelation site for the immobilization of histidine tagged proteins. Hydrogen terminated silicon was chlorinated with PCl₅ and alkylated with propenyl magnesium bromide at 135°C for 24 hours. Subsequently, the alkylated substrate was reacted with diazirine (generously provided by Alex Shestopalov Ph.D.) yielding an NHS modified monolayer. The resulting surface was reacted with lysine-N,N-diacetic acid and incubated with a Ni²⁺ solution then histidine tagged G3, providing the final substrate. This work was performed in close collaboration with Carleen Morris and Alexander Shestopalov.
Figure 42: Modification of H-terminated silicon with G3 via alkylation and carbene addition.

### 3.4 Hydrosilylation chemistry for MRFM studies

#### 3.4.1 Unbinding experiments

Our goal was to develop a new general functionalization method for force spectroscopy experiments that provides robust molecular systems that are both stable in aqueous environments and that do not degrade under mechanical strain. The molecular assemblies described in Figures 41 and 42 were used to immobilize lactose and G3 for molecular recognition force microscopy experiments on a custom 3-axis AFM using a previously reported automated force minimization protocol \( F_{\text{min}} \) described in Chapter 2.\(^{84}\) A series of unbinding experiments were conducted in order to directly compare the probability of binding, blocking efficiency, rupture forces and rupture
lengths generated using our new immobilization protocols to those results generated using the traditional aminosilanization protocol.

Initially, 200 force distance curves were generated at 20 different locations on the sample surface in the presence of PBS (pH 7.4). To confirm the specificity of the observed binding events, this experiment was repeated in the presence of soluble β-methyl lactose (10 mM in PBS pH 7.4). Analysis of force vs. extension plots was performed as described above (Chapter 2). All rupture forces >35 pN were considered to be resolved above noise. Unbinding events at extensions below 10 nm were considered non-specific adhesion.

3.4.2 Contact forces

Histograms of contact forces produced using the F_{min} routine are displayed in Figure 43. The most frequent contact force was ~60 pN, however higher contact forces (>100 pN) were often observed. We previously demonstrated the importance of using a data collection routine that minimizes contact forces to ensure that ruptures originate from specific, blockable interactions. Therefore, only force distance curves resulting from contact forces between 40 and 300 pN were considered in the analysis.
3.4.3 Probability of binding

Figure 44 depicts a representative force vs. extension plot for lactose-G3. The probability of observing a rupture event ($\rho_{\text{bind}} = \text{number of rupture-containing pulls/total pulls}$) is 0.41. To confirm the specificity of the observed binding events, soluble β-methyl lactose (10 mM in PBS pH 7.4) was introduced into the AFM liquid cell to prevent interactions between surface-bound G3 and immobilized lactose. Upon introduction of this blocking agent, $\rho_{\text{bind}}$ decreased to 0.10; a 76% blocking efficiency.
Figure 44: Representative force vs. extension plot of a typical rupture event; approach (green) and retract (black) curves shown. Inset: Binding probabilities for unblocked (PBS, pH 7.4) and blocked (β-methyl lactose, 10mM in PBS, pH 7.4) systems.

The goal of this work was to develop a general functionalization protocol for AFM binding studies. The traditional approach to AFM binding experiments requires careful optimization of functionalization conditions for each new molecular system. Our approach provides a general, simple method for covalent immobilization of a diverse variety of organic and biological molecules. The technique produces stable molecular systems through the formation of Si-C bonds and facilitates the formation of specific interactions between surface-immobilized G3 and immobilized lactose. Previously we investigated the probability of binding between G3 and lactose immobilized through traditional silane-based methods. Here, we achieved both higher
binding probabilities ($\rho_{\text{bind}} = 0.41$ vs. 0.33) and essentially identical blocking efficiencies using monolayers more stable and robust than those previously utilized.

### 3.4.4 Rupture forces and rupture lengths

Histograms of rupture forces and rupture lengths are displayed in Figure 45. The distribution of rupture forces for the lactose-G3 system is unimodal with the most frequently observed rupture forces falling between 60 to 80 pN. Rupture force histograms are in excellent agreement with those from lactose-G3 anchored by APTMS (Figure 33A) and in good agreement with previously reported forces of 50±10 pN using the same retraction rate.6

![Histograms of rupture forces and rupture lengths](image)

**Figure 45:** Rupture forces and rupture lengths for unblocked (PBS, pH 7.4) and blocked (10mM β-methyl lactose in PBS, pH 7.4) unbinding experiments for the lactose-G3 system using the $F_{\text{min}}$ data collection routine
Because G3 contains, at its N-terminus, a disordered 120 amino acid collagen-like-repeat (CLR), we estimated the total rupture length of immobilized lactose bound to a properly folded carbohydrate recognition domain with fully extended CLR and linkers to be ~63 nm. We assume that while force signatures at extensions between 10 and 70 nm potentially originate from the rupture of specific complexes, events at extensions >70 nm originate from either aggregation of non-specifically adsorbed material between the surface and tip or partial protein unfolding, assuming partially unfolded protein retains at least some affinity for its ligand.

Rupture length distributions for the unblocked and blocked experiments (Figure 45) are in excellent agreement with those reported for aminosilane anchored G3 and lactose (Figure 34A). Under minimal contact forces the rupture length histogram profile for aminosilane anchored lactose-G3 binding was unimodal with the centroid of the distribution at ~50 nm. Similar histogram morphology was observed using hydrosilylation chemistry. The most populated length bins are those between 20 and 60 nm, indicating that the majority of rupture lengths were within the total estimated length of a specifically bound lactose-G3 complex.

We determined previously that the biophysical significance of ruptures cannot be fully interpreted solely on the basis of rupture force and rupture length histograms and that blocking experiments remain essential for the interpretation of cantilever movement. Here, we have demonstrated essentially identical lactose-G3 rupture force profiles, rupture length profiles and blocking efficiencies using aminosilane chemistry.
and our novel monolayers directly bound to silicon surfaces and Si₃N₄ tips via stable Si-C bonds.

3.5 Conclusions

In conclusion, we have successfully demonstrated the first example of stable and efficient covalent immobilization of a binding partner to a silicon nitride AFM tip using hydrosilylation chemistry. We also took advantage of a new surface-immobilization technique that forms stable protective monolayers on silicon for attachment of histidine tagged proteins. Unbinding experiments demonstrated that these methodologies ensure that binding partners can orient properly to form specific blockable interactions between binding partners for biophysical studies. Finally, our technique can be optimized to distribute binding partners uniformly across the surface of the sample and tip and can be used as a general attachment technique for other molecular systems for MRFM studies ensuring the formation of systems robust to biophysical experimental conditions.
4. Vancomycin binding studies

4.1 Overview

The goal of this work is to develop molecular systems with which to study the binding of immobilized vancomycin to its cognate binding partner, L-Lys-D-Ala-D-Ala (KDADA). Our ultimate goal is the routine evaluation of thermodynamic parameters for immobilized binding partners through interpretation of the variation of the binary probability of binding as a function of soluble ligand concentration. The development of methodologies that produce stable ordered molecular systems on surfaces are of paramount importance towards achievement of this goal, and we will use the methodologies developed here will be applied to investigating the thermodynamics of multivalent vancomycin-KDADA systems.

Vancomycin is a glycopeptide antibiotic that inhibits cell wall biosynthesis in gram-positive bacteria. It is used clinically to treat severe staphylococcal infection.\textsuperscript{176} Gram positive bacteria rely on properly formed cell walls to maintain structural integrity; cell walls are composed of linear polysaccharide chains of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc). In a properly formed cell wall, these linear chains are periodically cross-linked by peptide chains comprised of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. Cross linking of the terminal D-ala to an adjacent GlcNAc-MurNAc polymer strand is catalyzed by transpeptidase enzymes. Vancomycin binds to the C-terminal KDADA of the sequence, competitively inhibiting transpeptidase binding. Vancomycin-KDADA binding precludes cross-linking and bacterial cells subsequently rupture under osmotic pressure.
Because of its clinical significance, complexation of vancomycin to peptides terminating in DADA is one of the most extensively studied model systems of non-covalent association in aqueous solution. Vancomycin-KDADA binding has been studied by Williams, Pratt, Whitesides, and others. Rao and coworkers reported a binding constant for the monovalent vancomycin-KDADA complex of $1.6 \times 10^{-6}$ M by isothermal titration calorimetry.

Whitesides and coworkers also used solution phase assays to demonstrate enhancements in binding of $\sim 10^3$ for the bivalent system ($K_d = 1.1 \times 10^{-9}$ M by affinity capillary electrophoresis), and $\sim 10^{11}$ for the trivalent system ($K_d = 4 \times 10^{17}$ M by displacement ITC) relative to the monomeric vancomycin-KDADA complex. Intermolecular binding, aggregation, and precipitation complicate the evaluation of multivalent affinities by solution phase assays. Affinity capillary electrophoresis is susceptible to errors induced by soluble aggregates which exhibit electrophoretic mobilities indistinguishable from those of the chelate-bound complex. Precipitation of insoluble aggregates within capillary tubes or within an ITC cell can further complicate such assays. Still, these massive reported enhancements in multivalent vancomycin-KDADA affinity provide a compelling reason to investigate mono- and multivalent systems using a single molecule approach that precludes intermolecular binding and aggregation.

To this end, we sought to develop efficient protocols for the rapid, robust, and uniform adsorption of oriented vancomycin and KDADA to surfaces and AFM cantilevers tips. Such methodologies will facilitate future evaluation of thermodynamic parameters.
for monovalent and multivalent systems comprising vancomycin and its cognate binding partner L-Lys-D-Ala-D-Ala (KDADA).

4.2 Immobilization of binding partners

4.2.1 Regiochemistry of tether attachment

The molecular assemblies for AFM binding studies were chosen based on the structure of the vancomycin-DADA complex and on the reactive moieties available for modification. Tight intermolecular binding is achieved by a series of five hydrogen bonds between the two partners (Figure 46). Apparent in Figure 46, the ε-amino group (green sphere) on KDADA, along with the C-terminal carboxylate (red sphere), and the primary amino group of vancosamine do not participate in key hydrogen bonding interactions. Furthermore, these positions can be modified using established synthetic techniques. 181, 188, 190-197
Figure 46: Vancomycin-KDADA complex. Reactive moieties to which tethers can be linked are denoted by spheres: vancosamine linkage (blue), C-terminal linkage (red), and $N^\varepsilon$-Lys-linkage (green).

Rao and Whitesides determined that C-terminal modified vancomycin binds $N^{\alpha,\varepsilon}$-diacetyl-L-Lys-D-Ala-D-Ala in solution. We therefore reasoned that tethering KDADA at the $N^\varepsilon$-Lys side chain and tethering vancomycin at the C-terminus would still permit productive binding. Additionally, Crowley and Boger determined that the deglycosylated vancomycin aglycon retains its ability to bind $N^{\alpha,\varepsilon}$-diacetyl-L-Lys-D-Ala-D-Ala in solution.\textsuperscript{194} We therefore reasoned that tethering vancomycin through the vancosamine moiety would also not prohibit binding.
Figure 47 depicts immobilized C-terminal-linked and vancosamine-linked complexes between tip-immobilized KDADA and sample-immobilized vancomycin. To our knowledge, no formal comparison of the thermodynamic parameters of KDADA binding to C-terminal-linked and vancosamine-linked vancomycin has been reported. However, previous studies have demonstrated that C-terminal linked and vancosamine-linked vancomycin-modified particles display measurable differences in their ability to bind KDADA-modified polystyrene beads\textsuperscript{198} and gram positive bacterial cells.\textsuperscript{199} These studies provided motivation to investigate both linkages by MRFM.
4.2.2 Immobilization strategies

Immobilization strategies were based on those described in Chapters 2 and 3, which had previously proven successful for MRFM studies. We reasoned that adaptation of these strategies would provide molecular assemblies with sufficient conformational flexibility to properly orient the system for binding and not interfere with productive ligand-receptor interactions.
Our strategy to covalently immobilize C-terminal-linked vancomycin and KDADA to surfaces and AFM tips, respectively, involved Huisgen 1,3-dipolar cycloaddition of alkyne derivatives to surface immobilized azide groups. Aminofunctionalization of tips and surfaces was accomplished using APTMS.

Our strategy to covalently immobilize vancosamine-linked vancomycin and KDADA to surfaces and AFM tips, respectively, involved conjugate addition of thiolated derivatives to surface immobilized maleimide groups. Three methods of aminofunctionalization of tips and surfaces were compared: etherification using ethanolamine, silanization using APTMS, and alkylation followed by diazirine carbene addition.

4.3 Materials for surface functionalization

4.3.1 Synthesis of soluble ligand

In order to competitively inhibit binding between immobilized vancomycin and KDADA we required a stable soluble ligand with high affinity for vancomycin. The tripeptide $N^\varepsilon$-diacetyl-L-Lys-D-Ala-D-Ala 25 has been shown by Rao and coworkers to bind both unmodified vancomycin$^{177}$ and bi-$^{182}$ and trivalent$^{180}$ derivatives, with dissociation constants ($K_d$) of $1.6 \times 10^{-6}$ M to $2.7 \times 10^{-6}$ M. Tripeptide 25 was synthesized from commercially available amino acids in six steps (Figure 48).

$N^\varepsilon$-protection of $N^\alpha$-acetyl-L-Lys with benzyl chloroformate was carried out under basic conditions to provide carboxylic acid 19. Carbodiimide coupling of Z-D-Ala with D-Ala-OtBu provided the fully protected dipeptide 20. Palladium catalyzed
hydrogenolysis of 20 provided free amine 21, which was coupled with carboxylic acid 19 to provide protected tripeptide 22. Palladium catalyzed hydrogenolysis revealed a free amine at the $N^\varepsilon$-position of 23, which was subsequently reacted with acetic anhydride in the presence of triethylamine to give bis-acetylated tripeptide 24. Acid-catalyzed deprotection of the C-terminal tert-butyl ester provided $N^{a,\varepsilon}$-diacetyl-L-Lys-D-Ala-D-Ala 25.

**Figure 48:** Synthesis of tripeptide ligand $N^{a,\varepsilon}$-diacetyl-L-Lys-D-Ala-D-Ala.

### 4.3.2 Synthesis of thiolated binding partners

Our strategy for covalent immobilization of vancosamine-linked vancomycin and KDADA to surfaces and AFM tips, respectively, involved conjugate addition of thiolated
derivatives to surface-immobilized maleimide groups. Thiolated $N^{\alpha}$-acetyl-L-Lys-D-Ala-D-Ala 29 was synthesized from mercaptopropionic acid in four steps (Figure 49). Mercaptopropionic acid was protected as the 2,4-dinitrophenyl thioether by reaction with 2,4-dinitrochlorobenzene to give 26. Carbodiimide-mediated coupling of 26 to the tripeptide 23 provided fully protected t-butyl ester 27. Acid catalyzed deprotection of 27 in a 1:1 mixture of trifluoroacetic acid and dichloromethane provided the carboxylic acid 28, which was reacted with β-mercaptoethanol and N,N-diisopropylethylamine to yield thiolated $N^{\alpha}$-acetyl-L-Lys-D-Ala-D-Ala 29.

![Chemical Structure](image)

**Figure 49: Synthesis thiolated $N^{\alpha}$-acetyl-L-Lys-D-Ala-D-Ala.**

A vancosamine-linked thiobutyl-vancomycin derivative 36 was also prepared. Regioselective modification of vancosamine required protection of the highly reactive secondary amine at the N-terminus of vancomycin (Figure 50). For vancomycin substrates, the regioselectivity of Boc-protection is highly dependent on both solvent
and base. Initial attempts to react vancomycin with di-tert-butyldicarbonate (Boc₂O) in DMF with triethylamine resulted in a mixture of vancosamine-protected, N-terminus-protected, and N,N’-bis-protected products in approximately 1:2:3 molar ratio (determined by HPLC analysis). Regioselective N-terminal Boc-protection was achieved by reaction of vancomycin with Boc₂O in a 1:1 mixture of dioxane/water with sodium bicarbonate. Molecular fragmentation during ESI mass spectrometric analysis confirmed the desired regiochemistry.

![Diagram](image)

**Figure 50: Regioselective N-Boc protection of vancomycin.**

The vancosamine-linked thiobutyl-vancomycin derivative 36 was synthesized from 30 in six steps (Figure 51). Tritylmercaptan was stirred with sodium hydride and then reacted with bromobutanioc acid in DMF to provide the carboxylic acid 31. PYBOP-mediated coupling of 31 to N,O-dimethyl hydroxylamine provided Weinreb amide 32. Reduction of 32 with lithium aluminum hydride followed by quenching with saturated sodium sulfate gave the aldehyde 33. Imine formation between Boc-protected vancomycin 30 and aldehyde 33 in DMF/MeOH (9:2) occurred exclusively at the
vancosamine amine to give 34. Reductive alkylation was completed by addition of sodium cyanoborohydride. Molecular fragmentation during ESI mass spectrometric analysis again confirmed the desired regiochemistry. Acid catalyzed removal of both trityl and Boc protecting groups provided the vancosamine-linked thiobutyl-vancomycin derivate 35, which was isolated by preparative HPLC. The purified thiol 35 was unstable in aqueous solution at room temperature. ESI-LCMS analysis displayed impurities consistent with spontaneous oxidation to disulfide 36 and deglycosylation of the thiol to give vancomycin aglycon. The thiol was therefore protected by quantitative oxidation to disulfide 36. Isolation by prep-HPLC provided pure material that was both stable in aqueous solution and readily reduced to the reactive thiol by tris (2-carboxyethyl) phosphine (TCEP).
4.3.3 Synthesis of alkyne modified binding partners

Our strategy for covalent immobilization of KDADA and C-terminal-linked vancomycin to AFM tips and surfaces, respectively, involved Huisgen 1,3-dipolar cycloaddition of alkyne derivatives to surface-immobilized azide groups. Alkyne terminated PEG₆ modified KDADA 41 was synthesized in five steps (Figure 52). Hexaethylene glycol stirred with sodium hyride then reacted with propargyl bromide to provide monosubstituted alcohol 37. Deprotonation with sodium hydride followed by reaction with tert-butyl bromoacetate provided tert-butyl ester 38. Acid catalyzed
deprotection in a 1:3 mixture of trifluoroacetic acid and DCM provided the carboxylic acid 39. Carbodiimide-mediated coupling with tripeptide 23 provided the tert-butyl ester 40, which was deprotected in a 2:3 mixture of trifluoroacetic acid and DCM to give the PEG₆-alkyne modified Nα-acetyl-L-Lys-D-Ala-D-Ala 41.

![Chemical diagram]

**Figure 52: Synthesis of PEG₆-alkyne modified Nα-acetyl-L-Lys-D-Ala-D-Ala.**

Synthesis of C-terminus linked vancomycin alkyne derivative 44 was accomplished in three steps from alcohol 37 (Figure 53). Conversion of alcohol 37 to azide 42 was accomplished with diphenyl phosphoryl azide and DBU. Staudinger reduction with triphenyl phosphine in a mixture of ammonium hydroxide and pyridine provided the amine 43. HBTU coupling of amine 43 to vancomycin in DMF provided C-terminal PEG₆-alkyne modified vancomycin 44.
Figure 53: Synthesis of C-terminal linked vancomycin alkyne

Heterobifunctional linker 50 (azido-PEG_{32}-acid) was synthesized (Figure 54) and used to covalently immobilize alkyne modified binding partners to amine modified surfaces. Hexaethylene glycol was monotosylated and then reacted with diphenylphosphoryl azide and excess sodium azide to form 46. Alkylation with tert-butyl bromoacetate provided the tert-butyl ester 47, which was subsequently deprotected under acidic conditions to produce 48. Carboxylic acid 48 was converted to the acid chloride using oxalyl chloride in the presence of catalytic DMF. Immediate reaction with benzotriazole formed the activated amide 49. Treatment with commercially available amino-PEG_{24}-acid (Quanta Biodesign) provided azido-PEG_{32}-acid 50.
4.4 Surface preparation and unbinding studies

4.4.1 C-terminal linkage

4.4.1.1 Click chemistry protocol for tip and sample modification

Our goal here is to investigate the binding properties of C-terminal linked vancomycin to tip-immobilized KDADA. Our immobilization strategy was based on the Huisgen 1,3-dipolar cycloaddition “click” reaction between terminal azides and alkynes. The Huisgen 1,3-dipolar cycloaddition has been thoroughly studied by Meldal\textsuperscript{200} and Sharpless,\textsuperscript{201} and the utility of “click” reactions for the immobilization of molecules to both inorganic surfaces and to living cells has been demonstrated by numerous groups.\textsuperscript{202-205}

Our motivation for implementing this immobilization strategy was provided by a temporary interruption of the commercial availability of heterobifunctional linker (maleimide-dPEG24-NHS Ester), which we had previously used for thiol immobilization.
We were compelled us to switch to azido-PEG$_{32}$-acid linker 50, which was used to selectively and covalently immobilize alkyne-modified binding partners.

Figure 55 describes the molecular assemblies used for modification of silicon nitride AFM tips T1 and silicon surfaces S1 using a silanization protocol. Plasma oxidized silicon nitride AFM tips and silicon surfaces were aminosilanized with APTMS and the resulting amine layers T2 and S2 were treated with DCC and coupled to a 15 nm heterobifunctional poly(ethylene glycol) tether comprised of azido-dPEG$_{32}$-acid 50. Diploar cycloaddition of alkyne-modified $N^\alpha$-acetyl-L-Lys-D-Ala-D-Ala 41 to azide-modified tips T3 provided T4, and reaction of C-terminal-linked vancomycin alkyne 44 to azide-modified surface S3 provided surface-immobilized vancomycin S4.
Figure 55: Click-based modification of Si$_3$N$_4$ tips with N$^\alpha$-acetyl-L-Lys-D-Ala-D-Ala and silicon surfaces with vancomycin via C-terminal linkage (red dot) using APTMS.

4.4.1.2 Click tip / sample unbinding experiments

Molecular recognition force microscopy experiments were conducted using a custom 3-axis AFM using the $F_{\text{min}}$ protocol described in Chapter 2.$^{54}$ Unbinding experiments were conducted using four tips T4 and four silicon samples S4 prepared according Figure 55. The probability of binding of each tip-sample system was determined.

For each experiment, 250 approach-retract cycles were performed at 25 different locations on the sample in the presence of phosphate buffer (5.0 mM, pH 7.0). Approach and retraction velocities were 200 nm s$^{-1}$, approach and retraction distances
were 200 nm, and dwell time was 1 sec. Experiments 1-3 (Figure 56) were performed on three separate tip-sample systems. Experiments 4a and 4b were performed using the same tip on two distinct regions of the same sample. Analysis of force vs. extension plots provided the probability of observing a rupture ($\rho_{\text{bind}} = \text{number of rupture-containing pulls} / \text{total pulls}$) for each experiment. All ruptures resolved above noise were included in data analysis, regardless of extension.

Evident from Figure 56, $\rho_{\text{bind}}$ varied from 0.02 to 0.24 across all tip-sample systems. Experiments 4a and 4b resulted in $\rho_{\text{bind}}$ ranging from 0.04 to 0.24. We concluded that the average $\rho_{\text{bind}}$ (0.09) was too low and the overall variability (standard deviation = 0.09) was too high to use this system for the probabilistic evaluation of the thermodynamics of immobilized vancomycin-KDADA binding. Because our approach is based on the variation of binary probability of binding as a function of soluble ligand concentration, it is essential to maximize the probability of forming specific non-covalent interactions in a reproducible manner.

![Figure 56: Probability of observing a bound interaction using click protocol](image)

Figure 56: Probability of observing a bound interaction using click protocol
We hypothesized that the $\rho_{\text{bind}}$ values obtained for C-terminal linked vancomycin were a function of the site and method of tether attachment, which may sterically preclude key contacts between ligand and receptor. Inspection of the bound vancomycin-KDADA complex (Figure 46) reveals a key hydrogen bond close in proximity to the site of C-terminal attachment. The distribution of $\rho_{\text{bind}}$ values may also reflect low yields in click chemistry or DCC coupling of PEG linker 50 to amine groups on the APTMS surfaces: hydrogen bonding of amines in APTMS layers to surface oxides has been shown to inhibit subsequent coupling reactions.\textsuperscript{145, 147, 154} We therefore sought both an alternative site of tether attachment and alternative immobilization strategies.

### 4.4.2 Vancosamine linkage

In order to test the hypothesis that $\rho_{\text{bind}}$ values are affected by the site and method of tether attachment, we investigated the binding of vancosamine-linked vancomycin to tip-immobilized KDADA. Inspection of Figure 46 reveals that no key hydrogen bonds between vancomycin and KDADA are close in proximity to the site of tether attachment on vancosamine. We previously reported covalent immobilization of thiolated saccharides to maleimide surfaces via conjugate addition (Chapters 2 and 3), and we adopted a similar strategy for the immobilization of thiolated KDADA 29 and thiobutyl vancomycin 36 via conjugate addition to heterobifunctional maleimide-dPEG24-NHS ester. Here we report the effect of three immobilization protocols on the probability of observing rupture events, on force curve morphology, and on rupture force and length distributions.
4.4.2.1 Etherification protocol for tip and sample modification

Figure 57 describes the molecular assemblies used for modification of AFM tips \( T_1 \) and silicon surfaces \( S_1 \) using an ethanolamine etherification protocol. Plasma oxidized silicon nitride AFM tips and silicon surfaces were heated under a solution of ethanolamine hydrochloride in DMSO with molecular sieves. Dehydration of SiOH groups with ethanolamine produces low surface density amino functionalization and highly diffuse reactive groups anchored by single Si-O-C bonds\(^ {21,38} \). The resulting amine layers \( T_2 \) and \( S_2 \) were coupled to a 10 nm heterobifunctional poly(ethylene glycol) (PEG) tether comprised of maleimide-dPEG\(_{24}\)-NHS ester (Quanta Biosdesign). Conjugate addition of thiolated \( \text{N}^{\alpha}\text{-acetyl-L-Lys-D-Ala-D-Ala} \) \( 29 \) to maleimide-modified tips \( T_3 \) provided tip-immobilized KDADA \( T_4 \). Conjugate addition of vancosamine-linked thiobutyl vancomycin, formed in situ by TCEP reduction of disulfide \( 36 \), to maleimide-modified surface \( S_3 \) provided surface-immobilized vancomycin \( S_4 \).
Figure 57: Modification of Si₃N₄ tips with thiolated N⁶-acetyl-L-Lys-D-Ala-D-Ala and silicon surfaces with thiobutyl vancomycin via vancosamine-linkage (blue dot) using ethanolamine protocol.

4.4.2.2 Ethanolamine tip / sample unbinding experiments

Molecular recognition force microscopy experiments were conducted using the $F_{\min}$ protocol with a single tip T₄ and silicon sample S₄ prepared according to the protocol described in Figure 57. All experiments were performed in the presence of phosphate buffer (5.0 mM, pH 7.0) with approach and retraction velocities of 100 nm s⁻¹, approach and retraction distances of 100 nm, and a dwell time of 1 sec. For experiment 1 (Figure 58), 80 approach-retract cycles were performed at 8 different locations on the sample surface. For experiment 2, 100 approach-retract cycles were performed in the same X,Y location on the sample surface. For experiments 3-5, the protocol for experiment 2 was repeated at different X,Y locations on the sample surface.
Force vs. extension plots were analyzed according to the protocol described in chapter 2. All rupture forces greater than 35 pN were considered, regardless of extension.

As shown in Figure 58, probing multiple positions in a single experiment (Exp 1) resulted in a $\rho_{\text{bind}}$ of 0.15. For experiments 2-5 the probability varied from 0.07 to 0.25, depending on lateral position of the tip on the sample. This positional variation is consistent with that observed in Figure 56 (experiments 4a and 4b). We concluded that the average $\rho_{\text{bind}}$ (0.16) was too low and the overall variability (standard deviation = 0.07) was too high to use this system for the probabilistic evaluation of thermodynamics of immobilized vancomycin-KDADA binding.

![Figure 58: Probability of binding using ethanolamine etherification protocol.](image)

Figure 59 depicts a force vs. extension plot and histograms of contact forces, rupture forces, and rupture lengths for experiments 1-5 (Figure 58). The majority of contact forces were below 300 pN. The most frequently observed rupture forces were
between 60 and 80 pN and the majority of rupture lengths were less than the overall length of the fully extended molecular system (~25 nm).

![Force vs. extension plot and histograms of contact forces, rupture forces, and rupture lengths for experiments 1-5 (Figure 58)](image)

**Figure 59**: Force vs. extension plot and histograms of contact forces, rupture forces, and rupture lengths for experiments 1-5 (Figure 58)

### 4.4.2.3 Silanization protocol for tip and sample modification

Figure 60 describes the molecular assemblies used for modification of AFM tips **T1** and silicon surfaces **S1** using a silanization protocol. Plasma oxidized silicon nitride AFM tips and silicon surfaces were aminosilanized with aminopropyltrimethoxy silane (APTMS) and the resulting amine layers **T2** and **S2** were coupled to a 10 nm heterobifunctional poly(ethylene glycol) tether comprised of maleimide-dPEG<sub>24</sub>-NHS ester. Conjugate addition of thiolated N<sup>α</sup>-acetyl-L-Lys-D-Ala-D-Ala **29** to maleimide-modified tips **T3** provided tip-immobilized KDADA **T4**. Conjugate addition of vancosamine-linked thiobutyl vancomycin, formed in situ by TCEP reduction of disulfide **36**, to maleimide-modified surface **S3** provided surface-immobilized vancomycin **S4**.
Figure 60: Modification of Si₃N₄ tips with thiolated Nα-acetyl-L-Lys-D-Ala-D-Ala and silicon surfaces with vancosamine-linked thiobutyl vancomycin using a silanization protocol.

4.4.2.4 APTMS tip / sample unbinding experiments

Molecular recognition force microscopy experiments were conducted using the Fₘᵢₙ protocol with a single tip T₄ and silicon sample S₄ prepared according Figure 60. All experiments were performed using approach and retraction velocities of 100 nm s⁻¹, approach and retraction distances of 100 nm, and a dwell time of 1 sec. Force vs. extension plots were analyzed according to the protocol described in chapter 2. All rupture forces greater than 35 pN were considered, regardless of extension.

Figure 61 displays the ρₜₙᵢₙ results of several unbinding experiments. In an effort to localize the tip above immobilized vancomycin receptors, multiple positions on the sample surface were probed (10 pulls per position) until ruptures were observed in >5
pulls at a single position (X,Y position 1). For experiment 1, 225 approach-retract cycles were performed at X,Y position 1. $\rho_{\text{bind}}$ at this position was 0.44.

![Figure 61: Probability of binding using APTMS protocol. Concentration of $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala (25) are shown in parentheses](image)

This protocol was repeated in order to localize the tip above a new group of receptors (X,Y position 2). For experiment 2a, 100 approach-retract cycles were performed at X,Y position 2. $\rho_{\text{bind}}$ at this position was 0.29. In order to determine if the ruptures resulted from specific vancomycin-KDADA binding, experiment 2b (100 pulls, X,Y position 2) was performed in the presence of soluble ligand $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala 25 (1 μM in 5 mM phosphate buffer, pH 7.0). Experiment 2c was performed using the same protocol in the presence of 25 (100 μM in 5 mM phosphate buffer, pH 7.0). $\rho_{\text{bind}}$ for experiments 2b and 2c was 0.55 and 0.03, respectively, indicating that rupture events were specific in nature. These results imply the binding constant for the system, i.e. the concentration of 25 which results in $\rho_{\text{bind}}$ equal to $\frac{1}{2}$ maximum $\rho_{\text{bind}}$, lies between $10^{-6}$ and $10^{-4}$ M. This value is similar in magnitude to the solution-phase binding
constant reported for the monovalent vancomycin-KDADA complex\textsuperscript{177} ($1 \times 10^{-6}$ M) and to that of soluble vancomycin bound to immobilized KDADA ($2.7 \times 10^{-6}$ M).\textsuperscript{189}

Not only were binding events successfully blocked by soluble ligand, but binding was restored after soluble ligand was flushed from the system. Experiment 2d was performed using the same protocol as experiments 2a-2c after washing the liquid cell with buffer ($4 \times 50 \mu$L). The resulting $\rho_{\text{bind}}$ was 0.44, which is in good agreement with the average $\rho_{\text{bind}}$ of experiments 2a and 2b (0.42).

Figure 62 depicts a force vs. extension plot and histograms of contact forces, rupture forces, and rupture lengths for experiment 1 (Figure 61). The majority of contact forces were below 200 pN. The most frequently observed rupture forces were between 60 and 80 pN and all rupture lengths were less than the overall length of the fully extended molecular system (~25 nm).

![Figure 62: Force vs. extension plot and histograms of contact forces, rupture forces, and rupture lengths for experiment 1 (Figure 61)](image)
Although these results suggest that this molecular system produces probabilities sufficiently high to probe the thermodynamics of vancomycin-KDADA binding, the probabilities were not reproducible. Replication of experiment 1 with a new tip and sample resulted in $\rho_{\text{bind}}$ variation between 0.09 and 0.28 in the same X,Y position. A series of 12 similar AFM binding experiments using multiple tips and samples resulted in $\rho_{\text{bind}}$ variation between 0.05 and 0.11.

A possible explanation for the high degree of $\rho_{\text{bind}}$ variability is incomplete surface reactions leading to variable ligand and receptor surface densities at the interface between different tips and samples. We attempted to drive the reactions which produced tips T3, T4 and samples S3, S4 (Figure 60) to completion by incubation at 37 °C for 3-7 h. AFM binding experiments ($F_{\text{min}}$, 100 pulls at a single X,Y position) consistently resulted in $\rho_{\text{bind}}$ values greater than 0.9 (Figure 63), however the morphology of the force vs. extension plots (Figure 63) was inconsistent with those of the unheated molecular system (Figure 62).

**Figure 63: Force vs. extension plots with evidence of APTMS polymerization**

Whereas all force curves for the unheated system displayed single or double ruptures at lengths less than 25 nm, the heated system resulted in force curves that
frequently contained >3 ruptures at rupture lengths greater than 30 nm. A potential cause of this behavior may be heat-induced desorption and polymerization of the APTMS underlayer during surface preparation. This explanation is consistent with literature reports of water-mediated polymerization of APTMS resulting in polydisperse aggregates with variable degrees of cross linking.\textsuperscript{141, 142, 145, 147, 152} The high $\rho_{\text{bind}}$ values likely resulted from complexation between multiple polymers bearing singly- or multiply- immobilized vancomycin and KDADA groups instead of the desired interactions between single immobilized vancomycin and KDADA.

4.4.2.5 Alkylation protocol for surface modification

We reasoned that the density of binding partners might be maximized and polymerization might be minimized by incorporating a reactive monolayer anchored to the surface by stable Si-C bonds. We have previously described a method for alkylation of chlorinated silicon and subsequent formation of reactive NHS esters (Chapter 3). The technique simultaneously protects silicon from degradation in biological environments and provides a reactive site for the immobilization of thiolated vancomycin.

Silicon $<111>$ S1 was cleaned with nanostrip, etched with HF (5% aq. solution), and then chlorinated with PCl$_5$ to form surface S2 (Figure 64). Alkylation with propenyl magnesium bromide 16 at 135°C for 24 hours formed S3. The alkylated substrate was reacted with diazirine 17 (generously provided by Alex Shestopalov Ph.D.) yielding a reactive NHS monolayer. The resulting surface S4 was treated with N-Boc ethylene diamine to form S5. Acid catalyzed deprotection and coupling of the resulting amine layer to maleimide-dPEG$_{24}$-NHS ester provided S6. Conjugate addition of vancosamine-
linked thiobutyl vancomycin, formed in situ by TCEP reduction of disulfide 36, to maleimide-modified surface S6 provided surface-immobilized vancomycin S7.

Figure 64: Modification of silicon surfaces with vancosamine-linked thiobutyl vancomycin using an alkylation protocol.

4.4.2.6 APTMS tip / Alkyl surface unbinding experiments

Molecular recognition force microscopy experiments were conducted to investigate the effect of heat on the APTMS tips and alkyl surfaces. Two vancomycin-modified silicon samples were prepared according to the alkylation protocol (Figure 64). Sample S7A was prepared at room temperature and sample S7B was prepared by
heating the thiol conjugate addition to 37 °C for 5 h. Two KDADA tips were prepared according to the silanization protocol (Figure 60). Tip T4A was prepared at room temperature and tip T4B was prepared by heating the thiol conjugate addition to 37 °C for 7 h. All unbinding experiments were performed using the F_{min} protocol with approach and retraction velocities of 100 nm s\(^{-1}\), approach and retraction distances of 100 nm, and a dwell time of 1 sec. Force vs. extension plots were analyzed according to the protocol described in chapter 2. All rupture forces greater than 20 pN were considered, regardless of extension.

Histograms of contact forces are shown in Figure 65. The majority of contact forces were below 250 pN and the most frequently observed contact force for the unheated and heated systems was 100 pN and 160 pN, respectively.

![Figure 65: Contact force histograms using for experiments using (A) unheated system T4A-S7A and (B) heated system T4B-S7B](image)

Figure 66 shows important differences in \( \rho_{\text{bind}} \) values, morphology of force vs. extension plots, and histograms of rupture forces and lengths for the unheated system (T4A-S7A) and heated system (T4B-S7B). \( \rho_{\text{bind}} \) for the heated system (0.35) was more than double that of the unheated system (0.15) and it is clear that force curve morphology is fundamentally different between the two data sets. Force curves for the
unheated system were similar in morphology to those of the unheated APTMS system (Figure 62) with single ruptures predominating (>85%). All rupture lengths were less than the overall length of the fully extended molecular system (~25 nm). In contrast, force curves for the heated system differed qualitatively from all other molecular systems utilized in this study. Single ruptures predominated (>95%) and the majority of rupture lengths were greater than 30 nm. These elongated rupture lengths are consistent with heat induced polymerization of the APTMS underlayer on the tip. Because vancomycin is anchored by a highly stable alkyl molecular system, we assume that polymerization did not occur on the silicon sample. This assumption is supported by the difference in force curve morphology between Figure 63 and Figure 66B.
Figure 66: $\rho_{\text{bind}}$, force vs. extension plots, rupture forces, and rupture lengths for unheated system T4A-S7A (column A) and heated system T4B-S7B (column B)

Rupture force histograms also support our assumption of heat induced polymerization of the APTMS underlayer on the tip. The distribution of rupture forces for the unheated system was bimodal with the most frequently observed rupture force between 60 and 80 pN and a second maximum between 120 and 140 pN. For the heated system, the unimodal distribution is populated by lower forces, predominantly 40 to 80 pN in magnitude. Because rupture forces for a given molecular interaction at a given loading rate have been shown to depend on both affinity and stiffness of the molecular system,\textsuperscript{111} and since both systems comprise the same binding partners, it
follows that there is a difference in stiffness between the heated and unheated molecular systems. Polymeric structures on the tip could account for this difference in stiffness.

4.5 Conclusions

We have successfully demonstrated several protocols for tethering oriented vancomycin and its cognate binding partner L-Lys-D-Ala-D-Ala to surfaces and AFM tips. Unbinding experiments demonstrated that traditional methods for forming low surface density amine layers (silanization with APTMS and etherification with ethanolamine) provided molecular constructs which displayed probabilities of binding that were too low and showed overall variability too high to use for probabilistic evaluation of thermodynamics parameters. Instability and heat–induced polymerization of APTMS layers on tips and surfaces also prohibited their utility.

Alkylation of chlorinated silicon provides a more reliable, stable molecular system anchored by Si-C bonds that facilitates attachment of vancomycin and is capable of withstanding prolonged exposure to heated organic and aqueous environments. It follows that covalent immobilization of KDADA to silicon nitride AFM tips via Si-C bonds using hydroisilylation chemistry (described in Chapter 3) will be similarly advantageous and should be pursued during future studies.

These methods offer great promise for probabilistic evaluation of thermodynamic parameters characterizing immobilized binding partners. Our ultimate goal is formal comparison of the thermodynamics of KDADA binding to C-terminal-
linked and vancosamine-linked vancomycin and vancomycin oligomers. These studies will permit unambiguous determination of the role of multivalency in ligand binding, using an experimental configuration in which intermolecular binding and aggregation are precluded.
5. Experimental

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. 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). Surfaces functionalized with G3 were visualized by contact mode lateral force microscopy (scan rate 4 Hz) and by tapping mode force microscopy (scan rate 1 Hz) on a Veeco D3100 microscope using DNP silicon nitride probes with a 0.12 N/m spring constant. 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).

5.1 The effect of contact force on molecular recognition force microscopy

5.1.1 Synthesis

Synthesis of Mercaptopentyl Lactose 2. Synthesis of mercaptopentyl lactoside 2 was based on a previously reported method.⁶
(3DAC005) **S-Acetyl-Mercaptopentanyl-heptabenzoyl lactoside (9).** A sample of 1-pentenyl-heptabenzoyl lactoside 8 (50mg, 44μmol) was combined with thiolacetic acid (63μL, 880μmol) and AIBN (0.5mg, 4.4μmol) and THF (1mL) in a quartz cuvette. The solution was stirred under fume hood lights for 3.5h then condensed to an oil. Chromatography on silica gel (10-40% EtOAc in hexane) provided pure 9 (98% yield). \(^1\)H NMR (300MHz, CDCl\textsubscript{3}): \(\delta = 7.88-8.04\) (m, 12H), 7.70-7.75 (d, \(J = 7.4\)Hz, 2H), 7.11-7.66 (m, 21H), 5.7-5.85 (m, 3H), 5.35-5.50 (m, 2H), 4.88 (d, \(J = 8\)Hz, 1H), 4.68 (d, \(J = 8\)Hz, 1H), 4.60 (m, 1H), 4.49 (dd, \(J = 7.8, 4.4\)Hz, 1H), 4.25 (t, \(J = 9.5\)Hz, 1H), 3.65-3.91 (m, 5H), 3.38-3.48 (m, 1H), 2.58 (m, 2H), 2.25 (s, 3H), 1.15-1.54 (m, 6H). \(^{13}\)C NMR (300MHz, CDCl\textsubscript{3}): \(\delta = 196.08, 166.09, 165.80, 165.65\) (m), 165.46, 165.39, 165.03, 133.77, 133.61 (m), 133.42, 130.23, 129.99 (m), 129.88, 129.83, 129.66, 129.09, 128.87 (m), 128.76, 128.62, 128.47, 101.39, 101.20, 77.5 (m), 76.31, 73.23, 73.12, 72.02, 71.63, 70.12, 67.77, 62.66, 61.33, 30.80, 29.25, 29.00, 25.22; MS (ESI) calcd for C\textsubscript{68}H\textsubscript{62}O\textsubscript{19}S \([M + Na]^+\) 1237.4 found 1236.8; ir (thin film): \(\nu = 3063.5\) (weak), 2940.3 (weak), 1731.0, 1687.0, 1601.6, 1451.3, 1315.0, 1269.6, 1177.0, 1095.0, 1069.6, 1027.1, 708.9.

(3DAC007) **Mercaptopentanyl lactoside (2).** A solution of sodium methoxide (0.2M in MeOH) was degassed with argon and added to a sample of the thioester 9 which slowly
went into solution over 1.5h while stirring under argon. The reaction was neutralized to pH 6 with formic acid and condensed to a white solid, which was triturated with a 30% solution of MeOH in CH₂Cl₂. The triturate was condensed to a white solid, 2 (14mg, 96% crude yield). MS (ESI) calcd for C₁₇H₃₂O₁₁S [M + Na]⁺ 467.2 found 466.8.

**Synthesis of Mercaptopentyl Mannose 1.** A full description of the synthesis of mercaptopentyl mannoside 1 can be found.

**Synthesis of NTA-maleimide Linker 3**

Synthesis Nα-bis-carboxymethyl-Nε-3-maleimido-propionyl lysine 3, described below, was based on a previously reported method.²⁰⁶

![Cbz-NTA-lys-tris(trimethylsilyl)ester (11)](image)

**Cbz-NTA-lys-tris(trimethylsilyl)ester (11).** To a stirred solution of Cbz-NTA-lysine (1.0g, 2.5mmol) with EDC (1.70g, 8.8mmol) in N,N-dimethylformamide (10mL) was added TMSEOH (2.2mL, 15.1mmol) followed by DMAP (462mg, 3.8mmol). The solution was stirred at room temperature under argon for 16h, then condensed to ½ volume and poured into ether. After washing with brine (3x), the organic layer was dried over sodium sulfate and condensed to a clear oil. Chromatography on silica gel (0-15% EtOAc in Hexane) provided pure 11 (902mg, 50% yield). ¹H NMR (300MHz, CDCl₃): δ = 7.2-7.38 (m, 5H), 5.07 (s, 2H), 4.95-5.05 (m, 1H), 4.05-4.25 (m, 6H), 3.58 (s, 4H), 3.38 (t, J = 7.4Hz, 1H), 3.05-3.22 (m, 2H), 1.2-1.7 (m, 6H), 0.91-1.01 (m, 6H), 0-0.05 (m, 27H). ¹³C NMR (300MHz, CDCl₃): δ = 172.89, 171.51, 156.44, 136.79, 128.48, 127.99, 66.45, 64.68,
62.78, 62.69, 52.81, 40.82, 30.03, 29.39, 23.05, 17.56, 17.34, -1.48. MS (ESI) calcd for 
C_{33}H_{60}N_{2}O_{8}Si_{3} [M + Na]^+ 719.3 found 719.3. ir (thin film): ν = 3379.5, 2953.3, 2898.3, 1727.7, 1525.2, 1455.0, 1249.8, 1158.1, 859.9, 837.1.

(3DAC047) NTA-lys-tris(trimethylsilyl)ester (12). A solution of 11 (870mg, 1.25mmol) in anhydrous ethanol (10mL) was stirred under argon. To this was added a spatula of palladium on carbon. The flask was evacuated and refilled (3x) with hydrogen gas, then stirred for 18h under an atmosphere of hydrogen. The mixture was filtered through celite and condensed to give pure 12 (700mg, 99% yield). \(^1\)H NMR (300MHz, CDCl\(_3\)): δ = 4.05-4.25 (m, 6H), 3.72 (bs, 2H), 3.57 (s, 4H), 3.37 (t, J = 7.4Hz, 1H), 2.74 (t, J = 6.9Hz, 2H) 1.2-1.75 (m, 6H), 0.91-1.01 (m, 6H), 0-0.05 (m, 27H). \(^1\)C NMR (300MHz, CDCl\(_3\)): δ = 172.91, 171.56, 64.81, 62.81, 62.69, 52.80, 41.29, 31.59, 30.20, 23.16, 17.58, 17.37, -1.47. MS (ESI) calcd for C_{25}H_{54}N_{2}O_{6}Si_{3} [M + H]^+ 563.3 found 563.3. ir (thin film): ν = 2953.5, 2898.6, 1745.2, 1250.1, 1159.8, 1061.9, 1042.6, 937.1, 860.7, 837.3.

(3DAC053) N-maleoyl-β-ala-NTA-lys-tris(trimethylsilyl)ester (13). To a stirred solution of 12 (170mg, 0.306mmol), N-maleoyl-β-alanine (62mg, 0.367mmol), and hydroxybenzotriazole (50mg, 0.367mmol) in CH\(_2\)Cl\(_2\) at 0°C was added EDC (70mg, 0.367mmol). The solution was stirred at 0°C for 0.5h, then at room temperature for 15h. The solution was diluted
with CH$_2$Cl$_2$ and washed with water. The aqueous layer was extracted with EtOAc (2×) and the combined organic layers were dried over sodium sulfate and condensed to an oily solid. Chromatography on silica gel (20-40% EtOAc in Hexane) provided pure 13 (137mg, 63% yield). $^1$H NMR (300MHz, CDCl$_3$): $\delta = 6.70$ (s, 2H), 6.28-6.38 (m, 1H), 4.08-4.24 (m, 6H), 3.84 (t, $J=7.1$Hz, 2H), 3.59 (s, 4H), 3.41 (t, $J=7.4$Hz, 1H), 3.1-3.22 (m, 2H), 2.54 (t, $J=7.2$Hz, 2H), 1.34-1.76 (m, 6H), 0.93-1.06 (m, 6H), 0-0.05 (m, 27H). $^{13}$C NMR (300MHz, CDCl$_3$): $\delta = 173.06$, 171.64, 170.49, 169.88, 134.21, 64.25, 62.89, 62.77, 52.88, 39.16, 34.55, 34.44, 29.54, 28.13, 22.75, 17.57, 17.33, -1.48. MS (ESI) calcd for C$_{32}$H$_{59}$N$_3$O$_9$Si$_3$ [M + Na]$^+$ 736.4 found 736.4. ir (thin film): $\nu$ =3376.4, 3308.7, 3096.3, 2953.6, 2898.7, 1715.9, 1652.6, 1548.1, 1409.2, 1250.2, 1173.8, 1042.5, 936.8, 860.7, 764.7, 696.1.

(3DAC059) **N-maleoyl-β-alaa-NTA-lys (3).** To a stirred solution of 13 (70mg, 0.098mmol) in CH$_2$Cl$_2$ (2mL) at 0°C was added TFA (2mL) dropwise under argon. The solution was stirred for 5h at 5°C. Anhydrous toluene (8mL) was added and the cloudy solution was immediately condensed. The remaining solid was triturated with CH$_2$Cl$_2$, dissolved in D$_2$O, and filtered to give pure 3 (41mg, 100% yield). $^1$H NMR (300MHz, D$_2$O): $\delta = 6.89$ (s, 2H), 3.95-4.38 (m, 5H), 3.78 (t, $J=6.6$Hz, 2H), 3.22 (bs, 2H), 2.49 (t, $J=6.0$Hz, 2H), 1.80-2.06 (m, 2H), 1.32-1.62 (m, 4H). $^{13}$C NMR (300MHz, D$_2$O): $\delta = 173.55$, 172.72, 171.75, 170.18, 134.62, 67.29, 54.63, 38.94, 34.96, 34.74, 27.96, 26.79, 23.25.
5.1.2 Expression and purification of G3 and KDPG aldolase

Expression and Purification of Murine His<sub>6</sub>-tagged Galectin-3. The his<sub>6</sub>-tagged galectin-3 was expressed and purified as described by Snyder et al<sup>6</sup>. The gene for wild type galectin-3 was obtained by polymerase chain reaction (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 was transformed into BL21 (DE3) cells. A 5 mL portion of Terrific Broth (TB) containing Kanamycin (Kan) (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 600 nm (OD<sub>600</sub>) reached 0.6-0.8 and expression was induced for 3 h using 125 mg/L 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 ethylenediaminetetraacetic acid (EDTA, 1 mM) in sodium phosphate (50mM, pH 7.5) for 8 h, followed by dialysis against sodium phosphate (50 mM, pH 7.5) for 24 h to remove imidazole and EDTA.

Expression and Purification of His<sub>6</sub>-tagged 2-Keto-3-deoxy-6-phosphogluconate (KDPG) Aldolase A plasmid containing the gene KDPG-pet36, which encodes his<sub>6</sub>-tagged KDPG aldolase, was chemically transformed into BL21 CodonPlus (DE3)-RIL cells. The transformation mixture was plated onto a Luria Broth with kanamycin (LB/Kan, 50 mg mL<sup>-1</sup>) plate and incubated overnight at 37°C. A single isolated colony from the LB/Kan plate was introduced into 50 mL of Terrific Broth with kanamycin (TB/Kan, 50 mg mL<sup>-1</sup>).
The culture was incubated at 37°C for 16 h 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 adding isopropyl β-D-1-thiogalactopyranoside (IPTG) 230 mg per liter of cell culture. The induced culture was shaken at 37°C for an additional 4 h. 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.\(^{207}\)

5.1.3 Surface functionalization and AFM binding studies

**Functionalization of Silicon Nitride AFM Tips.** AFM tips (NP, Veeco, nominal spring constant of 120 pN/nm) were rinsed thoroughly in methanol and water, 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.\(^{73}\) Tips were placed in 50 mL glass bottles containing 2 mL vials of freshly distilled 3-aminopropyl-trimethoxysilane (APTMS, 30 µL) and triethylamine (10 µL) under an atmosphere of argon. After 2 h of incubation, the APTMS and triethylamine were removed. The bottle was again flushed with argon and sealed for 2 days. Aminofunctionalized tips were rinsed with dichloromethane and immersed in a solution of NHS-PEG24-Mal (Quanta Biodesign, 2 mM in dichloromethane) with pyridine (1% w/v) for 1 h, 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 h at 37 °C, rinsed with water, and dried under argon.⁶

**Functionalization of Silicon Samples.** His₆-tagged galectin-3 was chelated to the surface using a protocol similar to that of Schmid et al.²⁰⁶ 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 SiO₂ 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 for 1 h with 0.2 mg/mL 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 NiSO₄ (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 his₆-galectin-3 (50 µM) in sodium phosphate buffer (50 mM, pH 7.5) or his₆-KDPG aldolase (50 µM) in HEPES buffer (20 mM, pH 7.5) for 1 h, then immediately rinsed with bind buffer followed by PBS (pH 7.4).

**AFM 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) (Rivera et al, ASME 2009). For each experiment, 250 force curves were generated using an automated data collection routine (Rivera et al, ASME2009). Sample was brought into contact with tip, held for a dwell time (DT) of 1 sec, 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. For force trigger experiments, the photodetector was calibrated prior to data collection. 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 a carried out in the presence β-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.

### 5.2 A general and efficient functionalization technique for AFM molecular recognition studies

All reagents and solvents were purchased from Sigma-Aldrich and used as supplied. Thin-layer chromatography was performed on Merc Silica Gel 60 F254 aluminium plates using iodine vapors or CAM stain1 for spot visualization. Column chromatography was performed using Silicycle Silica-P Flash Silica Gel. ¹H and ¹³C 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 prepared 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 AxioImager microscope at 10 and 20X magnification. Automated pulling experiments were carried out using ORC8 cantilevers (Veeco Prpbes) 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) (Rivera et al, ASME 2009).

5.2.1 Synthesis

Mercaptopentanyl lactoside (2). Saccharide 2 was prepared according to the procedure described above (Section 5.1.1).

Boc-aminoalkene (14). Commercially available alkene 14 was prepared according to previously published protocols.170,173

NHS-diazirine (17). 2,5-Dioxopyrrolidin-1-yl 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoate 17 was prepared from (4-bromophenyl)methanol in 9 steps following the previously published protocol.170,173

5.2.2 Surface functionalization and AFM unbinding experiments

Silanization of oxidized silicon and silicon nitride. Silicon and silicon nitride chips were aminosilanized with APTMS using the procedure described in Section 5.1.3

Hydrosilylation of H-terminated silicon and silicon nitride. Alkene 14 was used to form Boc- and NH2-terminated SAMs on silicon nitride. Freshly prepared silicon and silicon nitride chips (~ 1×1 cm) were exposed to a 5% aq. HF solution for 5 minutes and dried under a stream of nitrogen to remove oxide layer. Neat 14 (10 µl) was then 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/cm² at 2 cm distance) for 30 minutes 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 25% TFA in DCM for 30 min and by rinsing the resulting surfaces DCM and ethanol.

**Immobilization of G3 on silicon <111>.** A silicon <111> chip (~1×1 cm) was washed with ethanol and water and blow-dried with filtered argon to remove dust particles. The substrate was then oxidized in Nanostrip 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 minutes 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 hour 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 hours 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 minutes. The propylene-functionalized chip was subsequently reacted 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 hour 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 water and ethanol. 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, 20mM 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.

**Functionalization of Silicon Nitride AFM Tips.** AFM tips (ORC8, Veeco, nominal spring constant of 100 pN/nm) were functionalized with alkene 14 according to the hydrosilanization procedure described above. Boc-protected tips were rinsed with dichloromethane and deprotected in a solution of TFA / DCM (1:1 v/v), rinsed with DCM and ethanol, and then immersed in a solution of NHS-PEG24-Mal (Quanta Biodesign, 2 mM in dichloromethane) with triethylamine (1% w/v) for 1 h, rinsed with ethanol and water, and dried under argon. The cantilevers were next submerged in an aqueous solution of mercaptopentyl lactoside 2 (2.25 mM) for 4 h at 37 °C, rinsed with water, and dried under argon.⁶
AFM unbinding experiments. Were performed according to the $F_{\text{min}}$ data collection protocol described in Section 5.1.3.

5.3 Vancomycin binding studies

5.3.1 Synthesis

(1dac008) $N^\alpha$-Acetyl-$N^\epsilon$-Carbobenzoxy-L-lysine (19). To a stirred solution of $N^\alpha$-Acetyl-Lys (2.0 g, 10.6 mmol) in NaOH (aq, 1 N, 10.6 mL) at 0°C was added benzyl chloroformate (1.8 mL, 12.75 mmol) and NaOH (aq, 1 N, 12.8 mL) concurrently in four equal portions over 1 h. The reaction was stirred an additional 1.5 h at 0°C, then water was added until the cloudy white mixture became clear. The solution was washed with ether, acidified with HCl (aq, 6 N), to pH 1, and extracted with EtOAc. The EtOAc extracts were dried (Na$_2$SO$_4$) and condensed give 3.2 g of 6 (94% yield) as a white foam. 1H NMR (300 MHz, CDCl$_3$): $\delta$ 1.4-1.6 (m, 4H), 1.65-1.98 (m, 2H), 2.02 (s, 3H), 3.1-3.3 (m, 2H), 3.95-4.7 (m, 1H), 4.95-5.2 (m, 3H), 6.7-6.9 (m, 1H), 7.25-7.4 (m, 5H). MS (ESI) calcd for $C_{16}H_{22}N_{2}O_{5}$Na [M + Na]$^+$ 345, found 345.1.

(1DACS006) N-Carbobenzoxy-D-Alanyl-D-Alanine tert-Butyl Ester (20). To a mixture of Z-D-Ala-OH (3.1 g, 13.8 mmol) and D-Ala-OtBu (2.5 g, 13.8 mmol) in CH$_2$Cl$_2$ (50 mL) was added EDC (3.3 g, 17.3 mmol) then HOBT (2.33 g, 17.3 mmol). After stirring for 16 h
under Ar(g), the mixture was diluted with CH₂Cl₂ and the organic phase was washed with HCl (aq, 1 N), followed by NaHCO₃, then brine. After drying over Na₂SO₄, the organic layer was condensed and the crude material was purified by column chromatography on silica gel (35% EtOAc in hexane) to give 3.25 g of 20 (68% yield). \(^1\)H NMR (300 MHz, CDCl₃): δ 1.3-1.42 (m, 6H), 1.43-1.5 (s, 9H), 4.15-4.3 (m, 1H), 4.42 (q, 1H), 5.1 (s, 2H), 5.35 (bd, 1H), 6.45 (bd, 1H), 7.25-7.4 (m, 5H). MS (ESI) calcd for C₁₈H₂₆N₂O₅Na [M + Na]^+ 373, found 373.2.

(1DAC010) D-Alanyl-D-Alanine tert-Butyl Ester (21). To a solution of 20 (2.7 g, 7.7 mmol) in EtOH (25 mL) under Ar(g) was added Pd/C (~200 mg). The reaction flask was evacuated and back-filled with H₂(g). The mixture was stirred for 16 h, then filtered through celite and condensed to give 1.6g of 5 (95% yield). \(^1\)H NMR (300 MHz, CDCl₃): δ 1.4-1.55 (m, 15H), 4.25-4.4 (m, 2H), 7.98 (d, 1H). MS (ESI) calcd for C₁₀H₂₀N₂O₃H [M + H]^+ 217, found 217.

(1DAC012) N⁰-Acetyl-N⁶-Carbobenzoxy-L-Lysyl-D-Alanyl-D-Alanine tert-Butyl Ester (22). To a stirred mixture of the acid 21 (1.85 g, 5.7 mmol) in CH₂Cl₂ (75 mL) with EDC (1.36g, 7.1 mmol) and HOBT (0.96 g, 7.1 mmol) under Ar(g) was added the amine 5 (1.49 g, 5.7 mmol). Stirring continued for 16 h. The mixture was then diluted with CH₂Cl₂ and
washed with HCl (aq, 1 N), NaHCO₃ (aq), then brine and dried over Na₂SO₄. Removal of solvent gave 2.75 g of 22 (93% yield). ¹H NMR (300 MHz, CDCl₃): δ 1.2-1.35 (m, 6H), 1.4 (s, 9H), 1.41-1.85 (m, 6H), 1.92 (s, 3H), 3.1 (m, 2H), 4.25-4.95 (m, 3H), 5.02 (s, 2H), 6.4 (bd, 1H), 6.64 (bd, 1H), 6.84 (bd, 1H), 7.2-7.35 (m, 5H). MS (ESI) calcd for C₂₆H₄₀N₄O₇H [M + H]^+ 521.6, found 521.4.

(1DAC021) **Nα-Acetyl-L-lysyl-D-Alanyl-D-Alanine tert-Butyl Ester (23).** The Cbz-protected tripeptide 22 (0.75 g, 1.44 mmol) was dissolved in EtOH (20 mL) with Pd/C under Ar(g). The reaction flask was evacuated and back-filled with H₂(g). After stirring 16 h, the mixture was filtered through celite and condensed to 0.555 g of a white solid 23 (99% yield). ¹H NMR (300 MHz, DMSO-d₆): δ 1.15-1.65 (m, 19H), 1.8 (s, 3H), 3.3 (bs, 2H), 4.0-4.4 (m, 3H), 8.1 (td, 3H). MS (ESI) calcd for C₁₈H₃₄N₄O₅H [M + H]^+ 387, found 387.3.

(7DAC040) **Nαɛ-Acetyl-L-lysyl-D-alanyl-D-Alanine tert-Butyl Ester (24).** To a slurry of 23 (0.70 g, 1.8 mmol, 1 eq.) in dioxane (25 mL) under Ar (g) was added triethylamine (0.34 mL, 2.5 mmol, 1.5 eq.). To this was added a solution of AcCl (0.13 mL, 1.8 mmol, 1 eq.) in dioxane (3 mL) in a dropwise manner. The mixture was stirred for 4 h, then quenched
with MeOH and condensed to a white solid. ESI-LCMS showed complete conversion to 24. The crude material was taken directly to the next step.

(7DAC041) \textit{N}^{\alpha,\varepsilon}-Acetyl-L-lysyl-D-alanyl-D-Alanine (25). To a stirred slurry of 24 (0.78 g, 1.8 mmol) in DCM (10 mL) under Ar (g) was added TFA (10 mL). TLC (10% MeOH in DCM) showed the reaction was complete after 1 h. The mixture was condensed to an oil. Upon trituration with diethyl ether (50 mL) the product became a free flowing solid which was isolated by filtration and purified by preparative HPLC on an Agilent Prep-C18 column. Gradient: 5-40% MeOH in water (0.2% formic acid modifier) over 20 min. Fractions were condensed and stripped from MeOH to give 25 as a white solid (460 mg, 68% yield over two steps). \textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O): \(\delta\)

(4DAC019c) (2,4-Dinitrophenyl)thiopropionic Acid (26). To a solution of thiopropionic acid (2.5 g, 24 mmol, 1 eq.) and 2,3-dinitrochlorobenzene (5.73 g, 28 mmol, 1.2 eq.) in MeOH (75 mL) under Ar(g) was added triethylamine (6.55 mL, 47 mmol, 2 eq.). The solution became bright yellow. After stirring for 12 h, the reaction was condensed to a solid, which was dissolved in EtOAc and filtered to remove remaining solid impurities. The EtOAc layer was washed with HCl (1 M, aq.), then H\textsubscript{2}O, dried over Na\textsubscript{2}SO\textsubscript{4} and condensed to an impure solid. The material was dissolved in NaHCO\textsubscript{3} (sat., aq.) and
washed with diethyl ether (2×). The aqueous layer was acidified to pH = 1 and product extracted with MeOH / EtOAc (1:4). The organic layer was dried over Na₂SO₄ and condensed to give pure 26. (6.08 g, 25% yield) as a yellow solid. ¹H NMR (300 MHz, acetone-d₆): δ

(4DAC027) N°-2,4-Dinitrophenylthiopropylamido-N°-Acetyl-L-lysyl-D-alanyl-D-Alanine tert-Butyl Ester (27). To a solution of 26 (0.70 g, 2.6 mmol, 1eq.) and 23 (0.99 g, 2.6 mmol, 1eq) in DMF/DCM (40 mL, 1:1 mixture) under Ar(g) was added EDC (0.59 g, 3.1 mmol, 1.2 eq.) followed by HOBt (0.42 g, 3.1 mmol, 1.2 eq.). The mixture was stirred overnight, then condensed to ½ volume. The remaining solution was poured into iPrOH/EtOAc (1:4) and washed with HCl (1 M, aq.), NH₄Cl (sat., aq., 2×), then brine (3×). The organic layer was condensed to give 27 (1.49 g, 90% yield) as a yellow solid. ¹H NMR (300 MHz, DMSO-d₆): δ

(4DAC028c) N°-2,4-Dinitrophenylthiopropylamido-N°-Acetyl-L-lysyl-D-alanyl-D-Alanine (28). To a stirred suspension of 27 (1.3 g, 2 mmol) in DCM under Ar(g) was added TFA in a dropwise manner. The cloudy yellow solution was stirred for 1 h and then condensed to a yellow oil. Trituration with diethyl ether (4 × 20 mL) provided the pure carboxylic acid 28 (1.11 g, 96% yield) as a yellow solid. NMR (300 MHz, DMSO-d₆): δ
(4DAC033c) **N⁵-Thiopropylamido-N²-Acetyl-L-lysyl-D-alanyl-D-Alanine (29).** The DNP thioether 28 (0.50 g, 0.89 mmol) was dissolved in a mixture of MeOH (7 mL), diisopropylethyl amine (1 mL), and β-mercaptoethanol (2 mL). A deep red solution formed while stirring for 5 h under Ar(g). The mixture was condensed to an oil and precipitated with diethyl ether. The solid wax isolated by filtration and washed with diethyl ether and DCM. Chromatography on silica gel using MeOH (10%) and formic acid (1%) in DCM gave 29 as a white solid with silica gel impurities (0.26 g, 70% yield). A small portion of the product was purified by preparative HPLC on an Agilent Prep-C18 column. Gradient: 5-90% MeOH in water (0.2% formic acid modifier) over 30 min. NMR (500 MHz, D₂O): δ

(7DAC066) **N-terminal Boc-protected Vancomycin (30).** A solution of vancomycin hydrochloride (0.60 g, 0.40 mmol, 1eq.) in dioxane (5 mL) and water (10 mL) was cooled in an ice bath. To this was added NaHCO₃ (0.068 g, 0.81 mmol, 2eq.) followed by di-tert-
butyl dicarbonate (0.097 g, 0.44 mmol, 1.1 eq.) as a solution in dioxane (5 mL). The
solution was warmed slowly to room temperature overnight and then condensed to a
solid. The material was purified by preparative HPLC on an Agilent Prep-C18 column.
Gradient: 5-90% MeOH in water (0.2% formic acid modifier) over 40 min. The product
30 (0.42 g, 67% yield) was obtained as a white solid. NMR (500 MHz, D$_2$O) was
consistent with published spectra.$^{191}$

![Chemical structure](image)

(1BHG005) 4-(Tritylthio)butyric Acid (31). Trityl mercaptan (8.5 g, 31 mmol, 1 eq.) was
added to a stirred suspension of NaH (2.48 g, 62 mmol, 2 eq., 60% dispersion in mineral
oil) in DMF (50 mL) which cooled in an ice bath under Ar(g). To this was added 4-
bromobutyric acid (5 g, 31 mmol, 1eq.) as a solution in DMF (10 mL) in a dropwise
manner. The mixture was stirred for 30 min and then condensed and diluted in EtOAc.
The organic layer was washed with NH$_4$Cl (sat., aq.), dried over Na$_2$SO$_4$ and condensed.
Chromatography on silica gel using MeOH (5% in DCM) provided the pure acid 31 (3.4 g,
31% yield). NMR (300 MHz, CDCl$_3$) was consistent with published spectra.$^{209}$

![Chemical structure](image)

(9DAC008) N-Methoxy-N-methyl-4-(tritylthio)butanamide (32). A solution of the acid
31 (1.46 g, 4.0 mmol, 1 eq.) and PyBoP (2.29 g, 4.4 mmol, 1.1 eq.) in DMF (20 mL) was
stirred for 10 min under Ar(g). To this was added a solution of \( N,O \)-dimethylhydroxylamine hydrochloride (0.43 g, 4.4 mmol, 1.1 eq.) in DMF (10 mL) in a dropwise manner. DIEA (2.1 mL, 12 mmol, 3 eq.) was added and the mixture was stirred for 12 h and then condensed and diluted in EtOAc (30 mL). The organic layer was washed with brine (2 ×) and \( \text{NH}_4\text{Cl} \) (sat., aq., 2 ×), then dried over \( \text{Na}_2\text{SO}_4 \) and condensed. Chromatography on silica gel using 5% EtOAc in DCM provided the amide 32 (1.50 g, 94% yield) as a clear oil. NMR (300 MHz, CDCl\(_3\)) was consistent with published spectra.²⁰⁹

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\text{S} & \hspace{1cm} \text{H}
\end{align*}
\]

(9DAC009) 4-(Tritylthio)butanal (33). A solution of amide 32 (1.5 g, 3.7 mmol, 1 eq.) was dissolved in diethyl ether under Ar(g) and cooled in an ice bath. To this was added LiAlH\(_4\) in one portion. The mixture was stirred, under cooling, for 1 h and then 2 mL of saturated \( \text{Na}_2\text{SO}_4 \) was added dropwise. The mixture bubbled, then became a white/grey slurry, which was filtered and condensed to an oil. The oil was dissolved in DCM/hexane (1:1), dried over \( \text{Na}_2\text{SO}_4 \) and loaded directly onto a silica gel column. Chromatography using DCM/hexane (1:1) provided the aldehyde 33 (0.78 g, 60% yield). NMR (300 MHz, CDCl\(_3\)) was consistent with published spectra.²⁰⁹
To a solution of aldehyde 33 (0.11 g, 0.32 mmol, 1.5 eq.) in DMF (5 mL) was added a solution of N-terminal Boc-protected vancomycin 30 (0.34 g, 0.22 mmol, 1 eq.) in DMF (10 mL) and MeOH (2 mL). The mixture was stirred for 10 h and then a solution of NaCNBH₄ (0.28 g, 4.4 mmol, 20 eq.) in DMF (4 mL) was added. ESI-LCMS showed the reaction to be complete after 18 h of stirring. The solution was condensed to 1-2 mL and, while stirring, THF (20 mL) was added slowly to precipitate the product as a white solid. The solids were isolated by filtration, washing with THF to remove residual aldehyde and NaCNBH₄. The product 34 (0.41 g, 99% yield) was shown to be pure by ESI-MS.
was stirred under Ar (g) in an ice bath. To this was added trifluoroacetic acid, dropwise, and the mixture was stirred for 30 min. DMSO (5 mL) was added and the solution was warmed to room temperature. Our original hope was that the disulfide would form, but ESI-MS showed only 10% conversion after 48 h of stirring. The material was purified by preparative HPLC on an Agilent Prep-C18 column. Gradient: 5-40% MeOH in water (0.2% formic acid modifier) over 40 min. The fractions were kept cold to prevent deglycosylation. Lyophilization of fractions provided the fully deprotected 35 (0.13 g, 43% yield) as a white solid which was immediately taken to the next step.

(9DAC005) Vancosamine Linked 4-Thiobutyl-Vancomycin Disulfide (36). Thiol 35 was dissolved in DMSO. ESI-MS showed complete conversion to the disulfide after 24 h. The reaction was diluted with water (10 mL) and purified by preparative HPLC on an Agilent Prep-C18 column. Gradient: 5-30% MeOH in water (0.2% formic acid modifier) over 30 min. The disulfide 36 (0.090 g, 70% yield) was isolated and stripped from MeOH to give a white solid.

(6DAC083) Propargyl-PEG₆-OH (37). A solution of hexaethylene glycol (2.0 g, 7.1 mmol, 1 eq.) in THF (20 mL) was stirred under Ar (g) in an ice bath for 20 min. To this was
added a solution of propargyl bromide (0.93 g, 7.8 mmol, 1.1 eq.) in THF (10 mL) in a dropwise manner. The mixture was warmed to room temperature over 12 h, then condensed to a solid. Chromatography on silica gel using ethyl acetate and then 10% methanol in DCM provided the monoalkylated 37 (53% yield) as a clear oil.

(7DAC011) Propargyl-PEG₆-tert-Butyl Ester (38). To a solution of 37 (1.0 g, 3.1 mmol, 1 eq.) in DMF (10 mL) was stirred under Ar (g) in an ice bath. Sodium hydride (137 mg of 60% dispersion in mineral oil, 3.4 mmol, 1.1 eq.) was added and the mixture was stirred for 15 min. A solution of tert-butyl bromoacetate (1.4 mL, 9.4 mmol, 3 eq.) in DMF (10 mL) was added in a dropwise manner and the resulting slurry was warmed to room temperature over 12 h and then condensed and poured into a mixture of ethyl acetate and NH₄Cl (sat. aq.). After washing with NH₄Cl (sat. aq., 3 ×) and brine (1 ×) the organic layer was dried over Na₂SO₄ and condensed. Column chromatography on silica gel using ethyl acetate provided pure 38 (42% yield) as a clear oil.

(7DAC015) Propargyl-PEG₆-Acid (39). Tert-butyl ester 38 (550 mg, 1.3 mmol) was dissolved in DCM (9 mL) under Ar (g) and trifluoroacetic acid (3 mL) was added in a dropwise manner. The solution was stirred for 45 min and then condensed. Column chromatography on silica gel using 10% methanol, 2% acetic acid, 88% DCM, provided pure 39 (88% yield) as a clear oil.
To a solution of amine 23 (118 mg, 0.31 mmol, 0.9 eq) in DMF (10 mL) was added EDC (77 mg, 0.40 mmol, 1.2 eq.), hydroxybenzotriazole (54 mg, 0.40 mmol, 1.2 eq.), triethylamine (56 µL, 0.40 mmol, 1.2 eq.), and then acid 39 (127 mg, 0.34 mmol, 1 eq). The solution was stirred under Ar (g) for 40 h and then condensed to a brown oil. Chromatography on silica gel using 5-10% methanol in DCM provided pure 40 (83% yield) as a clear wax.

To a stirred solution of 40 (140 mg, 0.19 mmol) in DCM (3 mL) under Ar (g) was added TFA (2 mL) in a dropwise manner. After stirring for 2 h, the solution was condensed and eluted from silica gel using 10% methanol, 2% acetic acid, 88% DCM. The resulting cloudy solid was dissolved in water and purified by preparative HPLC on an Agilent Prep-C18 column to give pure 41 (80% yield). Gradient: 5-90% MeOH in water (0.2% formic acid modifier) over 40 min.

To a solution of 37 (0.49 g, 1.5 mmol, 1 eq.) in DMF (12 mL) under Ar (g) was added diphenyl phosphoryl azide (6.5 µL, 3.1 mmol, 2 eq.) and sodium azide (0.2 g, 3.1 mmol, 2 eq.). The reaction was transferred to a microwave
vial and irradiated (CEM Discovery Microwave, 200 W, 70°C, 60 min), then diluted with DCM and washed with brine (5 ×), dried over Na2SO4 and condensed. Chromatography on silica gel using 80% ethyl acetate in hexane provided pure 42 (67% yield).

(7DAC002) Propargyl-PEG6-Amine (43). To a stirred solution of 42 (0.29 g, 0.84 mmol, 1 eq.) in pyridine (25 mL), was added triphenylphosphine (1.1 g, 4.2 mmol, 5 eq.) and then NH4OH conc. (15 mL). The mixture was stirred for 12 h and concentrated to a solid. Chromatography on silica gel using 10% methanol in DCM (300 mL), followed by 20% methanol, 0.5 % NH4OH conc., 79.5% DCM provided pure 43 (94% yield) as a clear oil.

(7DAC003) C-Terminal-linked Azido-PEG6-Vancomycin (44). To a stirred solution of 43 (22 mg, 67 µmol, 2 eq.) and vancomycin HCl (50 mg, 34 µmol, 1 eq.) in DMF (5 mL) under Ar (g) was added HBTU (19 mg, 51 µmol, 1.5 eq.) and then triethylamine (9.4 µL, 67 µmol, 2 eq.). The mixture was stirred for 20 h then condensed, dissolved in water, and purified by preparative HPLC on a Phenomenex-C18 column to give pure 44 (80% yield) as a white solid. Gradient: 5-55% MeOH in water (0.2% formic acid modifier) over 50 min. LCMS (ESI) calculated for C81H102Cl2N10O29 [M + H]+ 1749.6, found 1749.6.
(6DAC013) **Tosyl-PEG₆-OH (45).** A solution of hexaethylene glycol (10 g, 35 mmol, 1 eq.) was stirred in DCM (300 mL) in an ice bath under Ar (g). To this was added Ag₂O (12.3 g, 53 mmol, 1.5 eq.), tosyl chloride (7.4 g, 39 mmol, 1.1 eq.), and then potassium iodide (1.2 g, 7.1 mmol, 0.2 eq.). The mixture was stirred for 2 h, filtered through celite, and condensed to a clear oil. Chromatography on silica gel using 2-4% methanol in DCM provided pure 45 (60% yield). ¹H NMR (300 MHz, CDCl₃) was consistent with published spectra.⁹⁷

(6DAC087) **Azido-PEG₆-OH (46).** In a microwave reaction vial was combined 45 (3.3 g, 7.6 mmol, 1 eq.), sodium azide (0.98 g, 15 mmol, 2 eq.), and NaHCO₃ (5 mL of 5% m/v aqueous solution) in methanol (15 mL). The mixture was irradiated (CEM Discovery Microwave, 200 W, 115 °C, 30 min), condensed to an oily solid, dissolved in ethyl acetate and filtered. The filtrate was condensed and purified by column chromatography on silica gel using 1-5% methanol in DCM to give pure 46 (71% yield). LCMS (ESI) calculated for C₁₂H₂₅N₃O₆ [M + Na]⁺ 330.2, found 330.1.

(7DAC009) **Azido-PEG₆-tert-Butyl Ester (47).** A solution of 46 (0.8 g, 2.6 mmol, 1 eq.) in DMF (10 mL) was cooled in an ice bath under Ar (g) and sodium hydride (208 mg of a 60% dispersion, 5.2 mmol, 2 eq.) was added. After stirring for 10 min, a solution of tert-
butyl bromoacetate (1.2 mL, 7.8 mmol, 3 eq.) in DMF (10 mL) was added and the mixture was warmed to room temperature over 24 h. The solution was condensed and poured into a mixture of ethyl acetate and NH₄Cl (sat. aq.). After washing with NH₄Cl (sat. aq., 2 ×) and brine (1 ×) the organic layer was dried over Na₂SO₄ and condensed. Column chromatography on silica gel using ethyl acetate provided pure 47 (57% yield) as a clear oil.

(7DAC014) Azido-PEG₆-Acid (48). To a solution of 47 (0.6 g) in DCM (9 mL) was added trifluoroacetic acid (3 mL) in a dropwise manner. The solution was stirred for 45 min. and then condensed and purified by column chromatography on silica gel using 20% methanol, 2% acetic acid, 78% DCM to give pure 48 (90% yield) as a clear oil. LCMS (ESI) calculated for C₁₄H₂₇N₃O₈ [M + Na]⁺ 388.2, found 388.2.

(7DAC026) Azido-PEG₆-Benzotriazole Amide (49). To a solution of 48 (100 mg, 0.27 mmol, 1 eq.) in DCM (10 mL) was added oxalyl chloride (190 µL, 2.2 mmol, 8 eq.) followed by DMF (4 drops). After stirring or 4 h under Ar (g), then solution was condensed and place under high vacuum for 2 h. The mixture was dissolved in DCM (10 mL) and benzotriazole (65 mg, 0.55 mmol, 2 eq.) and triethylamine (96 µL, 0.55 mmol, 2 eq.) were added. After stirring for 12 h under Ar (g), the solution was condensed,
dissolved in diethyl ether, washed with HCl (1 M, 5 x), NaHCO₃ (sat. aq.), brine, dried over Na₂SO₄ and condensed to give crude 49 (50% yield).

(7DAC031) Azido-PEG₃₂-Acid (50). To a solution of crude 49 (56 mg, 0.10 mmol, 1 eq.) in DCM (10 mL) under Ar (g) was added a solution of amino-PEG₂₄-acid (Quanta Biodesign, 115 mg, 0.10 mmol, 1 eq.) and triethylamine (28 µL, 0.20 mmol, 2 eq.) in DCM (5 mL). The mixture was stirred for 12 h at room temperature and then condensed. Column chromatography on silica gel using 15% methanol, 2% acetic acid, 83% DCM provided pure 50 (60% yield) as a waxy solid. LCMS (ESI) calculated for C₁₉H₃₆N₄O₁₀ [M + Na]⁺ 1516.7, found 1516.6.

5.3.2 Preparation of tips and samples

Silanization with APTMS. Silicon <100> and silicon nitride AFM tips (ORC8, Veeco probes, nominal spring constant 100 pN/nm) were oxidized in a plasma ashcer (100 W, 5 min) and then aminosilanized with APTMS according to the procedure described in Section 5.1.3 to provide amine terminated surfaces.

Etherification with Ethanolamine. AFM tips (ORC8, Veeco probes) and silicon <100> surfaces were oxidized in a plasma ashcer (100 W, 5 min) and then dried at 110 °C for 20 min under vacuum. The surfaces were then immersed in a solution of ethanolamine HCl (3.3 g) in anhydrous DMSO (6.6 mL) and heated to 100 °C in the presence of activated molecular sieves (4 Å) for 17 h and then rinsed with DMSO, water, and ethanol to provide amine terminated surfaces.
**Alkylation and diazirine insertion.** A silicon <111> chip (~1×1 cm) was washed with ethanol and water and blow-dried with filtered argon to remove dust particles. The substrate was then oxidized in Nanostrip 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 minutes 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 hour 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 hours 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 minutes. The propylene-functionalized chip was subsequently reacted 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 hour at room temperature. The NHS-functionalized surface was washed with dichloromethane, isopropanol, and dried under argon and reacted with N-Boc ethylene diamine (16 mM, 50 µL in 10 mL of DCM) for 4 h. Deprotection in TFA / DCM (1:1 v/v) for 30 min provided amine terminated surfaces.
**Immobilization of KDADA alkyne (41) to AFM tips via “click” reaction.** APTMS modified AFM tips (ORC8, Veecoprobes) were reacted with a solution of Azido-PEG32-Acid 50 (1.7 mM), DCC (2.4 mM), triethylamine (7.2 mM) and hydroxybenztriazole (4.8 mM) in DMF for 5 h. The tips were then rinsed with DCM and ethanol and then incubated in a solution of KDADA alkyne 41 (6 mM), trisbenzyl triazolyl amine (4 mM), CuSO4 (3 mM), and sodium ascorbate (4 mM) in ethanol / water (1:1, v/v) for 20 h. The tips were then rinsed with ethanol, water, and dried under Ar (g).

**Immobilization of C-terminal-linked vancomycin alkyne (44) to silicon via “click” reaction.** APTMS modified silicon <100> surfaces were reacted with a solution of Azido-PEG32-Acid 50 (1.7 mM), DCC (2.4 mM), triethylamine (7.2 mM) and hydroxybenztriazole (4.8 mM) in DMF for 17 h. The surfaces were then rinsed with DCM and ethanol and then incubated in a solution of vancomycin alkyne 44 (3 mM), trisbenzyl triazolyl amine (4 mM), CuSO4 (3 mM), and sodium ascorbate (4 mM) in ethanol / water (1:1, v/v) for 20 h. The surfaces were then rinsed with ethanol, water, and dried under Ar (g).

**Immobilization of thiolated KDADA (29) to AFM tips.** Amine modified AFM tips (ORC8, Veesoprobes) were incubated in a filtered solution of Maleimide-PEG24-NHS ester (Quanta Biodesign, 3 mM) and triethylamine (10 mM) in DCM for 1 h to 24 h at room temperature or 37 °C. The tips were then rinsed with DCM, ethanol, dried under Ar (g) and incubated in a solution of thiolated KDADA 29 (12 mM), and TCEP (6.4 mM) in water for 1 h to 24 h at room temperature or 37 °C. The tips were then rinsed with water, ethanol, and dried under Ar (g).
Immobilization of vancosamine-linked thiolbutyl vancomycin (36) to silicon. Amine modified silicon surfaces were incubated in a filtered solution of Maleimide-PEG24-NHS ester (Quanta Biodesign, 3 mM) and triethylamine (10 mM) in DCM for 1 h to 24 h at room temperature or 37 °C. The surfaces were then rinsed with DCM, ethanol, dried under Ar (g) and incubated in a solution of vancomycin disulfide 36 (0.8 mM), and TCEP (3.5 mM) in DMF / water (1:1, v/v) for 1 h to 24 h at room temperature or 37 °C. The surfaces were then rinsed with water, ethanol, and dried under Ar (g).

5.3.3 AFM unbinding experiments:

AFM unbinding experiments. Were performed according to the F\textsubscript{min} data collection protocol described in Section 5.1.3.
6. References


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7. Biographical Sketch

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EDUCATION

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<td>Ph.D. Candidate in Chemistry</td>
<td>Duke University, Durham, NC</td>
<td>Prof. Eric J. Toone</td>
<td>2004 - Present</td>
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<tr>
<td>Certificate of Pharmacology</td>
<td>Duke University, Durham, NC</td>
<td>Prof. Cynthia Kuhn</td>
<td>2005 - Present</td>
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<tr>
<td>Bachelor of Science in Chemistry</td>
<td>Allegheny College, Meadville, PA</td>
<td>Prof. Shaun S. Murphree</td>
<td>1995 - 2000</td>
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WORK AND RESEARCH EXPERIENCE

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<tr>
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<td>2004 - Present</td>
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<td>i) Synthesis and covalent immobilization</td>
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<tr>
<td>Advanced Scientist: Discovery Chemistry</td>
<td>Scynexis Inc., Durham, NC</td>
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<td>Synthesis and purification of small molecule libraries</td>
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<td>Kilogram-scale GMP manufacturing of active pharmaceutical ingredients</td>
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<td>Supervisor: Marc W. Andersen Ph.D. (Currently of Aptuit Consulting)</td>
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ENTREPRENEURIAL INTERESTS

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**Duke Startup Challenge – Team Member, PurAxis, Healthcare and Life Sciences**
Semi-Finalist 2010

**Duke Startup Challenge – Founder, Solvent Solutions, Consumer Products**
Elevator Pitch 2007

**AWARDS and FELLOWSHIPS**

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**MANUSCRIPTS IN PREPARATION**


**PUBLICATIONS**


**POSTER PRESENTATIONS**