Label-free Biomolecular Sensing Using Single Color Near-Null Reflective Interferometry and Brewster Angle Straddle Interferometry

By

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Curriculum Vitae

The author was born in Wuhan, Hubei, China in 1980. She attended Peking University in 1998 and graduated with a Bachelor of Science degree in 2002. She came to the University of Rochester in the fall of 2002 and started graduate studies in Chemistry. She received a Sherman Clarke Fellowship from 2003 to 2005 and an Elon Huntington Hooker Fellowship in 2006. She pursued her research in biomolecular sensing using Single Color Near-Null Reflective Interferometry and Brewster Angle Straddle Interferometry under the guidance of Professor Lewis J. Rothberg. She received a Master of Science degree from the University of Rochester in 2004.
Abstract

Reflective Interferometry uses reflectivity changes at an interface functionalized with molecular probes to detect label-free biomolecular binding. In this thesis, we report Single Color Near-Null Reflective Interferometry (sc-NNRI) that attaches target molecules to the surface and alters the effective thickness of an anti-reflective coating formed by thermal oxidation of a silicon wafer to remove destructive interference of the reflected waves. The thermal oxide thickness is adjusted for precise interference using layer-by-layer electrostatic self-assembly of polyelectrolytes to which the molecular probes can be bound covalently. Reflectivity increases of over a factor of 100 are observed for binding of 2.5 nm of streptavidin to biotinylated polyelectrolytes, considerably more sensitive than surface plasmon resonance detection. Theoretical modeling is in agreement with the experimentally observed reflectivity increases and suggests the sensitivity is at present limited by the roughness of the oxide.

We report another novel reflective interferometric method, Brewster Angle Straddle Interferometry (BASI), to detect chemical binding at an interface. The interference layer consists of the thin native oxide on silicon, and we utilize nearly opposite phase shifts of light at the oxide/water and oxide/silicon interfaces to achieve near-complete destructive interference. We measure selective binding of thrombin in solution to DNA aptamers covalently bound to the oxide. The technique can be used to detect and quantitate surface binding of less than 1 Å of material, sensitivity similar to that of surface plasmon resonance imaging and sc-NNRI. Results are in quantitative agreement with what is predicted theoretically. The method is very convenient to
implement since it utilizes unmodified silicon wafers as substrates and is extremely insensitive to both probe light bandwidth and collimation.

We used under water BASI to detect biomolecular bindings at a water/substrate interface. We measure selective binding of a 47-mer fragment of Has-ra mRNA in solution to six different designed 2’-O-methyl RNA probes covalently immobilized on the silicon oxide surface. The technique can be used to quantitate surface binding since the value of the reflectivity minimum is parabolic with the layer (oxide plus adsorbents) thickness. We were able to see increases in reflectivity of 33%, which is due to binding of 7.5 angstroms of 47-mer Has-ra mRNA with specific probe M 3291 on the surface. The limitation of detection (LOD) is estimated to be ~1.0 angstrom based on signal to noise ratio ~3.
Acknowledgement

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The faculty in and out department are acknowledged for their assistance. I sincerely thank Prof. Douglas H. Turner, Prof. Man Kit Ng and Prof. Thomas H. Foster for serving on my doctoral committee. Their constructive feedback and comments at various stages have been significantly useful in shaping the thesis up to completion.

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I dedicate this thesis to my father, mother, and all other family members.
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Chapter 1

Introduction

1.1 History of Biomolecular Sensing Techniques

Since two decades ago, biosensing techniques have become more and more popular for scientific and industrial research in various fields, such as pharmaceutical applications, environmental monitoring, health care and clinical diagnostics, food analysis and safety. A biomolecular sensor is an analytical tool to selectively identify a specific analyte during physiological and/or biochemical interaction by detecting a signal proportional to the concentration or amount of the analyte. The interaction is between a pair, the known biomolecule and the analyte, which are generally called probe and target molecules respectively.

Figure 1.1 Component diagram of a biomolecular sensor.
One implementation that illustrates the general idea of a biomolecular sensor is shown in Figure 1.1. In this case the presence of the target on the biochip is converted to an electric signal by the transducer. Then the signal is amplified, processed and output as the final data, which can be used to determine the concentration or amount of the target.

The concept of biomolecular sensors was first demonstrated by Leland C. Clark in 1962\(^1\). Since then, the feasibility of biomolecular sensors has been attracting many researchers to study a wide range of biochemical parameters, using a variety of approaches. These approaches include mechanical, electrochemical and optical techniques.

Examples of mechanical approaches include devices such as microbalance\(^2-4\) and micro-cantilevers\(^5-6\) that can measure the increasing mass due to the target binding in the biomolecular interaction. Electrochemical methods study the reactions that take place at the interface between electrodes and the electrolyte. The four main types of electrochemical methods are potentiometry\(^7\), voltammetry\(^8-10\), coulometry and conductimetry,\(^11-12\) among which voltammetry is more widely used for biomolecular sensing.

For optical biomolecular sensors, there are also several different types. Some common optical techniques involve light absorption measured by some traditional analytical methods such as UV-Vis spectroscopy and etc. The specific interaction between probe and target molecules causes a change in light absorption, which is used to detect the amount of the target. For example, Van Duyne’s group was able to detect streptavidin down to one picomolar concentrations by using biotinylated surface-confined Ag nanoparticles.\(^13\)
The second type of optical techniques either uses an optical label that is attached to the target during sample preparation, or introduces a sandwich assay. An optical label is generally a fluorescent tag that binds to the target molecules. A sandwich assay consists of two recognition steps. In the first step, a biomolecule immobilized on the surface is allowed for binding with an analyte of interest. In the second step, a secondary molecule flows over the sensor surface to bind with the previously captured analyte. The two separate recognition steps favor enhanced sensitivity and specificity. For example, Enzyme-Linked ImmunoSorbent Assay (ELISA)\textsuperscript{14} detects fluorescence caused by the second molecule, a type of enzyme that specifically binds the target antibody captured on the surface by the immobilized antigen.

The third type of optical techniques is based on changes in surface properties of the biochips. For example, surface plasmon resonance (SPR)\textsuperscript{15-18}, interferometry\textsuperscript{19-25} and elipsometry\textsuperscript{26-30} are able to detect minute changes in refractive index or thickness that occur when target molecules bind to the probes molecules on the surface, so they generally do not need target labeling or a sandwich assay.

1.2 Goals for Chip-based Biomolecular Sensors

With the development of many different chip-based detection techniques, there are a few factors we have to consider to build a high-quality biomolecular sensor. The first factor involves the biochip manufacturing. For different substrate materials, there are different methods for high-throughput patterning and surface chemistry. The second factor is a complex evaluation of a biomolecular sensor. It considers the substrate materials, biomolecular recognition pairs, and the transducer types. All of them together determine the quality of a biomolecular sensor’s sensitivity, selectivity and stability.
1.2.1 High-throughput Patterning

Nowadays it is a trend to fabricate arrays of sensors after the success of single analyte sensors, due to the large demands for high-volume and high-throughput screening in the pharmaceutical and clinical industry. Many patterning techniques, including spot arraying and photolithography, have been used to fabricate such arrays of biomolecules, which are also called microarrays.

The spot arrayer is a high-capacity instrument which is developed to deposit many samples in parallel. The liquid is drawn from multi-well plates by capillary forces in hollow steel tips and dotted onto slides. Since different solutions are held in parallel tips on the movable head at the same time, it is very efficient for the arrayer to spot on the slides at a high rate. Multiple slides with multiple solution samples can be arrayed within a couple hours, and each sample can optionally be dotted one to three times. The volume of the liquid is very small (several nL in general) but sufficient for delivery to all of the slides.

The uniformity of an array’s grid largely depends on the arrayer’s ability to precisely and accurately move to a new location. A system including an optical table or some other vibration-dampening system is recommended. Variations in spot size and shape can be caused by a variety of other factors, including the robot’s dwell time on the substrate, the substrate’s coating, the solution viscosity, and environmental issues, such as humidity and temperature. In addition, contaminants are a big problem because particulate matter can interfere with the deposition of spots or create false readings. The other critical component of the array system is the set of pins, pen tips, or quills that transfers the sample from a micro-well plate or other receptacle to the slide. An
alternative to using pins, pen tips, and quills is the relatively new inkjet-style printing. Inkjet printing technology can reproducibly dispense spheres of fluid with diameters of 25-100µm (10pl to 0.5nl). The printing heads deposit small volumes of material and permit high spot densities.\textsuperscript{31-32} Because it is a non-contact method, it eliminates the problem of pins being deformed during spotting, which can affect the reproducibility of spots. It is also very flexible, in some cases, allowing on-site synthesis of probes.

Microcontact printing (μCP)\textsuperscript{33-34} is another spotting technique, which is used to form patterned micrometer-scale spots of self-assembled monolayers (SAMs). The different molecules are transferred by elastomeric polydimethylsiloxane (PDMS) stamps to different regions on the surface of the substrate by contact. Figure 1.2 shows the scheme of μCP. After PDMS stamp is soaked with “ink”, it is put onto the substrate and the “ink” is transferred from the stamp onto the surface. After stamp removal, a SAMs pattern is formed and provides chemical functionalities on the regions with the “ink”. This technique is advantageous because it is simple and easy to use without complicated tools and facilities that might be required for a spot arrayer. Since the SAMs might contain regions terminated by different chemical functionalities, they can also facilitate on-site synthesis of probes.
Photolithography is a process used in microfabrication to selectively remove parts of a thin film or the bulk of a substrate and generate a pattern. This fabrication technique was developed by Affymetrix (Santa Clara, CA), and is used to produce their GeneChips®. The scheme is shown in Figure 1.3. The process begins by coating a 5” × 5” quartz wafer substrate with a light-sensitive chemical compound that prevents coupling between the surface and the first nucleotide of the DNA probe being created. Lithographic masks are used to either block or transmit light onto specific locations of the surface. The surface is then flooded with a solution containing either adenine, thymine, cytosine, or guanine, and coupling occurs only in those regions on the glass that have been deprotected through illumination. The coupled nucleotide also bears a light-sensitive protecting group, so the cycle can be repeated. In this way, the microarray is built as the probes are synthesized through repeated cycles of deprotection and coupling. The process is repeated until the probes reach their full length, usually 25 nucleotides. Commercially available arrays are typically manufactured at a density of over 1.3 million
unique features per array. Depending on the demands of the experiment and the number of probes required per array, each quartz wafer can be diced into tens or hundreds of individual arrays.

1.2.2 Surface Chemistry

Surface chemistry is the study of chemical reactions at interfaces. It is closely related to surface functionalization, which aims at modifying the chemical composition of a surface by incorporation of selected elements or functional groups that produce various desired effects or improvements in the properties of the surface or interface. For chip-based biomolecular sensors, the modified surfaces are used to immobilize bio-related molecules as probes to detect target biomolecular analytes.

Figure 1.3 Affymetrix uses a unique combination of photolithography and combinatorial chemistry to manufacture GeneChip® Arrays.
Glass, silicon and gold are the main three types of surface used in chip-based biomolecular sensors. Researchers popularly use glass as the substrate, since glass attachment chemistries for biomolecules are well developed to bind a large range of useful organic molecules. These chemistries can also be used for silicon since silicon surface is typically terminated by SiO₂.

The covalent attachment of organofunctional molecules to glass surface can be achieved with two steps. The scheme is shown in Figure 1.4. The first step is to generate silanol groups. This is relatively easier following piraha (30% H₂O₂: 98% H₂SO₄ = 3:7 volume ratio) etching of the glass surface under conditions that fully hydrolate the surface. The following step involves formation of Si-O-Si-R bonds to covalently bind moieties that are used for attachment of probe biomolecules. Since the silane coupling agents possess various functional groups, which behave as the linkers to attach the probe biomolecules, such a silylation reaction can easily graft silicon surface with a huge variety of molecular functionalities. For example, carboxyethylsilanetriol (sodium salt) can be used to immobilized on silicon surface and leave its carboxyl groups for further attachment with aminated biomolecules. On the other hand, silylation can be used to form SAMs consisting of long-chain hydrocarbons (Si-O-Si-(CH₂)ₙCH₃, n=10-18)³⁵ that change the wettability of silicon surface drastically and make it strongly hydrophobic. In this thesis we will use hydrophobic patterning to define hydrophilic regions to bind different probe molecules.
Figure 1.4 Scheme of silylation reaction and attachment of biomolecules. (a) chemistry of silylation and attachment of biomolecules; (b) molecular structure of silane coupling agent.

Layer by layer (LBL) electrostatic self-assembly (ESA) has become another approach to immobilize different capture molecules on silicon surface.\textsuperscript{36-37} LBL-ESA was initially introduced by Decher and co-workers.\textsuperscript{38-40} The protocol for LBL-ESA is shown in Figure 1.5. Polycation and polyanion layers are sequentially adsorbed on a substrate to form a polyelectrolyte complex, while the substrate is repeatedly dipped into their dilute solutions of each.
Structures of some commonly used polyelectrolytes are shown in table 1.1.

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<thead>
<tr>
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<th>PAH</th>
<th>PAA</th>
<th>PDAC</th>
<th>SPS</th>
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<tbody>
<tr>
<td>Poly</td>
<td>Poly (allylamine hydrochloride)</td>
<td>Poly (acrylic acid)</td>
<td>(Poly diallyldimethyl ammonium chloride)</td>
<td>(Poly sodium styrenesulfonate)</td>
</tr>
<tr>
<td></td>
<td>[H_2C\text{-CH}<em>{n}\text{-CH}</em>{2}\text{-CH}\text{-NH}_3^+\text{Cl}^-]</td>
<td>[H_2C\text{-CH}_{n}\text{-COOH}]</td>
<td>[\text{N}\text{Cl}^-]</td>
<td>[\text{O}\text{S}\text{O}^{\text{-Na}^+}]</td>
</tr>
</tbody>
</table>

Table 1.1 Molecular structures of polycation PAH, PDAC and polyanion PAA and SPS.

Notice that some weak polyelectrolytes, PAH and PAA for instance, possess functional groups of amino and carboxyl groups respectively that could be used for covalently binding with probe biomolecules modified by the corresponding linkers, carboxyl and amino groups, respectively.\(^ {41}\)

Gold films are another popular material for chip-based biomolecular sensors. For instance, surface plasmon resonance uses gold as the substrate. The first step to modify gold surfaces is to self assemble a monolayer of organofunctional molecules. Different
from LBL-ESA on silicon surface, the self-assembly reactions generate covalent bonds instead of electrostatic attraction. Most of the time, the desired functional groups can be derivatized with thiols that bind covalently to gold. The self assembly on gold is similar to silylation reaction on silicon, which can also produce monolayers and at the same time a large variety of functional groups. Figure 1.6 shows an example of self-assembling a monolayer of 16 carbon molecules with carboxyl groups\textsuperscript{42} at the end to further attach biomolecules.

![Figure 1.6 An example of gold surface chemistry.](image)

### 1.2.3 Sensitivity

Sensitivity has often been expressed as a signal level only, for example, a certain number of counts with no mention of the noise, but sensitivity should always be specified as a signal-to-noise ratio (SNR), which is the ratio of the signal produced by an analyte to the root mean square (rms) noise level. For analytical measurements, sensitivity determines the detection limit of an analyte.

It is always the case in life science, biomedical and environmental research that people work at very low concentration levels. Scientists, clinicians, food technologists
and environmentalists all have an interest in generally increased sensitivity and decreased limits of detection (LOD) for a range of analytes.

UV/Vis detection is broadly used in chip-base biomolecular sensors because of its simplicity and flexibility. However, the limit of detection (LOD) for UV/Vis is only $10^{-5} - 10^{-6}$ M for very good absorbers. Fluorescence spectroscopy is recognized as one of the most sensitive instrumental techniques. It measures the intensity of the sample emission. Compared with absorption spectroscopy, UV/Vis for instance, which measures the difference between two relatively large signals, the fluorescence signal is measured above a low background level and its sensitivity is much higher. It is as much as 1000 times more sensitive than absorption spectroscopy. A sensitive fluorescence spectrometer will be able to perform experiments at a low concentration for the fluorescing species.

A number of instrumental contributions determine the sensitivity of a fluorescence spectrometer. They include source intensity, efficiency of the optical system, spectral bandpass of the monochromators, and efficiency of the detector. It is the combination of all these factors that determines the sensitivity of a fluorescence spectrometer. With the development of the technique, substrate engineering is one of the aspects that could improve fluorescence detection limit. It was reported that sub-picomolar and attomolar for proteins and DNA respectively was detected by Hahm’s group by using a highly sensitive fluorescence method on nanoscale ZnO platforms.

Surface plasmon resonance is an alternative to fluorescence and also widely used due to its label-free feature. Surface plasmons, also known as surface plasmon polaritons, are surface electromagnetic waves that propagate parallel along a metal/dielectric (or metal/vacuum) interface. Surface plasmon resonance (SPR) occurs when surface plasmons are exited by an incident beam of light of appropriate wavelength
at a particular angle. A minimum in the reflectivity is observed at certain angle that is denoted as an SPR angle. This critical angle is very sensitive to the dielectric properties of the medium adjacent to the surface apart from its dependence on the wavelength and polarization state of the incident light. In particular, the resonance condition is extremely sensitive to the refractive index of the sample in contact with the metal surface because the optical electric fields are localized to within \( \sim 250 \text{ nm} \) from the gold surface.

When binding of the target on the surface occurs, the refractive index adjacent to the surface changes, and this causes a change in SPR angle. After scanning angle of incidence, imaging SPR\textsuperscript{16-17, 45-48} measures the change of reflectivity at the fixed optimal angle of incidence. The reflectivity change is proportional to the analyte concentration within the detection range. These are called direct assays. By the use of such simple direct assays, a LOD of 100 pM has been reported for bovine serum albumin (BSA, M.W. \( 67 \times 10^3 \)) by Lee et al.\textsuperscript{49} These are most useful for detection of molecules with molecular weight (MW) > 10 kDa, because smaller molecules have insufficient mass to effect a measurable change in the refractive index. A sandwich assay could also be utilized for SPR detection in order to achieve lower LOD. It can be employed for detection of molecules with MW >5000 Da, but those molecules have to possess multiple epitopes such as proteins, bacteria and viruses. In addition, labeling of the secondary antibody is preferred in most of the sandwich assays and hence they usually utilize chemiluminescence or fluorescence methods instead of SPR.

1.2.4 Selectivity

Generally, for chip-based biomolecular sensors, a high degree of analyte selectivity can be achieved by using the specificity of surface-attached ligands and
passivation of the sensor surface to reduce non-specific binding. In addition, if the biomolecular sensor is capable of probing label-free complex mixtures, the unknown effects to the binding specificity caused by the tag is eliminated. This is especially desirable for clinical samples without prior purification and tagging.

Given the ability to efficiently suppress non-specific binding, biomolecular sensor’s selectivity depends on the properties of the biorecognition pairs used for analyte binding. Antibodies, due to their versatility, commercial availability and high affinity with antigens, are widely reported biological recognition molecules used in affinity-based biomolecular sensors. Another example is DNA hybridization sensors, which offer considerable high-throughput potential for obtaining specific sequence information in a faster way compared with traditional hybridization assay. The strong interaction between the complementary nucleic acid strands is the basis for these nucleic acid hybridization devices. One is immobilized on the chip and called probe, and the other is the analyte nucleic acid called target. A DNA chip is a powerful and useful tool for biomedical analysis such as single nucleotide polymorphisms (SNPs), allowing parallel detection of many different target molecules at once. Differences in thermal stability of duplexes are used to distinguish SNPs. It can be very small between a perfectly matched and single-mismatched double-strand, so it is very important to increase selectivity. There has been considerable recent work to find molecular strategies for increasing selectivity to such small genetic differences. 50-53
1.2.5 Stability

The stability of a biomolecular sensor refers to how well the biomolecules can be stabilized to provide the specificity of response. Basically there are two types of stability: shelf-stability and operational-stability.

Shelf-stability may be defined as the retention of activity for a biomolecular sensor when stored under particular conditions after manufacture. The stability of the labile biomaterial in biomolecular sensor devices is often one of the key factors as to the commercial viability of that sensor device. Examples of low-temperature and sterile storage are some ways to keep biomaterial stable.

Operational-stability may be defined as the retention of activity of a biomolecular sensor when in use. It is even more widely discussed in the literature, since it is related to the reusability and the operating lifetime of a biomolecular sensor. Scientists are working to regenerate the surface-bound probes after each assay. These regeneration schemes include thermal and chemical methods, such as prolonged incubation in hot water, concentrated acidic or basic solutions or buffers. They are commonly used to dissociate the recognition pairs. The bound targets are released and the immobilized probes are reinstated for another assay.

Though both types of stabilities are important parameters and shelf-stability is always required, sometimes the operational stability is irrelevant, such as in the manufacture of single use, disposable biomolecular sensors. However, in many cases the lifetime of a biomolecular sensor can be important because it has to be used several times. For example, if the price is so high that disposing the biomolecular sensor is unacceptable, the reusability is crucial.
1.3 Motivations for Single Color Near-Null Reflective Interferometry (sc-NNRI) and Brewster Angle Straddle Interferometry (BASI)

Chip-based biomolecular sensors are aiming for the goals of being high-throughput, easy to manufacture, sensitive, selective and stable. It is further desirable to have good operation simplicity and low cost. Since the surface attachment chemistry for probe molecules and molecular recognition chemistry are well studied, transducers are the main focus for us to develop a high-quality detection method. To date, some major categories have been explored to accommodate such a transducer to achieve the goals described above, including mechanical, electrochemical and optical systems. Mechanical transducers could be very sensitive, but might be difficult to microarray and need a relatively long time to stabilize. Electrochemical transducers are more widely used due to their operation simplicity, but they might be destructive and affect the sensors’ operational-stability. Optical transducers, for example, fluorescence transducers are even more popular due to their high sensitivity and visual read-out results, but they normally require tagging of analytes and relatively expensive imaging systems. Label-free approaches such as ellipsometric\textsuperscript{26-30} and interferometric\textsuperscript{19-25} methods that detect spectral shifts are also widely practiced, but they still tend to require a relatively complicated detection system. In addition, if array imaging is desired, they might be expensive to implement. Recently, the most popularly used approach, Surface plasmon resonance, has proven to be label-free and able to realize imaging versions.\textsuperscript{16-17, 44-48} However, it is typically sensitive to adsorption of only a few Angstroms of material, remains relatively expensive, has limited dynamic range and is difficult to interpret quantitatively.
1.3.1 Development of Wavelength Scanning Reflective Interferometry (ws-RI)

Considering all the goals of chip-based biomolecular sensors, ws-RI was developed by previous researchers in our group, to partially overcome the challenges that are faced by the methods mentioned above. Ws-RI is based on the destructive interference in the reflected intensity of s-polarized light from an anti-reflection coated silicon substrate. A minimum in reflectivity occurs for light source at a certain angle of incidence. At the same time, the wavelength of the light source and the coating of certain thickness for the coating on the substrate surface are related and paired to satisfy the requirement of minimum reflectivity. The wavelength for minimum reflectivity at the local surface has a red shift after specific binding of biomolecules that causes an apparent increase in thickness of the coating. By scanning the probe wavelength, the wavelengths of minimum reflectivity for different spots on the surface are determined and used to infer the surface topology.

Previous research demonstrated specific and label-free binding between probe/target pairs of DNA/DNA,\textsuperscript{54} PNA/DNA and Aptamer/Protein.\textsuperscript{55} As low as 0.1 nm variance of surface topology due to binding target molecules on the surface was measured, which is caused by the coverage of target molecules on the sensing surface.

Compared with SPR, ws-RI provides an absolute minimum shift $\Delta \lambda = 3.2$ nm when binding 1.7 nm biomaterial, using the optimized geometries.\textsuperscript{54, 56} Although the shift is similar to the shift in the minimum SPR signal, it is much easier for RI to determine the deeper minimum than SPR.\textsuperscript{54}
1.3.2 Motivations to Develop sc-NNRI

The biggest advantages of ws-RI are its ability of being label-free and sensitive. Unfortunately, the light source has to meet the requirement of being scannable and possessing narrow bandwidth, which has a number of negative effects. First, the sensitivity of the technique is affected by the source bandwidth. Second, multiple measurements at a variety of wavelengths are needed which are time-consuming for data collection. Third, the scannable source (a lamp and spectrometer for example) could be expensive. In the case of using the lamp and spectrometer as a tunable source, precise collimation of the light to meet the interference condition is very difficult. This both reduces sensitivity and requires better CCD cameras for detection when throughput is sacrificed to obtain directionality.

Using a simple laser as a light source has the potential to greatly alleviate problems with collimation, monochromaticity, scanning time and expense. These considerations motivate our work on sc-NNRI. The set up has the potential to be simple and the measurement to be fast, because the only two measurements are the images before and after adding target biomolecules. Also sc-NNRI should in principle be more sensitive than ws-RI since improved monochromaticity and collimation enable us to better meet the condition for destructive interference.

Although sc-NNRI has potentially major advantages to be more sensitive, simpler, faster and cheaper than ws-RI, we unavoidably had to face two challenges. First, precise control of the coating thickness is required to satisfy perfect destructive interference at a specific laser wavelength, because our substrate of choice thermal oxides on Si cannot achieve this currently. Second, the interferometric imaging system with coherent sources can introduce substantial spatial variation of intensity even in the absence of topological
features on the biochip surface. Our approaches\textsuperscript{41} to meet these challenges will be
detailed in Chapter 3. Another group also recently reported this simple approach as
arrayed imaging reflectometry (AIR).\textsuperscript{57-58}

1.3.3 Motivations to Develop BASI

While use of a laser does an excellent job of addressing the bandwidth and
collimation problems for sc-NNRI, it does not help with coherent problems when using
imaging cameras as detectors that save time for substrate scanning. Also, inhomogeneity
of the layers (coating, probe and target) turns out to be the true limit to sensitivity for sc-
NNRI. Considering this, the reduction of sensitivity in choosing BASI gives all the
benefits of incoherent sources with little BW requirement while the actual sensitivity for
practical systems is about the same (See Chapter 4). In consequence no surface scanning
is required and imaging by a camera is more convenient. In addition, without critical
thickness control the reproducibly-coated native 2 nm SiO\textsubscript{2} can just work as the
interference layer.

All the discussion above is based on \textit{ex situ} measurements, but biomolecular
sensors are also powerful tools to monitor \textit{in situ} binding chemistry and study binding
kinetics in aqueous media. However, this has not yet been realized for ws-RI and sc-
NNRI. From the previous discussion, we know that ws-RI and sc-NNRI basically use s-
polarized light and a convenient geometry for the coated thermal oxides on silicon, but
for under water detection using sc-NNRI, near grazing incidence probing is required. The
measurement scheme is shown in Figure 1.7. Although theoretical calculation proves its
feasibility, it is very hard to satisfy such a steep angle of incidence, even with a prism
coupling light into the set up.
Figure 1.7 Geometry of sc-NNRI for under water analyte binding.

Besides the problem of introducing light at the required angle onto the interface of water/substrate, sc-NNRI also requires control over either the wavelength of the light (for ws-RI) or the oxide thickness (for sc-NNRI). These two challenges make sc-NNRI inconvenient, and motivated us to work on a new invention for reflective interferometry, BASI.

Fortunately, BASI alleviates the problems discussed above with sc-NNRI and can be implemented experimentally with comparable sensitivity. The principle is similar to sc-NNRI except that we exploit the fact that reflected light in p-polarization undergoes a 180 degree phase shift at angles of incidence above the Brewster angle while it experiences no phase shift at angles of incidence below. We are able to choose conditions where reflection at the water/oxide interface of a silicon wafer is above its Brewster angle and the companion reflection at the oxide/silicon interface is below its Brewster angle. Since the incidence angle is chosen in this way, we called the technique “Brewster Angle Straddle Interferometry”. With p-polarization, we expect near-perfect interference when the oxide coating thickness approaches zero and the two reflection coefficients have
equal magnitude. The uniform thin native oxide on silicon wafers can then be utilized as the “anti-reflection coating” since it is small (~2nm) compared to the wavelength of the probe light. While BASI is less sensitive than sc-NNRI in principle because the interference condition cannot be made exact, its sensitivity is highly tolerant of imperfect monochromaticity and source collimation so that it does not compromise measurement quality using incoherent sources or broadband light such as filtered lamps or light-emitting diodes. Even more importantly, no engineering of the oxide thickness is necessary and the thin, and highly reproducible native oxide on silicon is a suitable anti-reflection coating that works satisfactorily as a reference allowing quantitation of target binding. As an added bonus, more moderate angle of incidence works to enable near-perfect destructive interference for under water measurements, because working with p-polarized probe light (i.e. the polarization in which there is a Brewster angle) at oblique incidence serves to reduce the high reflectivity of the silicon. In this circumstance, it is easier for BASI to achieve good imaging efficacy under water than with the thick oxide coatings for sc-NNRI.

1.4 Aim of This Study

In this work, two principles of reflective interferometry will be utilized for biomolecular sensing.

Chapter 2 gives the details of the theory of reflective interferometry and the measurement schemes for ws-RI, sc-NNRI and BASI.

In Chapter 3, our work will be focused on sc-NNRI, which uses an s-polarized laser source. Because the thickness of the anti-reflection coating is critical when the wavelength of the source is not tunable, we will show that augmenting the thickness
using layer-by-layer electrostatic assembly of polyelectrolyte can be done to make the interference coating thickness nearly ideal for a given laser wavelength. Using a laser to probe the surface allows for highly collimated and monochromatic light so that high sensitivity can be achieved. We are able to see increases in reflectivity of 2 orders of magnitude due to binding of a 2.5-nm layer of streptavidin on a biotin-functionalized silicon substrate. Our present limit of detection (LOD) level is less than 0.12 nm. We compared sc-NNRI with SPR using the optimized SPR geometry. Theoretically sc-NNRI has an enormous advantage over SPR in dynamic range and sensitivity due to the depth of the reflectivity minimum near conditions of perfect destructive interference. While practical sc-NNRI cannot approach the theoretical limit, it nevertheless promises to be at least 1 order of magnitude more sensitive than SPR although sc-NNRI is simpler to implement.

In Chapter 4, our work will be focused on under water BASI (uw-BASI), which uses p-polarized light. We utilize the fact that reflected light undergoes a 180° phase shift for angles of incidence above the Brewster angle for a first interface but no phase shift at a second interface where it is incident below the Brewster angle. This property enables us to use the very thin and flat native oxide on silicon as an interference layer, which provides us a very convenient substrate design. We find that the measurement configuration we use is particularly insensitive to probe light bandwidth and collimation. We have implemented BASI for under water detection using a prism coupling scheme to inject light into a chamber. Our apparatus is sensitive to average thickness changes of less than 1 Angstrom and unlabeled thrombin detected by BASI under water will be demonstrated as an example. Compared with SPR, BASI has the comparable sensitivity but its practical implementation has considerable advantage over SPR and sc-NNRI since
the measurement configuration is particularly insensitive to probe light bandwidth and collimation.

In Chapter 5, an application of uw-BASI will be demonstrated by detecting a 47-mer fragment of mutant Ha-ras messenger RNA. We immobilized six designed 9-mer 2’-O-methyl-RNA probe sequences, two of which are modified with TTT TTT TTT spacers, on the silicon substrate in an array format. Using BASI, we are able to detect the bindings between the mRNA hairpin and its specific probes which are complementary to single-stranded regions exposed on the mRNA hairpin. We hope that these demonstrations of reflective interferometry to detect biomolecules will inspire further work in biological research, health care, diagnostics, pharmaceutical screening and environmental monitoring applications.

Chapter 6 is a summary of all the significant results of research presented in this thesis and suggestions for future research directions to develop and apply reflective interferometry.
Chapter 2

Theory and Measurement Schemes for Reflective Interferometry

2.1 Fundamental Optics

Our implementation of Reflective Interferometry is based on destructive interference of polarized light from anti-reflection coated silicon substrates. The basic idea and the theoretical model are shown in Figure 2.1.

![Diagram of Reflective Interferometry](image)

Figure 2.1 Basic idea of Reflective Interferometry. Light is polarized before it is incident on the substrate. After it is reflected from the surface, the light intensity is collected as the signal. Denoted by $n$ are the refractive indices of the measurement ambient, coating and the silicon respectively. $\theta$ are the propagation angles of the beams in the three media. The total reflection is the sum of all the beams reflected from surface.
To deal with the multiple reflections of polarized light, we use an analytic solution based on the summation of all reflected beam components. In principle, there are an infinite number of reflected and transmitted beams, and the beams transmitting out of the coating layer attenuate as they propagate in the coating.

The following is the terminology for the calculation.

<table>
<thead>
<tr>
<th>$n$</th>
<th>Refractive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\theta$</td>
<td>Angle of incidence</td>
</tr>
<tr>
<td>$d$</td>
<td>Thickness of coating</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength of incident light</td>
</tr>
<tr>
<td>$r$</td>
<td>Fresnel Reflection Amplitude</td>
</tr>
<tr>
<td>$t$</td>
<td>Fresnel Transmission Amplitude</td>
</tr>
<tr>
<td>$E$</td>
<td>Electric field of light</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Incidence</th>
<th>Beam of incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$</td>
<td>Reflected beam</td>
</tr>
<tr>
<td>1</td>
<td>Ambient medium</td>
</tr>
<tr>
<td>2</td>
<td>Medium of coating</td>
</tr>
<tr>
<td>3</td>
<td>Medium of substrate</td>
</tr>
</tbody>
</table>

$i_j$ denotes the medium from which medium the beam is incident on the interface and $j$ stands for the medium of the other side of the interface.

$\beta$ is the optical thickness of the film, given by

$$\beta = 2\pi n_2 (d/\lambda) \cos \theta_2 = 2\pi (d/\lambda) \sqrt{(n_3^2 - n_2^2 \sin^2 \theta_3)}.$$  

Equation 2.1

Each successive reflected beam obeys the formula:

$$E_n^r = t_{23} t_{32} (r_{23})^{n-2} (r_{32})^{n-1} e^{-i(2n-2)\beta}.$$  

Equation 2.2
Notice that the Fresnel reflection coefficients are dependent on the polarization of the light source. They are functions of angle of incidence and the refractive indices of the media \( i \) and \( j \) at the interface. For s-polarized incident light,

\[
    r_s(ij) = \frac{(n_i \cos \theta_i - n_j \cos \theta_j)}{(n_i \cos \theta_i + n_j \cos \theta_j)}. \tag{2.3}
\]

For p-polarized incident light,

\[
    r_p(ij) = \frac{(n_i \cos \theta_i - n_j \cos \theta_j)}{(n_i \cos \theta_i + n_j \cos \theta_j)}. \tag{2.4}
\]

Summing all the reflected beams (Equation 2.2) gives the total reflection

\[
    E_{total}^r = E^{incidence} \left( r_{32} + r_{21}e^{-i2\beta} \right) / \left( 1 + r_{32} r_{21}e^{-i2\beta} \right). \tag{2.5}
\]

So the ratio of the reflectivity over the incident light intensity is

Reflection coefficient \( R = (E_{total}^r / E^{incidence})^2 \)

\[
    = \left| (r_{32} + r_{21}e^{-i2\beta}) / (1 + r_{32} r_{21}e^{-i2\beta}) \right|^2. \tag{2.6}
\]

The Fresnel Reflection Amplitudes \( r_{32} \) and \( r_{21} \) are dependent on the refractive indices and angle of incidence, and the optical thickness \( \beta \) is dependent on the refractive index of the coating layer, angle of incidence, wavelength and the thickness of the coating. In order to achieve perfect destructive interference,

Reflection coefficient \( R = 0. \) \tag{2.7}

This makes it clear that the optimal parameters for destructive interference include six factors, the refractive indices of three media, angle of incidence, wavelength and the coating thickness.

### 2.2 Optimization for Near-Null Reflective Interferometry (NNRI)

From the calculations discussed above, in order to achieve destructive interference, in other words, near-zero reflectivity, the refractive indices, angle of
incidence, wavelength and coating thickness have to satisfy certain conditions. All of these factors are discussed in detail below.

2.2.1 Angle of Incidence

A computation of the optimal conditions for destructive interference is straightforward and the results are easy to rationalize physically. We can solve for perfect interference,

\[ R_s = 0. \]  

Equation 2.8

Certain conditions must be satisfied to cancel the s-reflection from the first and the second surfaces. The conditions are \( r_{32} = r_{21} \) and \( 2\beta = m\pi \ (m = 1, 3, 5\ldots) \), when the reflections at the first interface and the second interface have equal magnitude and travel optical distances that differ by a half wavelength.

Let us first consider \( r_{32} = r_{21} \). The angle of incidence is highly dependent on refractive indices of the three media. We solve Equation 2.8 to get the optimal incident angle when \( 2\beta = m\pi \ (m = 1, 3, 5\ldots) \),

\[ \theta_{\text{min}} = \arcsin \sqrt{(n_1^2 - n_2^4 / n_3^4) / (n_3^2 + n_1^2 - 2n_2^2)}. \]  

Equation 2.9

For the air/SiO\(_2\)/Si system under consideration and \( \lambda = 660 \) nm, in solving the equation analytically, it makes sense to simplify by neglecting the imaginary part of the refractive index of Si since it is small when using red probe wavelengths far below the direct band gap of silicon. The refractive indices of the three media are respectively \( n_3 = 1 \), \( n_2 = 1.4563 \) and \( n_1 = 3.8251 \). The optimal incident angle is calculated to be 70.6°. Larger angles do not work since the reflection from the first surface becomes too large to be cancelled by the second surface reflections. When the angles are smaller, the back
reflection from the highly reflective silicon is too large to be cancelled by the front face reflection.

2.2.2 Ratio of \( d/\lambda \) (\( H \))

In addition to finding the angle for near-perfect destructive interference, we must also meet the phase shift condition of Equation 2.8. This is \( 2\beta = m\pi \) \((m = 1, 3, 5...),\) which determines the ratio of the coating thickness \( d \) to the light wavelength \( \lambda \). The case of particular interest, however, here is that of \( m = 1 \) (the thinnest practical oxide) where we attempt to satisfy \( 2\beta \approx \pi \), so

\[
H = (d/\lambda)_{\text{min}} = 1 / (4n_2\cos\theta_2) = 1 / (4\sqrt{n_2^2 - n_3^2 \sin^2 \theta_3}).
\]

Equation 2.10

Since the optimal incident angle is fixed at 70.6°, and we still use the same structure model, the ratio \( H \) could be calculated through the values of the refractive indices of the top two media and the angle of incidence. It turns out that \( H \) also has to be a fixed value, and this value is 0.2253. The basic physics explains the result quite well, since at that moment, optical distances traveled by the two reflected beams differ by a half wavelength.

We assumed the refractive indices of the three medium at wavelength 660 nm and obtained the optimal angle of incidence, 70.6°, and the optimal \( H \), 0.2253. Actually, the refractive indices are functions of wavelength, so certain wavelengths are corresponding to certain optimal angles and \( H \)'s, which means perfect destructive interference could happen at any wavelength, the corresponding angle of incidence and coating thickness.
2.3 Optimization for Brewster Angle Straddle Interferometry (BASI)

Near-Null Interferometry exploits the near-zero reflectivity when \( r_{12} \approx r_{23} \) and \( \beta \approx \pi/2 \). The optimal conditions are \( \theta_3 = 70.6^\circ \) and \( H = 0.2253 \) when \( \lambda = 660 \text{ nm} \). Optimal conditions may vary versus probe wavelengths. Are these conditions the only solutions to Equation 2.7? And is s-polarization the only option to achieve destructive interference? It turns out that zero reflection will also result when \( 2\beta \approx m\pi \) (\( m = 0, 2, 4 \ldots \)) and \( r_{32} \approx -r_{21} \). For judicious choice of angle, it is possible to realize \( r_{32} \approx -r_{21} \) for p-polarized light as long as \( n_3 < n_2 < n_1 \) or \( n_3 > n_2 > n_1 \). Physically, the reason is that there is a 180° phase flip in the reflected electric field for p-polarized light when the angle of incidence is above the Brewster angle and no phase shift for angles below the Brewster angle. When the refractive indices are ordered as above, it is always possible to choose an incident angle above the Brewster angle for one interface and below it for the other so that the signs of the reflection coefficients are opposite and the magnitudes are equal. This underlying principle is the reason that we have also called this method as “Brewster Angle Straddle Interferometry (BASI)” though it is easily seen to be p-polarized RI.

2.3.1 Angle of Incidence

Let us solve for the optimal conditions analytically. The magnitudes of the two reflection coefficients have to be equal and the signs have to be opposite, which means \( r_{32} \approx -r_{21} \). This implies that there is a 180° phase flip in the reflected electric field for p-polarized light, and that the reflections at the first interface and the second interface have
nearly equal magnitude. Since the reflection coefficients of p-polarized light are different from those of s-polarized light, we obtain a different result,

$$\frac{n_3\cos\theta_2 - n_2\cos\theta_3}{n_3\cos\theta_2 + n_2\cos\theta_3} = -\frac{(n_2\cos\theta_1 - n_1\cos\theta_2)}{(n_2\cos\theta_1 + n_1\cos\theta_2)}. \tag{2.11}$$

The angle of incidence is then given by

$$\theta_{\text{min}} = \arcsin \sqrt{(n_3^2 - n_1^2) / ((n_3^4 / n_1^2) - n_1^2)}. \tag{2.12}$$

The calculated optimal angle of incidence is 75.3°, assuming the same refractive indeces for calculating optimal angle of incidence for NNRI. Similarly as that for Near-Null Interferometry, the angle of incidence for BASI at minimum reflectivity is still dependent on the indices, but differently it is only dependent on $n_3$ and $n_1$. Even though the optimal angle of incidence for BASI is not dependent on $n_2$ as long as $n_3 < n_2 < n_1$ or $n_3 > n_2 > n_1$, the absolute value of reflectivity minimum does depend on $n_2$.

### 2.3.2 The Coating Thickness

The criterion that $2\beta \approx m\pi \ (m = 0, 2, 4 \ldots)$ can be met just as for Near-Null Interferometry using thermally grown oxides on silicon with precisely tailored thickness ($d \approx m\lambda/4n_2\cos\theta_2$). In the case of particular interest, $m = 0$, we attempt to satisfy $2\beta \approx 0$ with an interference layer comprised of the native oxide on silicon ($d_{\text{native}} \approx 2 \text{ nm} << \lambda$). Due to the $\sim 2$ nm native oxide, longer wavelengths will exhibit deeper minima as $2\beta$ is smaller. While the finite oxide thickness introduces a small phase shift between reflections from the two interfaces that prevents perfect destructive interference, the practical advantages of using the native oxide as an interference layer are overwhelming, because no thermally grown SiO$_2$ and no critical thickness coating are needed.
2.3.3 BASI and NNRI Comparison of Reflectivity Dependence on Wavelength and Angle of Incidence

Shown in Figure 2.2 is the comparison of reflectivity of s-polarized light and p-polarized light dependent on wavelength for BASI and NNRI respectively. For BASI, considering the 2 nm SiO\textsubscript{2}, relatively longer wavelength (or redder light) produces lower reflectivity (see Figure 2.2a). Even though the non-zero coating results in imperfect destructive interference and the reflectivity is not as low as that of NNRI, the wavelength dependence is tolerant in a relatively big range. In other words, no critical wavelength control or thickness engineering is required as for NNRI.

Figure 2.2 BASI and NNRI Reflectivity of p, s-polarized light respectively versus wavelength. (a) BASI Reflectivity of p-polarized light versus wavelength, when angle of incidence is fixed at optimal value $75.3^\circ$ and the coating is 2 nm native SiO\textsubscript{2}. (b) NNRI Reflectivity of s-polarized light versus wavelength, when $\theta$ is fixed at optimal value $70.6^\circ$ and the coating is 148.7 nm SiO\textsubscript{2}. The center wavelength is 660 nm, we utilize $n_3 = 1$, $n_2 = 1.4563$ and $n_1 = 3.8251$ as the refractive indeces of three media respectively, neglect
the imaginary parts and assume no variations of refractive indices in the wavelength range. The reason we do not get perfect destructive interference $R_s = 0$ is our plot is coarse-grained and there is rounding error.

Shown in Figure 2.3 is the comparison of reflectivity of $s$-polarized light and $p$-polarized light dependent on angle of incidence for BASI and NNRI, respectively. For BASI, even though the reflectivity is not as low as that of NNRI, it is relatively more tolerant of angle of incidence in the plot range, which means control of angle and probe beam divergence are less critical than those for NNRI.

![Graphs showing reflectivity for BASI and NNRI](image)

Figure 2.3 BASI and NNRI Reflectivity of $p$, $s$-polarized light respectively versus angle of incidence. (a) BASI Reflectivity of $p$-polarized light versus angle of incidence, when wavelength is 660 nm and the coating is 2 nm native SiO$_2$. (b) NNRI Reflectivity of $s$-polarized light versus angle of incidence, when wavelength is 660 nm and the coating is 148.7 nm SiO$_2$. The center wavelength is 660 nm, we utilize $n_3 = 1$, $n_2 = 1.4563$ and $n_1 = 3.8251$ as the refractive indices of three media respectively, neglect the imaginary parts and assume no variations of refractive indices in the wavelength range. The reason we do not get perfect destructive interference $R_s = 0$ is our plot is coarse-grained and there is rounding error.
2.4 Measurement Schemes of NNRI and BASI

We have outlined the theoretical considerations in achieving interference minima above and now we turn to a discussion of how to exploit these minima to detect molecular binding at the air/oxide or water/oxide interface.

The following sections describe the journey from wavelength scanning RI (ws-RI), to single color NNRI (sc-NNRI) to BASI as we try to develop the most practical implementation of reflective interferometry for biomolecular sensing and to illustrate the tradeoffs between various formats.

2.4.1 Measurement Scheme of ws-RI

In our discussion of NNRI optimization, the ratio $H (d/\lambda)$ was a fixed number, meaning that the minimum reflectivity occurs when $d$ is a fixed fraction of the probe wavelength. Shown in Figure 2.4 is a theoretical illustration of how ws-RI detects the binding event occurring at the substrate surface. The reflectivity curve before attachment of target molecules to the surface is calculated, shown as the red curve. The blue and the green curves represent the calculated reflectivity after binding event with an averaged thickness increase of 2 nm and 6 nm respectively.
Figure 2.4 Theoretical illustration of how ws-RI detects the binding on the substrate surface. The red curve is the reflectivity of the surface before binding events. The blue and green curves stand for the intensity for the same surface after binding 2-nm or 6-nm layers of target molecules. The assumption for the original surface is a 150 nm coating of SiO₂ and the probe molecules. The angle of incidence is 70.6°. In this calculation, we also assume that the refractive indices of both probe and target molecules are identical to that of the oxide.

The experimental measurement scheme involves two steps. For a given spot, the first step is to measure its reflectivity versus wavelength from 660 nm to 700 nm. This generates a curve before binding, for example the red curve in the illustration. The wavelength of the reflectivity minimum for the red curve can be determined and is 666.2 nm. Similarly, once the wavelengths of the reflectivity minima after biomolecular binding are determined, the shift of the wavelength minima will give an estimate for the amount of target molecules attached to the surface by converting $\Delta \lambda$ to $\Delta d$, given $\Delta d = H \times \Delta \lambda$. For the blue and green curves, their minima occur at 674.9 nm and 692.2 nm, respectively. Assuming refractive indices of 1.46, this implies 1.96 nm and 5.86 nm increases in thickness due to biomolecular binding, respectively.
2.4.2 Measurement Scheme of sc-NNRI

The measurement scheme used for ws-RI has several disadvantages. It requires a tunable source to provide a good match to the optical thickness of the interference coating. Also, implementation with a lamp and spectrometer is bulky and expensive, at the same time providing relatively poorly defined angle of incidence and large spectral bandwidth that affect the sensitivity. These factors motivated us to invent a single-color variation with a highly collimated and monochromatic laser.

Our version of single color NNRI (sc-NNRI) used a HeNe laser that is coherent and highly collimated compared to the source for ws-RI. The reflectivity images are measured before and after biomolecular binding and the reflectivity contrast is obtained by dividing the intensity of the two images. Illustrated in Figure 2.5 are the calculated logarithm of the reflectivity and the theoretical contrast for specific amount of binding molecules.

![Figure 2.5](image)

**Figure 2.5** Logarithm of reflectivity (left) versus coating thickness $d$ and theoretical contrast (right) for 1 nm binding with the change of coating thickness $d$. The calculations are based on the optimal conditions for $\lambda = 632.8$ nm, $\theta = 70.6^\circ$. The contrast of $R_s$ is calculated by dividing the light intensity after 1 nm binding by the background intensity.
at the certain coating thickness. We assume the refractive index of the binding layer is the same as oxide coating.

A monochromatic source, here a HeNe laser, unavoidably causes problems in imaging with a camera due to fringes and speckles associated with spatial coherence. This is the reason that we chose to collect all of the reflected light in an unexpanded laser beam and utilize a photomultiplier as the detector while scanning the substrate with a two-dimensional stepping motor, which could take about 10 minutes (see Chapter 3). In addition, when the wavelength is fixed, the coating thickness $d$ becomes particularly critical. This is the main challenge of sc-NNRI. The layer by layer electrostatic assembly method we used to engineer the coating thickness accurately is demonstrated in Chapter 3. Since inhomogeneity of the layers (coating, probe and target) turns out to be the true limit to sensitivity for sc-NNRI, the efforts in improving monochromaticity and collimation become less important for increasing detection sensitivity. A detailed discussion is included in Chapter 3.

2.4.3 Measurement Scheme of BASI

As pointed out above, sc-NNRI uses a laser as a probe source and this does an excellent job of addressing the bandwidth and collimation problems but makes it necessary to scan the substrate and implement photomultiplier as the detector. When using imaging cameras as the detectors, the LOD in sc-NNI was no better than with the lamp and spectrometer in ws-RI because of coherence problems. Since this ends up limiting sensitivity anyway, the reduction of sensitivity in choosing BASI gives all the benefits of incoherent sources with little BW requirement while the actual sensitivity for
practical systems is about the same (See Chapter 4). In addition, BASI uses the reproducible 2 nm native SiO₂ as the interference layer, so no critical thickness control is needed.

BASI measurements can be done in air or under water. Considering the goals of chip-based biomolecular sensors to detect real-time biomolecular binding, this is a great advantage. For sc-NNRI, it is possible to realize the measurement scheme for under water detection. The optimal geometry was briefly discussed in Chapter 1. In Equation 2.9 and 2.10, since the refractive index for the nominally ambient medium is water instead of air, both the angle of incidence and the ratio \( H \) have to be adjusted to satisfy the destructive interference. Figure 2.6 shows an example of utilizing sc-NNRI for under water measurement when the light wavelength is 632.8 nm.

![Figure 2.6 The ideal geometry of the light path on the substrate for under water sc-NNRI. The thickness of SiO₂ is denoted by \( d \). \( \lambda \) is the wavelength of light. When we use a HeNe laser for probe light and silicon as the substrate, the angle of incidence needs to be 85.7° for near-perfect destructive interference.](image)

Now the optimal incident angle is 85.7°, when the medium of incidence is water (refractive index is 1.33). And the ratio of film thickness to probe wavelength is 0.3115.
If we use a HeNe laser for sc-NNRI ($\lambda = 632.8$ nm), the coating thickness has to be strictly 197.09 nm.

It seems that we just need to change the experimental parameters. However, if we take a close look at this geometry, we will find a problem. If the angle at the water SiO$_2$ interface is to be 85.7°, the angle in air (where light source originally is coupled into the set up) has to be $\arcsin(1.33 \times \sin 85.7^\circ)$ degrees, which is impossible. The largest angle that can be achieved in water is the critical angle (48.8°).

To solve this problem, we could adjust the refractive index of the interference coating, replacing SiO$_2$ with a higher index material. Using the critical angle (48.8°) for air/water interface as incident angle for water/SiO$_2$ interface, we calculate the required refractive index of the coating. It is around 2.0. That is to say, practically we should choose a material with refractive index larger than 2.0. This method requires engineering the substrate surface with coatings of materials such as silicon nitride or titanium dioxide and either changing attachment chemistry or growing an overlayer of oxide. These are possible but not easy. One alternative way is to use a 45° prism on top of water and couple light to couple into the liquid at 85.7°. Figure 2.7 shows the basic idea of prism coupling.
Figure 2.7 A practical geometry of the light path for under water measurement. There is a 45° glass prism on top of the water. Included in the layer of substrate are coating of SiO₂ and silicon.

For BASI, the concern about steep angle of incidence turns out not to be a problem. From Equation 2.12, by changing the ambient refractive index to 1.33, the optimal angle of incidence in water for under water measurement of BASI is estimated to be 70.8°. Even though we still need prism coupling on the top, the angle (70.8°) is never as steep as required for sc-NNRI (85.7°).

2.4.4 Data Quantitation

Compared with sc-NNRI, BASI not only has the advantage of easy under water implementation, but also there is another feature of importance. BASI is quantitative in the sense that the magnitude of reflectivity can be used to infer the binding amount of biomolecules on the surface. The following is a detailed discussion of how BASI can be used to quantitate the binding amount of target binding to the substrate.

In Equation 2.6, taking $r_{32} \approx - r_{21} = r_o$, we can approximate the reflectivity near its interference minimum,
$R_{\text{min}} \approx r_o^2 \left| \frac{1 - e^{-2i\beta}}{1 - r_o^2} \right|^2$. \hspace{1cm} \text{Equation 2.13}

When we operate relatively near the Brewster angles of the respective interfaces as is the case for the implementation on the native oxide of silicon, the value of $r_o$ is much less than unity and we can take the denominator to be approximately unity so that

$$R_{\text{min}} \approx r_o^2 \left| 1 - e^{-2i\beta} \right|^2.$$ \hspace{1cm} \text{Equation 2.14}

Since $d \ll \lambda$, the value $\beta$ is much less than unity too so we can write

$$e^{-2i\beta} \approx 1 - 2i\beta.$$ \hspace{1cm} \text{Equation 2.15}

The minimum reflectivity can then be approximately evaluated at the angle where $r_{32} \approx -r_{21} = r_o$ and simply reduces to

$$R_{\text{min}} (d) \approx 4r_o^2 \beta^2 = \left( 16 \pi^2 r_o^2 n_2^2 \cos^2 \theta / \lambda^2 \right) d^2.$$ \hspace{1cm} \text{Equation 2.16}

The most important consequence of equation 2.16 is that we expect the value of the reflectivity minimum to be parabolic with $d$. To the extent that the refractive index of the adsorbates under study matches that of the oxide, we can write

$$R_{\text{min}} (d) / R_{\text{min}} (d_{\text{oxide}}) = (d/d_{\text{oxide}})^2.$$ \hspace{1cm} \text{Equation 2.17}

and infer the adsorbate thickness from its reflection intensity relative to that of the oxide whose thickness is known.
Chapter 3

Biomolecular Sensing Using Single Color Near-Null Reflective Interferometry

3.1 Introduction

Development of sensitive label-free platforms to detect biomolecules has facilitated biochemical research. The most widely used approach is surface plasmon resonance (SPR), which is typically sensitive to adsorption of only a few Angstroms of material, but remains relatively expensive, has limited dynamic range, and is difficult to interpret quantitatively. Ellipsometry and interferometric methods that detect spectral shifts are both very sensitive but expensive to implement, particularly if imaging of probe arrays is desired. We recently reported a simplified version of these that exploits conditions of nearly perfect destructive interference in reflectivity that can be achieved when the wavelength and incidence angle can be controlled precisely. A previous researcher in our group demonstrated the approach on submonolayer detection of DNA and referred to the method as Reflective Interferometry (RI).
ws-RI provides high sensitivity, excellent dynamic range, and the ability to infer the amount of target adsorbed quantitatively but requires a tunable source to provide a good match to the optical thickness of the interference coating. Implementation with a lamp and spectrometer is, however, bulky and expensive, also providing relatively poorly defined incidence angle and large spectral bandwidth that reduce the sensitivity. Here we report a single-color variation with a highly collimated and monochromatic laser that results in the ability to detect average adsorption of less than 1.2 Å. The apparatus is amenable to very inexpensive construction and could be made small and portable.

We demonstrate control of the thickness of the interference coating using layer-by-layer electrostatic self-assembly of polyelectrolytes\textsuperscript{59-61} applied to optimize the thickness for use with a HeNe laser. Functional groups on the polyelectrolytes also serve as suitable binding sites for probes with molecular recognition capability. The efficacy of the platform is demonstrated with well-documented biotin-streptavidin chemistry. Reflectivity changes larger than a factor of 10 are observed for binding 3Å of biotin on the polyelectrolytes and nearly a factor of 100 when binding less than 3nm of streptavidin to the biotin. We show that these measurements are in quantitative agreement with theoretical predictions that include non-idealities associated with imperfect surface roughness. Theoretical comparison with optimized SPR based on literature geometry\textsuperscript{47-48, 56} results suggests that this implementation of reflective interferometry is considerably more sensitive than SPR.
3.2 Experimental Section

3.2.1 Materials

Nominally undoped silicon wafers with thermally grown oxides of 134 nm were obtained from the Microelectronics Center at the Rochester Institute of Technology. Poly (allylamine hydrochloride) (PAH; average MW 70000 Aldrich), poly (sodium 4-polystyrenesulfonate) (SPS; average MW 70000 Aldrich), poly (acrylic acid) (PAA; average MW 100000 Aldrich), N-hydroxysuccinimide (NHS; Fluka), N-(3-dimethyl aminopropyl)-N’-ethylcarbodiimidehydrochloride (EDC; Sigma), biotinethylenediamine (Biotium), and streptavidin (Sigma) were used as received. Millipore-filtered water was used for all aqueous solutions and rinsing.

3.2.2 Layer-by-Layer Electrostatic Self-assembly (LBL-ESA)

Silicon wafers were diced into pieces of 2cm×4cm dimensions to be used as substrates. These were cleaned for 1h in boiling piranha solution, freshly prepared by mixing 3 volumes of 30% hydrogen peroxide and 7 volumes of 98% sulfuric acid. The substrates were rinsed repeatedly with water and dried under flowing nitrogen gas. Bilayers of charged polyelectrolytes were applied with arobotic HMS Programmable Slide Stainer (Carl Zeiss Inc.). Substrates were immersed into PAH solution (0.01M, pH 6.42) for 10min, followed by immersion while agitating in three separate bins of water for 2, 1, and 1min, respectively. The substrates were then immersed into SPS solution (0.01M, pH 6.40) for 10min followed by an identical rinse procedure. This protocol was repeated by a predetermined number of cycles as needed to augment the effective
thickness of the anti-reflection coating on the silicon wafer. For the final layer on the substrate, PAA solution (0.01M, pH 2.96) was used instead of PAH to produce relatively dense carboxyl groups for the biomolecular probe attachment chemistry. After completion of the polyelectrolyte layers, the substrates were dried in an oven (100°C) for 1h.

Figure 3.1 Wafer treatment procedures. The interference coating thickness is adjusted by polyelectrolyte self-assembly. A template is applied to create four distinct spots. Biotin receptors are attached to spots 1, 2, and 3 while spot 4 is left bare as a control. Streptavidin solution is applied to spots 1, 2, and 4. Spot 3 is used to measure biotin thickness while spot 4 is used as a control to measure non-specific binding of streptavidin.

3.2.3 Ellipsometric Measurements

The overlayer thicknesses on the silicon substrates were determined before and after layer-by-layer deposition by spectroscopic ellipsometry (JL Woollam M-2000). We used the silicon oxide refractive index from the literature and assumed the refractive index of the multilayer films to be 1.55 at 632 nm for modeling the ellipsometric data.
3.2.4 Surface Attachment Chemistry and Experimental Design for Streptavidin Detection

The steps involved in the preparation of the chips are summarized in Figure 3.1. After LBL-ESA, an elastomeric cover with patterned wells was used to mask the substrate so that different chemistry could be applied at different spatial locations. It was used to generate a pattern of four chemically distinct spots while the remainder of the substrate was untreated. Spots 1, 2, and 3 were then modified by applying 1.0 µL of a mixture of equal volumes of EDC (31 mM) and NHS (7.8 mM) along with 1.0 µL of 0.14 M biotin ethylenediamine in 4-morpholineethanesulfonic acid (MES) buffer solution (pH 6.5) for 8h. The elastomeric template was then removed, and the substrate was rinsed with water and dried under flowing nitrogen gas. Streptavidin (0.25 mg/mL in 10 mM PBS, pH 7.4) was applied to two of the three biotinylated spots (1 and 2) and to the untreated spot (4) for 45min to assess both specific and non-specific binding. This procedure necessitates careful placement of the analyte drop so that it registers with the underlying probe spots. We found that this was possible to do by hand when the positions of the spots were marked ruled lines on graph paper below the chip. Fortunately, the analyte drops did not spread significantly after being applied to the surface. Again, this was followed by rinsing with water and drying under flowing nitrogen gas.

3.2.5 Sc-NNRI Measurement

The reflectivity from the substrate of a collimated HeNe laser was measured for the interferometric detection. The beam was expanded and collimated with a telescope that was adjusted using a shear plate to optimize collimation. The divergence was measured to be 0.03°. The beam was set to be incident on the substrate at an angle near
the expected reflectivity minimum (\(\sim 70.9^\circ\)) with a polarizer in the beam path to enforce s-polarization. The substrate was rotated to adjust the incidence angle for minimum reflectivity. The reflected light was detected by a photomultiplier tube covered with a 75-\(\mu\)m-diameter aperture that determined the spatial resolution of the experiment. The probe laser was mechanically chopped, and phase-sensitive detection was used for convenience so that rigorous elimination of stray light in the laboratory was not necessary. The substrate was moved using a computer-controlled stepping motor assembly capable of two-dimensional translation so that a reflectivity map could be generated. The step size in the direction orthogonal to the beam propagation direction was adjusted to be approximately commensurate with the pinhole size to preserve the spatial resolution allowed by the pinhole, and the reflectivity at each point was measured with a 100ms time constant after waiting 300ms for the value to settle at a new point. Step sizes were chosen to be \(\sim 2.7 (=\tan (70.9^\circ))\) times larger in the direction along the beam propagation direction since the resolution in that direction is degraded by the oblique angle of incidence. An area of approximately 0.3cm \(\times\) 0.3cm could be scanned in less than 7min. Note that the method can also be implemented with a camera as the detector to accelerate detection but that signal-to-noise ratios are slightly reduced in that case. Camera images are complicated by interference since the laser is coherent and by diffraction from the array spots. Also, the dynamic range of a typical CCD camera (for example 1024:1, 10bits in bit depth for Orion Starshoot Deep Space Color Imaging Camera) is much less than that of a photodiode or photomultiplier tube.
3.2.6 Ws-RI Measurement

Verification that we can quantitatively determine the amount of material detected using sc-NNRI requires an independent measurement of the amount of biotin and streptavidin deposited on our chips using the chemistry outlined above. Therefore, two-spot chips were made using identical chemistry where one spot has biotin and the other bond biotin plus streptavidin. Interference coating thicknesses at the spot locations were measured by ws-RI in an apparatus where the wavelength could be tuned to map out the substrate topology. The optical apparatus and data analysis procedures for ws-RI have been described in previous literature.54

3.3 Results and Discussion

3.3.1 Theoretical Modeling of sc-NNRI with Ideal Geometry

Figure 3.2a illustrates the layer model we use to compute reflectivity. The refractive indexes of the silicon and silicon oxide are taken from the literature while all of the organic components including the polyelectrolytes, biotin, and streptavidin are taken to have refractive indeces of 1.55. A transfer matrix formalism is applied to compute reflectivity for s-polarized incident light. The conditions closest to complete destructive interference are achieved when the light source is strictly monochromatic and perfectly collimated, and the substrate coating is completely uniform with thickness determined by the probe wavelength. Figure 3.2b plots absolute reflectivity versus total thickness of the organic components where the wavelength is taken to be monochromatic (632.8 nm), the oxide thickness to be 134 nm, and we assume perfect collimation and layer uniformity.
As with other methods based on reflection such as surface plasmon resonance, the figure of merit for detection is the ratio of reflectivity with target binding relative to that without it. The extremely deep minimum allows for the sensitivity to be extremely high in principle, much higher than that achievable for SPR. For example, the data of Figure 3.2b suggest that a 2Å increase in coating thickness (e.g., due to target binding) can produce as much as a 4 order of magnitude increase in reflectivity. In this particular implementation of RI, the fundamental limitation has to do with the fact that the refractive index of the silicon substrate is complex due to absorption above its indirect band gap. With a non-zero absorptive part of the refractive index, perfect destructive interference cannot be achieved. Longer wavelengths will exhibit deeper minimums as one probes further from the direct band gap of the substrate.

![Figure 3.2](image)

Figure 3.2 (a) Geometry used to model reflective interferometry where $\lambda$ is the incident wavelength used to probe the structure and $\theta$ is the incident angle. The silicon substrate is coated with a thick oxide, which is in turn coated with self-assembled polyelectrolyte bilayers, a probe layer, and a target layer. The polyelectrolytes, probe, and target are all assumed to have refractive indices of 1.55 while literature values are used
for the other materials. (b) Transfer matrix computation of the structure reflectivity versus the total thickness of the layers above SiO₂. The probe wavelength is 632.8 nm, the incidence angle is 70.861°, and there is a 134-nm-thick oxide.

In practice, limitations on the achievable sensitivity using RI come from non-ideal conditions including finite spectral bandwidth and angular divergence of the light source as well as inhomogeneity and roughness of the overlayers on the substrate. For the implementation here with a source of light that is not tunable, the absolute thickness of the overlayers is also critical in maximizing the reflectivity contrast between regions of slightly different thickness. We define reflectivity contrast at a given position on the substrate to be $1 + \Delta R/R_o$, where $\Delta R$ is the increased reflectivity relative to that measured in a reference region, $R_o$. Figure 3.2b illustrates clearly that a strategy to fine-tune the overlayer thickness is necessary to achieve optimal results with sc-NNRI and is the motivation for developing layer-by-layer assembly to augment thermally grown oxides.

### 3.3.2 Theory of Practical sc-NNRI and Comparison with SPR

Detailed modeling of how the reflectivity contrast is degraded when the conditions for RI are non-ideal is included in the section 3.4. In particular, we model how the sensitivity of RI is degraded when the interference layer thickness is not optimal for the probe wavelength as well as how the sensitivity is decreased when there is finite source bandwidth, finite angular divergence, and spatial inhomogeneity in the interference layer thickness. We also model “ideal SPR”, where SPR is performed under literature conditions with a source that has perfect bandwidth and collimation and a substrate with the optimal thickness of gold and no chromium or titanium adhesion layer. Briefly, we find that 1nm of target adsorption doubles the reflectivity in SPR while, even
for realistic conditions (0.03° angular divergence and 1-nm-thickness inhomogeneity in the interference layer), the reflectivity would increase by a factor of $\sim 15$ in sc-NNRI. This factor is reduced to $\sim 5$ if the interference layer is also fabricated 0.5 nm too thick. Thus, we conclude that RI is superior to ideal SPR even for non-idealities considerably worse than what is realistically achievable. It is worth noting, as is clear from thinking about Figure 3.2, that thickness inhomogeneity in the interference layer means that it is much better for a substrate to be slightly too thick than for it to be slightly too thin in order to achieve best reflectivity contrast.

3.3.3 LBL-ESA Results

As is clear from the modeling in Figure 3.2, it is critical to modify the thickness of the interference coating to be precisely correct. Commercially available Si/SiO$_2$ wafers have thermally deposited SiO$_2$ layers generally only accurate to within a few nanometers (± 3-5% of the oxide thickness). Our strategy was to buy substrates substantially thinner than ideal for 632.8nm destructive interference and to augment the film to the desired thickness using LBL-ESA.

Deposition thicknesses obtained using LBL-ESA were assessed on silicon wafers using ellipsometry. Figure 3.3 presents thickness data versus the number of bilayers of PAH and SPS deposited where the error bars represent reproducibility over several deposition runs, typically better than 0.5 nm.
Figure 3.3 Ellipsometrically determined thickness of electrostatically assembled polyelectrolytes versus the number of deposited bilayers. Triangles denote values averaged over several runs while the error bars represent the standard deviation between runs. A linear fit to the data is shown as a dashed line.

We use PAA as a final layer so that its carboxyl acid groups are available for probe attachment and assume its thickness is the same as that for SPS as measured in Figure 3.3. The experimental data are well fit by a linear relationship between number of bilayers and thickness so that we can control the film growth and augment an oxide of measured thickness by the correct number of bilayers to compensate for variations in the thickness of the thermal oxide grown on the silicon wafer. We used three substrates with oxide thicknesses between 134 and 137 nm and augmented them with LBL-ESA by 6, 7, and 8 bilayers. The idea behind using three substrates was to deliberately create experimental circumstances corresponding approximately to the cases considered in the Supporting Information, where a substrate could be nearly exactly correct for destructive interference or too thin or too thick by ~0.5 nm. Ellipsometric measurement of the substrates before and after LBL-ESA gave us values of thickness for the three
interference layers that had 0.69 nm too little polymer, nearly exactly the correct polymer thickness, and 0.14 nm too much polymer before application of biotin or streptavidin.

3.3.4 Biomolecular Detection Results

Four-spot chips were made from these three substrates by the procedure described in the Experimental Section. Figure 3.4 shows the sc-RI contrast images of the three chips. The reflectivity contrast $1 + \Delta R/R_o$ is plotted where $R_o$ is the reflectivity of the background as measured by the average reflection from a corner of the sample far from the treated spots. The gray scale for contrast values is shown on the right side. The contrast images indicate that when the substrate thickness is exactly perfect, the reflectivity contrast $1 + \Delta R/R_o$ for streptavidin-treated regions with respect to the background averages 76 over the spots (Figure 3.4b, spots 1 and 2) with places where the contrast is over 100. For the biotin-only spot, the contrast is 17 (Figure 3.4b, spot 3), and for the non-specific binding of streptavidin on polyelectrolytes without biotin, a contrast of 5 (Figure 3.4b, spot 4) was observed. When the thickness is a little thinner or thicker, the streptavidin binding contrast decreased to 19 (Figure 3.4a, spots 1 and 2) and 37 (Figure 3.4c, spots 1 and 2), respectively, with much smaller changes in the control spot 4. It is interesting to note that biotin appears to be more easily observed on the chip that is slightly too thick. The reason for this has to do with the roughness (thickness inhomogeneity on length scales less than the instrument resolution of ~100 μm) being larger than the biotin thickness such that more regions of the chip with precise average thickness have regions that are too thin and contribute reflectivity contrast less than unity to the overall observed signal.
Figure 3.4 Reflectivity contrast maps of three treated substrates with spots oriented as in Figure 3.1. The scanned area is approximately 3mm × 3mm. The gray scale at the right conveys the reflectivity contrast $1 + \Delta R/R_0$, where $R_0$ is the reflectivity in an untreated corner of the substrate. Three different substrates that underwent identical chemical treatment as described in the text are scanned: (a) ~0.69 nm too thin; (b) nearly the correct thickness; (c) ~0.14 nm too thick.

### 3.3.5 Quantitative Comparison with Theory

Determining whether the reflectivity contrasts we observe agree with the theoretical prediction based on optical theory is tantamount to assessing whether SC-NNRI can be used to derive quantitative results for the amount of adsorbed material. We know the interference coating (background) thickness without probe or target from ellipsometry, the HeNe wavelength, and have measured the probe beam’s angular divergence (0.03°). For a full comparison of the data of Figure 3.4 with theory, we also need independent measurements of the amount of biotin and streptavidin bound to the spots and of the surface inhomogeneity. To determine the amount of biotin and streptavidin attached to the spots, we used ws-RI since thicknesses are easily extracted. To determine surface inhomogeneity, we measured the ratio of p-polarized reflection to s-polarized reflection for the substrate with oxide only and assumed that the
polyelectrolyte layers were approximately conformal. The p-polarized reflectivity is ~20% and insensitive to interference layer thickness so that it can be used to calibrate the absolute s-polarized reflectivity of the substrate. When the oxide thickness is known from ellipsometry and the angular divergence is measured, the coating thickness inhomogeneity can be extracted from the theory. This involves calculating the predicted s-reflectivity with the experimentally determined angular divergence and interference layer thickness for various thickness inhomogeneities and finding the one that matches the experimentally determined s-reflectivity.

3.3.6 Biotin and Streptavidin Thickness Determination

Figure 3.5a shows a schematic of a two-spot structure created on a control Si/SiO₂ substrate. Identical chemistry was applied to what was used for spot 3 (biotin only) and spot 1 (biotin and streptavidin) of the experimental substrates discussed previously. After LBL-ESA, 1.0μL droplets of biotin solution were applied to both spots 1 and 2 of the control substrate. Spot 2 was further exposed to the streptavidin solution, followed by thorough water rinsing and drying under flowing nitrogen gas. Figure 3.5b presents raw data of reflected intensity versus wavelength derived from three different spatial regions on the chip that represent background, the biotinylated spot, and the spot containing both biotin and streptavidin. The minimum reflectivity in those three regions can be derived from fitting to a parabola and occurs at 633.7nm, 635.7nm, and 646.7nm, respectively. The topology is extracted from theory using a transfer matrix model and illustrated in Figure 3.5c. We assumed that the refractive index of the biotin and streptavidin layers is 1.55, typical for organic materials far from resonance, and found that the average deposition of biotin in its spot was 3.5 ± 0.5 Å and streptavidin plus biotin in its spot was
26.5 ± 0.5 Å. Since the technique measures optical phase delays that are proportional to the product of path length traveled in a material and its refractive index, if our assumed refractive indexes are too low (high) by a few percent then our inferred adsorption thicknesses will be too high (low) by a few percent. Notice that the best reflectivity contrast between the streptavidin and biotin spots is around 5-6 when the biotin spot is close to its minimum reflectivity. Because the probe light is from a lamp and spectrometer, its bandwidth and collimation are poor relative to the laser used for sc-NNRI and so the contrast is correspondingly much worse than for laser probing as reported here.

Figure 3.5 ws-RI to determine biotin and streptavidin layer thicknesses. The experiment is done on a wafer with layers such as those of Figure 3.2. (a) Spot geometry with the left spot having only biotin applied and the right spot having biotin followed by streptavidin. (b) Two dimensional gray scale reflection topology of the substrate surface in the streptavidin sensing experiment. (c) Raw data on reflection versus wavelength and fits for
reflectivity in the labeled rectangular regions encompassed by the background (squares), biotin (circles), and biotin/streptavidin (triangles). The curves are compiled by extracting data from reflection taken at various wavelengths with a CCD camera.

3.3.7 Coating Thickness Inhomogeneity Determination

To estimate the coating thickness inhomogeneity, we measured the reflectivity ratio of p-polarized light versus that of s-polarized light from a bare wafer to determine the absolute s-reflectivity and compared with theory using inhomogeneity as the only adjustable parameter. With this procedure, we determined the inhomogeneity in the interference layer thickness to be 1.28nm, a value consistent with AFM profiles of selected spots on our wafers. We assumed that the inhomogeneity of each of the wafers we used was the same and that the polymer bilayers are conformal and do not change the inhomogeneity.

3.3.8 Comparison with Theory

Using this value of inhomogeneity, we calculated theoretical curves of \(1 + \frac{\Delta R}{R}\) versus target thickness for our three substrates using the measured thickness, probe beam divergence, and no adjustable parameters. Those results are plotted in Figure 3.6 and should be directly comparable to the experimental results in Figure 3.4. The predicted contrast for the streptavidin spots are 9, 53, and 50 for the substrates used, assuming combined deposition of 2.65nm biotin plus streptavidin. These correspond reasonably well with the experimental results of 17, 76, and 37. The approximate agreement implies that semiquantitative conclusions regarding amount of target binding can be made from sc-NNRI measurements. More important, it confirms our conclusion that spatial
inhomogeneity of the coating thickness is the present limitation on the sensitivity of the method. That sensitivity could reasonably be expected to improve by almost another order of magnitude with a smoother oxide (cf. section 3.4). Nevertheless, ideal SPR has contrast of only 10 for 3nm adsorbed material.

Figure 3.6 Theoretical curves for reflectivity contrast \(1 + \frac{\Delta R}{R_0}\) versus analyte thickness on each of the three substrates measured in Figure 3.4. The modeling uses the ellipsometrically measured interference layer thicknesses and the measured experimental parameters for angular divergence (0.03°) and inhomogeneity (1.28 nm). For comparison, the contrast for SPR with ideal thicknesses and no divergence or inhomogeneity is plotted using a literature geometry from Ref. 56 (see section 3.4).

The contrast values we measure for biotin are a factor of \~2 better than predicted by our theory for the correct thickness interference layer. This discrepancy may arise since we have treated thickness inhomogeneity as a flat distribution for computational convenience rather than a more realistic distribution such as a Gaussian. For very small adsorbate thickness (i.e., less than the inhomogeneity in the coating thickness) on
substrates with nearly exact average thickness of the interference layer, the detailed assumptions one makes about the inhomogeneity distribution strongly affect the reflectivity result.

3.3.9 Evaluation of Detection Sensitivity

We can also estimate the minimum detectable amount of analyte in a practical implementation of sc-NNRI from Figure 3.6. Of particular importance is how well we can determine the average reflectivity contrast over a spot. Assuming Gaussian statistics, if we sample N pixels in the spot, the error in the average reflectivity contrast will be the square root of the variance divided by the number of points sampled, $(\sigma^2/N)^{1/2}$. Using these values in Figure 3.4b, a randomly chosen region of the background gives reflectivity contrast $1 + \Delta R/R = 1.08 \pm 0.28$. The value of $1.08 \pm 0.28$ is determined by averaging a reference region in a corner of the substrate whose value is defined as unity and computing the standard deviation of the average ($\sim 10\%$) and applying a similar procedure to a region of the bare spot. Taking two standard deviation changes in the average as a measure of what we could realistically detect, we would need reflectivity contrast greater than 1.56 before we could say with confidence that additional adsorption had occurred on the wafer. Since the 0.28 value already contains the errors for both the measured spot and the reference region, it is approximately correct to conclude that regions exhibiting average reflectivities above around 1.56 (i.e. $1 + 0.28*2$) almost certainly have real increases in thickness over the bare substrate as this would be two standard deviations above the expectation for no adsorption. Combining this value with the theory of Figure 3.6 implies minimum detection limits of 1.2 Å of material. The assumption here is that we can confidently detect real topology when it is more than 2
standard deviations about the background reflectivity (i.e. reflectivity on the bare reference spot region). Thus, we need to estimate the standard deviation in the background reflectivity (note: the value of background reflectivity is 1 by definition). The critical assumption here is that the wafer is sufficiently flat that the background reflectivity that would be observed in the target region is approximately the same as is observed in the reference region. This assumption should be supported by data on bare wafers or experiments with two reference regions. This procedure overestimates the error in that we are assuming that the regions are really flat and that the variation measured by the instrument is not genuine topology.

The situation when looking at adsorption on areas other than the background (e.g., streptavidin target on biotin probe spots) is more complex since we also need to allow for spot-to-spot variations in the amount of bound probe. A similar analysis of the biotin spot gives an average reflectivity contrast of 10.5 ± 0.4. Conservatively, we could be confident that target adsorption has occurred if the average reflectivity contrast increased to 15, and this also leads to the conclusion that we could detect ~1Å binding of streptavidin. One nice consistency check on the validity of this approach to estimating the minimum thickness we could detect is that the two distinct experimental spots where we applied streptavidin have consistent reflectivity contrasts, measuring 45.3 ± 2.2 and 44.7 ± 2.8, respectively.

Biomolecular detection sensitivities are frequently reported in units of picograms per milliliter, and it is interesting to consider the potential detection sensitivity in these units when 1.2Å (120 pm) average adsorption is observable. We assume here that binding constants are high so that analyte present in the fluid will bind to the surface. Taking a 1mm spot, typical for spots sizes made by application of 1μL quantities of solutions for
probe attachment, the volume occupied by adsorbates (1\text{mm} \times 1\text{mm} \times 120\text{pm}) would be \(\sim 1.2 \times 10^{-12}\text{cm}^3\). With a density of 0.7 g/cm\(^3\), this would mean 0.8 pg of adsorbed material can be detected. Assuming that 1\(\mu\text{L}\) of analyte is applied, the sensitivity of sc-NNRI would translate to 0.32 pg/mL. We prefer to quote the sensitivity in terms of detectable average adsorbate thickness since this makes no assumptions about spot geometries or analyte volumes and is therefore directly comparable to theory and SPR.

3.4 Supplementary Data and Theory

3.4.1 Effects of Finite Bandwidth and Angular Divergence in the Probe Light

Practical limitations such as finite spectral bandwidth, angular divergence and spatial inhomogeneity of the interference layer thickness are easily added to the theory. We assume distributions with equal probability over the specified width in each case to compute the effects of each of these on reflectivity contrast. We also model SPR, choosing an implementation of SPR from the literature for reference (Figure 3.7) and optimizing the parameters for best contrast. For SPR, we assume ideal conditions, meaning zero spectral bandwidth and no divergence for the light source, no roughness for the gold surface and no chromium or titanium adhesion layer.
Figure 3.7 Geometry used for theoretical modeling of surface plasmon resonance for comparison with RI. $^{56}$

Figure 3.8 Modeling of RI contrast degradation with probe light spectral bandwidths and angular divergence. (a) Modeling of RI contrast degradation with probe light spectral bandwidths of 0.5 nm (solid squares) and 1.0 nm (open circles) with zero angular divergence. (b) Modeling of RI contrast degradation with probe light angular divergence of 0.05° (solid squares) and 0.1° (open circles) with zero probe bandwidth. Triangles plot the contrast for an equal amount of adsorbed target in SPR assuming a perfectly collimated and monochromatic source. Note that the reflectivity contrast for ideal RI is far off scale in each case. The structure and refractive indices for RI calculations are those of Figure 3.2 of the main text with 632.8 nm incident probe light while it is shown
in Figure 3.7 for SPR. The interference layer thickness is assumed to be homogeneous and exactly matched for the incident wavelength.

Figure 3.8 plots the reflectivity contrast versus thickness of bound target where we assume that the coating thickness with no adsorbed target has been adjusted to the destructive interference minimum for RI. The theory shows that small amounts of spectral bandwidth (Figure 3.8a) or angular divergence (Figure 3.8b) in the light source used to probe reflectivity degrade the contrast significantly from what would be predicted based on Figure 3.2 of the main text. Nevertheless, under the conditions of our experiment where the HeNe laser bandwidth is very small and the angular divergence is only 0.03°, the theory predicts reflectivity increases of more than three orders of magnitude for 1nm of target deposition when the substrate overlayer thickness is uniform and perfectly adjusted. In contrast, SPR even with a monochromatic and collimated source is predicted to be several orders of magnitude less sensitive. Note that we have also omitted the layer of Cr or Ti that is typically used to promote adhesion of Au in the SPR model. In practice, that thin layer is a significant limitation on SPR sensitivity.

3.4.2 Effects of Non-ideal Thickness and Inhomogeneity in the Interference Layer

For sc-NNRI, we must also consider the accuracy and the spatial homogeneity of the coating thickness on the length scale of the instrument resolution. Figures 3.9a, b illustrate the effects of these variables on the reflectivity contrast curves as a function of the amount of target deposited. For the purposes of isolating the relevant variables, we have taken the angular divergence and spectral bandwidth to be ideal. Introducing
inhomogeneity in the effective coating thickness degrades the reflectivity contrast significantly. As seen in Figure 3.9a, about 0.5nm inhomogeneity in the oxide layer reduces the contrast by as much as 5 orders of magnitude from the ideal case represented in Figure 3.2 of the main text and is a much greater practical limitation than collimation (cf. Figure 3.8b). Further increases in inhomogeneity reduce the contrast for RI even more but the results are still superior to those for ideal SPR with even as much as 2 nm inhomogeneity in the coating thickness. Several other features of importance for design of sc-NNRI experiments are evident from the modeling. Achieving nearly the exact coating thickness remains very important to the sensitivity but becomes less so as the interference layer becomes more non-uniform. This makes sense since one is averaging the optical properties over a variety of thicknesses when one introduces inhomogeneity.

Figure 3.9 Modeling of RI contrast degradation with interference layer inhomogeneity and interference layer thickness imperfectly matched to the probe wavelength. (a) Modeling of RI contrast degradation with interference layer inhomogeneity of 0.5nm (solid squares), 1.0nm (open circles) and 2.0nm (inverted open triangles). Exact thickness, zero probe angular divergence and bandwidth are assumed. (b) Modeling of RI contrast degradation with interference layer thickness imperfectly matched to the probe wavelength. The thickness is varied between optimal for destructive interference (solid
squares), 0.5nm too thick (open circles) and 0.5nm too thin (inverted open triangles). Perfect layer inhomogeneity, zero probe angular divergence and bandwidth are assumed. In each case, triangles plot the contrast for an equal amount of adsorbed target in SPR assuming a perfectly collimated and monochromatic source. Note that the reflectivity contrast for ideal RI is far off scale in each case. The structure and refractive indexes for RI calculations are those of Figure 3.2 of the main text with 632.8 nm incident probe light while it is shown in Figure 3.8 for SPR.

One can also see that erring on the side of making the coating too thick is superior and, for large inhomogeneity, coatings that are too thick can even become superior. The reason for this is that coatings that are too thin produce the opposite sign effect in $\Delta R/R$. In other words, increasing thickness due to target binding will result in reduced rather than increased reflectivity when the coating thickness is thinner than optimal. This can be seen clearly in the case of Figure 3.9b where, for target thicknesses less than ~ 1 nm, the contrast is predicted to decrease for the case where the coating is too thin as the target thickness increases. As the inhomogeneity increases, one is averaging in regions of the sample where this is the case so that increasing target thickness does not uniformly produce increasing reflectivity. Hence, thicker coatings where none of the regions are producing effects of the opposite sign become better as inhomogeneity is increased. These are important design considerations since achieving low inhomogeneity and good control over absolute coating thickness turn out to be the practical limitations of sc-NNRI.
3.5 Conclusions

We have developed and refined a simple method for measurement of molecular binding at interfaces using reflective interferometry where only a single-color probe source is required. We have implemented the approach using thermal oxides on silicon since they are very flat and easy to obtain. Silicon also has the advantage of being very weakly absorbing in the visible below its direct band gap so that near perfect destructive interference can be obtained. At the same time, silicon is opaque to visible light so that complications due to backside substrate reflections are avoided. Because the thickness of the antireflection coating is critical when the excitation source is not tunable, we have shown that augmenting the thickness using layer-by-layer electrostatic assembly of polyelectrolytes can be done to make the interference coating thickness nearly ideal for a given probe wavelength. Moreover, poly (acrylic acid) presents useful carboxyl groups that can be used to functionalize the surface with molecularly specific probes such as biotin through condensation chemistry. Alternate approaches to tailoring coating thickness such as controlled HF etching of the oxide are also viable.58

Using a laser to probe the surface allows for highly collimated and monochromatic light so that high sensitivity can be achieved. In fact, we are able to see increases in reflectivity of 2 orders of magnitude due to binding of a 3nm layer of biomolecules on the surface. This contrast is much better than what can be done with surface plasmon resonance. Our present minimum detectable level of adsorption is less than 0.12nm, which, for perspective, would correspond to roughly 2% monolayer of a typical 20-base-long DNA oligonucleotide. Further improvements seem likely with flatter oxide coatings on the silicon wafers. Theoretically RI has an enormous advantage over SPR in dynamic range and sensitivity due to the depth of the reflectivity minimum near
conditions of perfect destructive interference. While practical sc-NNRI cannot approach the theoretical limit, it nevertheless promises to be at least 1 order of magnitude more sensitive than SPR although sc-NNRI is simpler to implement.

We have shown that the amount of adsorption can be determined semiquantitatively once three experimental parameters are measured, the angular divergence of the laser, the inhomogeneity of the coating thickness, and the absolute coating thickness. The first is measured directly, the second by polarized reflectivity, and the third by ellipsometry. Using these as inputs to a transfer matrix theory of the reflectivity, the amount of material deposited inferred from reflectivity contrast agreed well with what was measured independently using ws-RI.

While many detection chemistries are sufficiently robust to withstand rinsing and drying, adaptation of sc-NNRI to working at interfaces in aqueous environments is of obvious importance for biomolecular sensing. Theoretical results for model geometries under water are very close to those under air. Physically, the close correspondence is easy to understand because the degree of destructive interference depends primarily on the path length difference between waves reflected at the interface between air or water and the oxide and waves reflected at the oxide/silicon interface. The main difference in the case of water is that the incidence angle for the light onto the substrate must be steeper to maintain balance in magnitude between the reflections from those two interfaces. The modification to probe under aqueous media is straightforward to implement and has been discussed previously.\(^{54}\)
Chapter 4*

Label-Free Sensing of Binding to Microarrays Using Brewster Angle Straddle Interferometry

4.1 Introduction

Fluorescent and chemiluminescent microarray formats for specific detection of biomolecules have played a central role in screening and discovery efforts in genomics and proteomics. Even so, their application has been limited due to the complexity and expense of labeling the targets of interest as well as lengthy readout protocols. In spite of lower sensitivity than fluorescent detection, label-free approaches such as imaging versions of surface plasmon resonance (SPR), ellipsometry, and interferometry are therefore still widely practiced. Our group and another recently reported imaging biomolecular arrays with a simple interferometric approach we called arrayed imaging reflectometry (AIR, the same as sc-NNRI) that improves upon SPR in sensitivity, exhibiting reflectivity changes of more than a factor of 10 upon binding of ~0.3 nm of biomolecules. Sc-NNRI exploits interference between reflections from the air/oxide and oxide/silicon interfaces of a silicon wafer with an ~150 nm thermally grown oxide.

* Blanket permission is extended by ACS to include my article Anal. Chem. 2007, 79, 7589 in this chapter.
Judicious choice of the incident angle and wavelength can be used with s-polarized probe light to obtain near complete destructive interference that is removed upon target binding. Unfortunately, meeting this condition requires precise control over either the probe wavelength or the oxide thickness. Expensive tunable sources with narrow bandwidth or exquisite control at the Angstrom level over the oxide thickness and homogeneity are required to achieve optimal sensitivity. Sc-NNRI also requires excellent source collimation so that the laser readout is preferable, but imaging with coherent sources introduces substantial spatial variation of intensity even in the absence of topological features on the microarray surface. Finally, while using reflection from functionalized thermally grown oxides on silicon is an extremely convenient format, near grazing incidence probing is required for detection underwater using sc-NNRI. Monitoring binding chemistry in aqueous media is essential for many important biomolecular binding pairs and for kinetic studies but has not yet been realized with sc-NNRI.

Here we report a similar reflective interferometric approach that alleviates the problems cited above with sc-NNRI and document an experimental implementation with comparable sensitivity. The principle is similar except that we exploit the fact that reflected light in p-polarization undergoes a 180° phase shift at incident angles above the Brewster angle while it experiences no phase shift at incident angles below the Brewster angle.69 We are able to choose conditions where reflection at the water/oxide interface of a silicon wafer is above its Brewster angle and the companion reflection at the oxide/silicon interface is below its Brewster angle. In this circumstance, when the reflection coefficients have equal magnitude, near-perfect destructive interference is predicted when the oxide coating approaches zero thickness. Straddling the Brewster
angles of the two interfaces enables us to utilize the robust and uniform thin native oxide on silicon as an approximate antireflection coating for p-polarized light. While this is less sensitive in principle than standard reflective interferometry, its sensitivity is highly tolerant of imperfect source collimation and monochromaticity so that using incoherent sources does not compromise efficacy. No engineering of the oxide thickness is necessary and even broadband light sources such as filtered lamps or light-emitting diodes can be used effectively. Working with p-polarized probe light at oblique incidence also serves to reduce the high reflectivity of the silicon substrate such that more moderate incidence angles at the water/oxide interface are required to achieve destructive interference, and imaging microarrays underwater is more easily achieved than with the thick oxide coatings in sc-NNRI.

In the following discussions, we outline the theory of Brewster angle straddle interferometry (BASI) and we document its sensitivity and its tolerance of angular divergence and spectral bandwidth in the probe light. We demonstrate detection of the protein α-thrombin using surface immobilized DNA aptamers that bind thrombin specifically. The sensitivity as measured in the amount of surface bound target is less than 1 Å, similar to those of SPR and sc-NNRI but with greatly relaxed requirements on the source and substrate design.
4.2 Experimental Section

4.2.1 Materials

Undoped silicon wafers were purchased from University Wafers. Compounds for surface functionalization of the native oxide were bought from a variety of sources, methyltrichlorosilane from Sigma-Aldrich, carboxyethylsilanetriol (sodium salt, 25% in water) from Gelest, \( N \)-hydroxysuccinimide (NHS) from Fluka, \( N \)-(3-dimethylaminopropyl)-\( N \)'-ethylcarbodiimide hydrochloride (EDC) from Sigma, and 4-morpholine ethane sulfonic acid (MES) from Sigma. All were used without further purification. Millipore-filtered water was used for forming all aqueous solutions and for rinsing. The 5’ C6-amino linker modified thrombin-binding DNA aptamer 5’- TTT TTT TTT TTT GGT TGG TGT GGT TGG (probe 1) and a nonbinding control DNA sequence 5’- TTT TTT TTT GGT TAA TGT AAT TGG (probe 2) were purchased from Integrated DNA Technologies. A sequence of nine thymines was appended to the standard thrombin binding aptamer and control sequences to facilitate target binding by inhibiting attraction between the probe and the native oxide surface.  

4.2.2 Probe Attachment Chemistry

Silicon wafers were diced into pieces of 1.3 cm × 2.8 cm dimensions to be used as substrates. These were cleaned for 1 h in boiling piranha solution, freshly prepared by mixing 3 volumes of 30% hydrogen peroxide and 7 volumes of 98% sulfuric acid. The substrates were rinsed repeatedly with water and dried under flowing nitrogen gas. The steps involved in the preparation of the chips are summarized in Figure 4.1. Photoresist
(Shipley 1805) was micropipetted by hand in a pattern of two rows of three spots each, with spacing of ~2 mm between the centers of adjacent spots. Volumes of 0.2 μL were applied to each spot and spread spontaneously to form ~1 mm diameter spots that were left 30 min to dry slowly in a humid environment. A thin, hydrophobic coating was attached covalently in regions unprotected by the photoresist by immersing the substrate in a mixture of 50 μL of methyltrichlorosilane and 10 mL of hexanes for 5 min at room temperature. Subsequently, the substrate was washed by dipping in 20 mL of acetone three times to remove the photoresist and expose the bare oxide surface in the designated spots. These spots were functionalized to present carboxyl groups by placing the patterned substrate into 420 μL of carboxyethylsilanetriol sodium salt (25% in water) and 8 mL of water where it was ultrasonicated for 10 min at room temperature. This was followed by a thorough water rinse, dried under flowing nitrogen, and baked for 30 min in a drying oven at 100°C. The resulting hydrophilic wells were modified according to the scheme described in Figure 4.1. Two wells remained bare and the remaining four wells were modified to attach the relevant DNA oligonucleotide probe sequences by applying 0.2 μL of a mixture of equal volumes of EDC (31 mM) and NHS (7.8 mM) along with 0.2 μL of the relevant probe at 40 μM concentration in 25 mM MES (4-morpholine ethane sulfonic acid) buffer solution (pH 6.5) for 8 h. The substrate was then rinsed with water and dried under flowing nitrogen gas.
4.2.3 Ellipsometric Measurements

In experiments to verify the quantitative nature of BASI, overlayer thicknesses on the silicon substrates were determined by spectroscopic ellipsometry (J. A. Woollam M-2000). We used the silicon oxide refractive index from the literature and assumed that the refractive indices of adsorbed materials were the same as that of SiO$_2$ for modeling the ellipsometric data.
4.2.4 BASI Measurement

The reflectivity of a collimated beam from an arc lamp transmitted through either a 650 nm bandpass or 660 nm long pass filter was measured for interferometric detection. The beam was collimated by two pinholes separated by a distance of 75 cm whose size could be varied to control angular divergence. The substrate was put on a Teflon sample stand and immobilized in a chamber formed by a sloping PDMS gasket around the edges and a right angle prism on top as illustrated in Figure 4.2a. Analyte could flow into and out of the chamber via ports in the Teflon substrate.

Figure 4.2 Flow cell used for BASI measurement, expanded view of the silicon substrate used for detection and model substrate used for analytical computations. (a) Flow cell used for BASI measurement. A PDMS gasket (shown as striped regions) with one side thicker than the other is placed on a Teflon surface surrounding the silicon substrate (solid black rectangle) while a prism (clear triangle) is used both to confine the liquid and to couple light into the water at an angle suitable for BASI. The incident light and reflection from the substrate are sketched as well as the reflection from the prism/water interface which is blocked by a beam stop. (b) Expanded view of the silicon substrate
used for detection. (c) Model substrate used for analytical computations in the discussion section. The layer thicknesses are not to scale.

The prism was used to couple light into the cell so that incidence angles of ~71° at the interface between the water and the substrate can be realized experimentally. Nearly complete destructive interference is predicted, and the ratio of p-polarized to s-polarized reflection we observe is consistent with theory. The asymmetry in the PDMS gasket allowed reflections from the substrate and from the prism/water interface to be spatially separated so that the former could be measured exclusively. Water or analyte solution was injected from the bottom of the sample stand to fill the sample chamber whose volume was approximately 0.8 mL. The sample cell mount was rotated to achieve an angle of incidence that minimized the substrate reflectivity with a polarizer in the beam path to enforce p-polarization. The reflected image was detected after a 0.5× Focal Reducer (Orion Telescopes and Binoculars) by an inexpensive CCD typically used for amateur astronomy, the Orion Starshot Deep Space Color Imaging Camera (Orion Telescopes and Binoculars). The CCD chip size is 8mm diagonal and the array is 752 × 582 in pixel and 8.6 × 8.3 in micron pixel. The Focal Reducer increases the imaging area by a factor of 4. The images were read by MATLAB software for display and analysis.

4.2.5 Target Binding Experiments

After probe attachment, the functionalized substrate was placed into the flow cell described above. The layer structure of the substrate is illustrated in Figure 4.2b and was used for the optical modeling. A syringe was used to inject ~1 mL of water into the flow cell, and a baseline image of the array is recorded prior to injection of the analyte.
Subsequently, the water is displaced by injection of 1 mL of thrombin (26 nM in 100 mM sodium chloride, 10 mM magnesium chloride, 10 mM sodium phosphate buffer with pH 7.14) solution. After 15 min, another image was recorded and then 1 mL of water was injected into the flow cell to help remove nonspecifically bound target. We used water for rinsing instead of the buffer, because the binding affinity between thrombin and TBA is so high that rinsing with buffer could hardly get rid of non-specific binding in a reasonable short period of time. After a second 15 min period, a final image was taken and analyzed for thrombin binding.

4.3 Results and Discussion

4.3.1 Approximate Analytical Model

The reflection $R$ from the structure shown in Figure 4.2b, c is given by a simple analytic expression,\(^69\)

$$R = \frac{\left|\left[r_{12} + r_{23} e^{-2i\beta}\right] / \left[1 + r_{12}r_{23} e^{-2i\beta}\right]\right|^2}{\text{Equation 4.1}}$$

where $r_{jk} = (n_j \cos \theta_k - n_k \cos \theta_j) / (n_j \cos \theta_k + n_k \cos \theta_j)$ are the Fresnel reflection coefficients for TM (p-polarized) light at the interface between layer j and k. $n_j$ and $n_k$ are the complex refractive indexes of the various layers while $\theta_j$ and $\theta_k$ are the propagation angles in the layers. We are using $i = \sqrt{-1}$ and $\beta = (2\pi/\lambda) n_2 d \cos \theta_2$, $\lambda$ is the incident wavelength and $d$ is the thickness of the interference coating (layer 2 in Figure 4.2c).

Our previous work on s-polarized reflective interferometry exploits the near null reflection that can be achieved by destructive interference when $r_{12} \approx r_{23}$ and $2\beta \approx \pi/2$.\(^54\)

It can be seen that zero reflection will also result when $2\beta \approx m\pi$ ($m = 0, 1, 2 \ldots$) and $r_{12} \approx$
For judicious choice of angle, it is possible to realize $r_{12} \approx -r_{23}$ for TM (p-polarized) light as long as $n_1 < n_2 < n_3$ or $n_1 > n_2 > n_3$. Physically, the reason is that there is a $180^\circ$ phase flip in the reflected electric field for p-polarized light when the incidence angle is above the Brewster angle and no phase shift for angles below the Brewster angle. When the refractive indexes are ordered as above, it is always possible to choose an incidence angle above the Brewster angle for one interface and below it for the other so that the signs of the reflection coefficients are opposite and equal. This underlying principle is the reason we have termed this method “Brewster angle straddle interferometry”. The additional criterion that $2\beta \approx m\lambda$ can be met just as in sc-NNRI using thermally grown oxides on silicon with precisely tailored thickness ($d \approx m\lambda/2n_2\cos\theta_2$). The case of particular interest here, however, is that where $m = 0$ so that we can attempt to satisfy $2\beta \approx 0$ with an interference layer comprised of the native oxide on silicon ($d_{\text{native}} \approx 2 \text{ nm} << \lambda$). While the finite oxide thickness introduces a small phase shift between reflections from the two interfaces that prevents near-perfect destructive interference, the practical advantages of using the native oxide as an interference layer are overwhelming and the sacrifices in sensitivity are small, as is illustrated in Chapter 2. The reflectivity minimum is parabolic with $d$,

$$R_{\text{min}}(d)/R_{\text{min}}(d_{\text{oxide}}) = (d/d_{\text{oxide}})^2 \quad \text{Equation 4.2}$$

We can infer the adsorbate thickness from its reflection intensity relative to that of the oxide (or other base layer) whose thickness is known. With the use of the native oxide on silicon (~2 nm) underwater, the absolute reflectivities for the substrate at a minimum angle are ~$10^{-5}$ so that BASI has a very large dynamic range. Comparable numbers for typical SPR are $10^{-34}$ and, while sc-NNRI can theoretically achieve $R_{\text{min}} < 10^{-9}$, in practice it is difficult to achieve even $10^{-5}$. 
4.3.2 Transfer Matrix Modeling of BASI

When molecular probes and targets attach at the water/oxide interface, the analytic model and expression are no longer valid. We can still rigorously model the optical properties of the structure in Figure 4.2b with a standard transfer matrix formalism accounting for a fourth layer between the water and oxide. The refractive indexes of the silicon and silicon oxide are taken from the literature while all of the organic components are taken to have identical refractive indexes. The figure of merit for target detection is the reflectivity contrast \( R_{\text{after}} / R_{\text{before}} \) = 1 + \( \Delta R/R \) where \( R \) is the reflectivity before exposure to the analyte and \( \Delta R \) is the observed increase in reflectivity with target binding. The theoretical reflectivity contrast for different target layer thicknesses is plotted in Figure 4.3 where we assume for simplicity a 2 nm thick native oxide and a probe layer 1 nm thick with a refractive index the same as that of the target. The analytical approximation assuming all materials have the index of SiO\(_2\) (i.e., Equation 4.6) is also plotted in the figure for reference, and analogous data for SPR and sc-NNRI are also shown. In each case, we have assumed "ideal" conditions where the layers are completely uniform in thickness and the light source is perfectly collimated and monochromatic. In the case of SPR, we have also assumed an ideal case where a gold film can be deposited with no Cr or Ti adhesion layer.
Figure 4.3 Reflectivity contrast \( 1 + \Delta R/R \) versus target thickness for sc-NNRI and SPR compared with both the analytic and transfer matrix theory for BASI. The calculations for sc-NNRI and SPR and the comparable BASI calculations assume probe and target refractive indexes of 1.55. Note that the value of theoretical reflectivity contrast for sc-NNRI has been reduced by over 4 orders of magnitude so that it can be presented on the same scale. The analytic approximation for BASI reflectivity is based on a three level model and therefore must assume probe and target refractive indexes equal to those for SiO\(_2\) (1.457). A full transfer matrix treatment where the same assumption is made is shown for comparison.

The data of Figure 4.3 show that the analytic model discussed above overestimates the reflectivity change on target binding. Neglecting the imaginary (absorptive) part of the silicon refractive index allows better destructive interference than is actually possible so that \( 1 + \Delta R/R \) is overestimated by \( \sim 30\% \). One can also see that deposition of materials with a refractive index typical of biomolecules (greater than that of SiO\(_2\)) increases the sensitivity considerably. Finally, the model predicts BASI to have sensitivity nearly the same as that of SPR for several nanometers average target binding while both are dramatically worse than the ideal case of sc-NNRI. In practice, however, the additional sensitivity predicted to be obtainable from sc-NNRI cannot be realized due
to inevitable layer thickness nonuniformity. We turn our attention to modeling non-ideal implementations since this is central to a critical evaluation of these approaches to label-free biomolecular sensing.

### 4.3.3 Reduction in Sensitivity Due to Divergence and Bandwidth of Probe Source

With the use of the transfer matrix formalism and model of Figure 4.2b, it is straightforward to simulate the degradation in reflectivity contrast \( 1 + \frac{\Delta R}{R} \) due to angular divergence or non-monochromaticity of the light used to read the arrays. Figure 4.4a, b illustrates the variation of \( 1 + \frac{\Delta R}{R} \) for a case where a 1 nm layer of probe molecules \((n = 1.55)\) is present and a 1 nm average thickness of target \((n = 1.55)\) binds to them.

![Figure 4.4](image.png)

**Figure 4.4** Theoretical reflectivity contrast of sc-NNRI, SPR, and BASI with 1 nm probe thickness and 1 nm target deposition for imperfect probe collimation. (a) and monochromaticity (b). For sc-NNRI, it is assumed that the near perfect destructive interference condition is met when the probe is on the substrate. Note that the reflectivity
contrast for sc-NNRI with zero bandwidth and zero angular divergence is off-scale, approximately $2.2 \times 10^4$ in this case.

Using sources with significant spectral bandwidth leads to a heavy penalty for sc-NNRI such that the large theoretical sensitivity advantage is lost for sources with >10 nm bandwidth. Implementations using lasers$^{41, 57-58}$ or narrowband filtering of broadband sources$^{54}$ are required to preserve the superiority of sc-NNRI while spectral requirements for the other techniques can be satisfied with light-emitting diodes (LEDs). However, the constraints on collimation render the use of LEDs difficult for SPR. The fact that BASI is also highly tolerant of angular divergence is an enormous advantage that allows it to be implemented in a more compact fashion with much less sophisticated sources and optics. For example, when using a source with 10 mrad (0.6°) divergence, BASI is predicted to be an order of magnitude more sensitive than SPR. If one requires a beam of 1cm diameter to image an array, 10 mrad corresponds to placing apertures separated by 1 meter to enforce adequate collimation. To the extent that these considerations drive one to choose lasers for SPR and sc-NNRI, the imaging problems associated with speckle and fringe patterns introduced by coherent sources further reduce sensitivity.

4.3.4 Reduction in Sensitivity Due to Layer Inhomogeneity

In our previous work, we showed that the primary limitation on sc-NNRI in practice is layer thickness inhomogeneity.$^{41}$ Since thin film structures for the three types of measurements compared in the previous section are considerably different, it is not possible to make direct comparisons analogous to those we made for source nonidealities in Figure 4.4. However, we can still compute how variation of a critical layer in the
structures decreases $1 + \Delta R/R$. For sc-NNRI, we analyze thickness inhomogeneity in the thermal oxide interference layer, for BASI in the native oxide and for SPR in the gold film. What we mean by inhomogeneity is thickness variation on the length scale of a resolution unit in the image, typically around 10 $\mu$m. We model it by averaging over a distribution of thicknesses. It is very difficult to produce ~100 nm thermal oxides with less than 1 nm roughness and, as shown in Figure 4.5a, this severely reduces the sensitivity of sc-NNRI. Our simulations of sc-NNRI also presume that the probe molecules form part of the interference coating such that the exact reflectivity minimum is obtained in the absence of the target. Achieving uniform deposition of probe molecules is also difficult and puts further constraints on sensitivity that were limiting in our prior work.41 In a practical implementation of sc-NNRI, it is necessary to "overshoot" the ideal interference layer thickness so that addition of target cannot cause reflectivity to decrease.41 This further degrades any advantage of sc-NNRI relative to BASI. As can be inferred from Figure 4.5b, c, SPR and BASI are little affected by realistic variation of their respective spacer layers. Given experimentally realistic nonidealities, we have found minimum detectable thickness changes on the substrate to be very similar to the best obtained using sc-NNRI and SPR. However, we think that the convenience of implementation in terms of the simplicity of the substrate and optical probe source weigh heavily in favor of BASI. We turn to experimental documentation of the method.
Figure 4.5 Theoretical modeling of the reflectivity contrast (a) sc-NNRI, (b) SPR, and (c) BASI as the thickness of the layer used for probe molecule attachment is made to have spatially inhomogeneous thickness on a length scale less than the lateral resolution of the measurement. The horizontal scales are in Angstroms and are chosen to be typical for what is realistic with state-of-the-art fabrication techniques. For example, 10-20 Å inhomogeneity is normal for the thermally grown oxides used in sc-NNRI, 5 Å for native oxides used in BASI, and 20 Å for gold films used for SPR. Note the difference in vertical scales and how critical the ability to achieve smooth layers is for sc-NNRI.

4.3.5 Experimental Data on Reflectivity Changes with Thickness

The theoretical predictions of reflectivity contrast can be tested simply with a stepped structure whose thickness is independently measured using spectroscopic ellipsometry. Half of a chip cut from a standard polished and cleaned silicon wafer was covered with photoresist. The chip was dipped in a bath to silanize the uncovered half and the photoresist was then removed by washing with acetone. The silanized half (left of Figure 4.6a, b) was measured by spectroscopic ellipsometry to be 3.38 nm and the bare
Figure 4.6 Experimental assessment of reflectivity contrast for a stepped substrate submerged underwater. (a) Reflected image at incidence angle with minimum reflectivity. (b) Analogous image at an angle 1.28° different. The angle of minimum reflectivity is assumed to be that derived from theory, and the value of 1.28° is derived from measurement external to the flow cell and corrected using Snell's law. (c and d) Horizontal cross-sections of the reflected intensity in bands across the center of images like those in parts a and b. (c) Illustrates the variation of reflected intensity with angle of incidence for comparison with the theoretical degradation of contrast with angular divergence. (d) Documents the reflected intensity using white light with a 650 nm bandpass filter (BP) or a 660 nm long pass filter (LP) for comparison with theoretical predictions of contrast degradation with source bandwidth.

Note that the data have not been corrected for intrinsic intensity variation across the probe beam although it would be straightforward to do if desired. We have measured
the beam intensity profile by inserting a half wave plate and using s-polarized reflection from the same substrate and the correction over the central region of importance to the thickness analysis is less than 10%. There is a clear and reproducible bright feature at the border where photoresist was present during silylation, perhaps due to the hydrophobicity of the silane causing it to form thicker layers near the photoresist polymer.

We assume that we have achieved the optimal alignment angle for BASI by minimizing reflectivity. The ratio of average reflectivity on the silanized plateau compared to the bare plateau is measured to be $1.70 \pm 0.13$ (Figure 4.6c, $0^\circ$ data) or $1.57 \pm 0.13$ (Figure 4.6d, BP data) while theory predicts a value of 1.74 based on the independently measured thicknesses. What we did here was to choose areas on either side of the peak that are in the region without substantial intensity variation (as judged by the s-polarized reflectivity profile) around 150 pixels wide and assume they are actually flat (this overestimates the error since the RI is probably measuring real topology). This gives values for the left side and right side with standard deviations. For example, the signal level in Figure 4.6c is about 3200 on the left and 1800 on the right. We used the actual numbers and computed standard deviations to guess error for each of these values. (e.g. $\sim 3200 \pm 100$ and $\sim 1800 \pm 150$). To estimate the error in the quotient (3200/1800), we used standard error combination (in the case of division, it is adding percent errors in the numerator and denominator to get percent error in the quotient). The error in the quotient is the percent error in the quotient times the quotient, of course. That’s where the $\pm 0.13$ comes from.

The measured ratio $1.70 \pm 0.13$ is in very good agreement with the predicted ratio 1.74, considering the need to average over large areas for our ellipsometric reference measurements. The theoretical simulation in this case assumes that the silane layer index
is equal to that of SiO₂ \( (n = 1.457) \) since this is also what we assume in modeling the ellipsometric data used to estimate thickness. Our conclusion is that one could rely on theoretical comparisons against a known bare oxide thickness to interpret experimental reflectivity in terms of thickness, something very difficult to do in SPR or sc-NNRI.

### 4.3.6 Experimental Data on Sensitivity Degradation with Increasing Divergence and Bandwidth

The same procedure was used for the angular divergence and bandwidth data. We are able to use the same substrate to ascertain empirically how the reflectivity contrast is degraded when we simulate increasing divergence by deliberately varying the substrate angle and recording data like those of Figure 4.6. At the optimal angle, the reflectivity contrast is \( 1 + \frac{\Delta R}{R} = 1.70 \pm 0.13 \) while deviations of 0.55° and 1.28° reduce this to \( 1 + \frac{\Delta R}{R} = 1.38 \pm 0.07 \) and 1.35 ± 0.06, respectively. These reflectivity contrast values and error bars are computed by averaging over the plateau regions assuming that they are completely flat and that we are not actually measuring topology. Therefore, the magnitude of the error is overestimated, probably by a considerable amount. Note that these angle changes are corrected using Snell's law to account for the fact that variation of the incidence angle in air by 1° actually only changes the incidence angle of interest at the water/SiO₂ interface by \( \sim 0.97° \) near the incidence angle of relevance. For the purpose of rough comparison with theory (Figure 4.3a), the values of deviation should be doubled so that they represent FWHM divergences of \( \sim 1.1° \) and 2.56°. The effect of increasing the source bandwidth (BW) can also be simulated by modifications of the spectral filter in the probe light. Figure 4.6d illustrates that there is little difference in contrast when we
change from a white light source filtered by a 10 nm bandpass filter around 650 nm \((1 + \Delta R/R = 1.57 \pm 0.13)\) to the white light source as transmitted through a 660 nm long pass filter \((1 + \Delta R/R = 1.618 \pm 0.08)\) where the effective bandwidth of the light is ~150 nm. This modification would completely destroy contrast in sc-NNRI or SPR (cf. Figure 4.3). The data of Figure 4.6 show that the decrease in reflectivity contrast with both increasing light bandwidth and divergence are in reasonable agreement with the theoretical predictions of a similar geometry in Figure 4.4. These give us good confidence in the validity of the theoretical comparisons with alternative methods which show that BASI can be expected to have sensitivity comparable to sc-NNRI or SPR and perhaps better when implemented using simple optics.

4.3.7 Detection of Thrombin Binding

Figure 4.7 presents data where we apply BASI to detection of thrombin using immobilized thrombin binding aptamer (TBA)\(^{72}\) on a silicon chip. The detailed attachment chemistry and patterning procedures were described in the Experimental Section. TBA (probe 1) was attached to spots 1 and 4, a similar control sequence without the G quadruplex (probe 2) was attached to spots 3 and 6, while spots 2 and 5 remained bare for reference. All measurements are made underwater as described in the Experimental Section. Three intensity images are shown, one before exposure to the 26 nM thrombin solution, one after exposure, and one after a rinse step.

We used the same procedure for thrombin binding data. We made a decision about which pixels represented the various spots (reference, control, and target) and assumed the spots are flat to compute a standard deviation over the spot which substantially overestimates the error. Before thrombin exposure, the probe attachment
spots exhibit $1 + \Delta R/R$ of $2.34 \pm 0.12$ (lower) and $1.74 \pm 0.10$ (upper) for the TBA with $1 + \Delta R/R$ of $2.45 \pm 0.16$ (lower) and $1.62 \pm 0.08$ (upper) for the control sequence, all measured relative to the bare reference spots. Again, these error bars are extremely conservative as the values are standard deviations of the reflectivity over central regions of the spots assuming that they are flat. Figure 4.7, however, clearly indicates that we are actually resolving topology associated with the spot. After thrombin exposure and rinsing, these values become $2.63 \pm 0.16$ (lower) and $1.92 \pm 0.11$ (upper) for the TBA and $2.53 \pm 0.17$ (upper) and $1.73 \pm 0.08$ (lower) for the control. These values confirm the thrombin binding indicated by inspection of Figure 4.7. The native oxide thickness for this substrate as determined by ellipsometry was 2.06 nm. Using the transfer matrix formalism and a refractive index of 1.55 for DNA, we calculate probe spot thicknesses of $10.8 \pm 0.8$ Å and $6.6 \pm 0.8$ Å (TBA spots) and $11.4 \pm 1.0$ Å and $5.6 \pm 0.6$ Å (control spots). Exposure to the 26 nM thrombin solution clearly increases the reflectivity of both sets of probe spots as well as the bare reference spot, but the control and bare spot reflectivities return nearly to their initial values after replacing the thrombin with water so that nonspecifically bound thrombin is removed from the substrate. After the rinse, peak changes $1 + \Delta R/R$ of $2.63 \pm 0.16$ and $1.92 \pm 0.11$ (TBA spots) and $2.53 \pm 0.17$ and $1.73 \pm 0.08$ (control spots) relative to the bare reference spots are observed. Using the transfer matrix formalism and a refractive index of 1.55 for both DNA and thrombin, we calculate spot thicknesses after thrombin binding of $12.6 \pm 1.0$ Å and $7.8 \pm 0.8$ Å (TBA spots) and $12.0 \pm 1.0$ Å and $6.4 \pm 0.6$ Å (control spots). Combined with the measurements with probe only, we infer thrombin binding of 1.8 and 1.2 Å on the TBA spots and 0.6 and 0.8 Å on the control spots. We conclude that we are able to detect 26 nM thrombin with a
signal-to-noise ratio of \(~3\), limited in this case by uncertainty due to nonspecific binding as evidenced by thickening of the control spot. Again, the error is greatly overestimated (much worse than for the flat regions above since these are clearly not flat spots), which is why there is obvious target signal to the eye in the figure but the computation suggests it is not clear whether there is target binding above the noise level. If we use the bare spot where reflectivity changes due to nonspecific adsorption of thrombin were within the noise as a reference, the signal-to-noise ratio for thrombin detection would be greater than 20. This is comparable to sensitivities in unamplified SPR\textsuperscript{73} and electrochemical methods\textsuperscript{74-76} using similar concentrations of target. Improved sensitivity as obtained with labeled reagents and enzymatic amplification\textsuperscript{77} could also be applied to further improve detection.
Figure 4.7 Detection of 26 nM thrombin solution. Images of reflectivity in the top row are from a single wafer. Left to right they are images prior to injection of thrombin solution, 15 min after injection of thrombin, and 15 min following injection of water to flush the thrombin. The plot at the bottom right is a cross-section of the reflected intensity in a horizontal band across the bottom row of spots. The schematic at the bottom left indicates that the left spots are functionalized with thrombin binding aptamer, the middle spots are the bare silane layer, and the right spots are functionalized with a control oligonucleotide sequence that does not specifically bind thrombin. Note that elliptical spots on the CCD with vertical elongation by a factor of $\sim \tan 70^\circ$ correspond to round spots on the substrate due to the oblique incidence of the probe light on the substrate.

4.3.8 Evaluation of Detection Sensitivity

The limit on the detection sensitivity of thrombin in the experiment of Figure 4.7 was placed by the nonspecific binding on the control spot. With an ideal control probe that elicited no nonspecific binding, it is useful to estimate the limit that would have been
placed by the BASI technique as implemented here. In that case, we can ask what the smallest thickness change observable would have been. On the basis of the reproducibility of the reference spot reflectivity, we can detect reflectivity changes $\Delta R/R$ greater than 3% with confidence (i.e., 3:1 signal-to-noise ratio). Because of the parabolic scaling of reflectivity with thickness (Equation 4.2), this corresponds approximately to changes of 1.5% in $\Delta d/d$ ($\delta R/R = 2 \times \delta d/d$) where $d$ is the thickness of the probe spot prior to target exposure and $\Delta d$ the increase in thickness. For a 4 nm probe spot height (e.g., a monolayer of DNA oligomer like TBA), this implies sensitivity to changes in thickness as small as 0.06 nm (0.6 Å). Note that this simple relationship allows quantitative determination of the amount of material deposited without complex calibration as would be necessary for sc-NNRI or SPR. For 1 mm spot diameters and material density of 0.7 g/cm$^3$, this corresponds to 32 pg adsorbed (~40 pg/mm$^2$) which is comparable to what is reported for SPR.

### 4.4 Conclusions

We have developed a simple interferometric method to detect binding of label-free molecules on arrays of probes capable of specific recognition chemistry. Our approach is designated BASI since we exploit the fact that reflected light undergoes a 180° phase shift for incidence angles above the Brewster angle for a first interface but no phase shift at a second interface where it is incident below the Brewster angle. This property enables us to use the very thin and flat native oxide on silicon as an interference layer, providing a very convenient substrate design. We find theoretically that the measurement configuration we use is particularly insensitive to probe light bandwidth
and collimation. These features give practical implementations of BASI considerable advantages over sc-NNRI and SPR.

We have implemented BASI for underwater detection of biomolecular binding at an interface using a prism coupling scheme to inject light into a suitably designed flow cell. The observed reflectivity changes with adsorbate thickness agree well with theoretical expectations. Our apparatus is sensitive to average thickness changes of less than 1 Å and it is straightforward to quantitate these using the native oxide thickness on the same silicon substrate as a reference. As an example, we have demonstrated detection of unlabeled thrombin using a DNA oligonucleotide aptamer immobilized in an array on a silicon wafer. Detection sensitivities were comparable to those obtainable by SPR and by standard enzymatic amplification methods (ELISA). We believe that the combination of high sensitivity, extremely simple implementation, and the ability to infer coverage quantitatively make BASI an excellent choice for array detection of unlabeled biomolecules.
Chapter 5

Secondary Structure Sensing of a 47-mer Fragment of Mutant Ha-ras mRNA Using Under Water BASI

5.1 Introduction

Antisense molecules are molecules interacting with complementary strands of nucleic acids to modify expression of genes. Cells naturally produce antisense nucleic acids that interact with complementary mRNA molecules and inhibit their expression. They have been used experimentally and clinically to bind with mRNA and prevent expression of specific genes. For example, in the United States, the Food and Drug Administration (FDA) has approved a phosphorothioate antisense oligo, fomivirsen (Vitravene), for human therapeutic use.

For an antisense sequence to be effective, the strand on the mRNA has to be available for hybridization in both sequence and structure. However, the mRNAs always have secondary or tertiary structures, which have been shown to affect the binding affinity between the antisense sequences and mRNA.\(^78\) This effect was further studied by Freier\(^79,80\) and other groups.\(^81,82,83,84\) They used traditional methods radioactive labeling and gel retardation to determine the thermodynamic and kinetic parameters, which provided solid evidence of the relationship between the affinity and mRNA secondary structure.
Here, we demonstrate in situ sensing of an RNA transcript corresponding to residues +18 to +64 of activated Ha-ras mRNA on an antisense nucleic acid chip with high sensitivity. The two reasons that we were interested in sensing of the binding between immobilized probes and this target were discussed by Freier’s group. First, the region of Ha-ras mRNA was predicted by RNA folding algorithms to be folded into a stable hairpin structure that would likely be the structure most frequently associated with an antisense oligonucleotide target site. Second, this 47-mer fragment of Ha-ras mRNA contains the site of a point mutation, codon 12, thought to be responsible for the transforming activity of mutant Ha-ras and therefore could be useful for antisense therapeutics. The sensing method we use is underwater Brewster Angle Straddle Interferometry (uw-BASI). We chose the same six probes, allowing us the ability to compare to Freier’s studies. All of the six antisense sequences are paired with different sites on the 47-mer fragment of mutant Ha-ras mRNA. Three of them are complementary to parts of the loop, two are complementary to parts of the stem, and one of them is complementary to a sequence spanning the loop and stem. The probe sequences were modified with a 2'-O-methyl group, which was demonstrated to increase the binding affinity when hybridizing with their complementary strands compared with either DNAs or RNAs, since 2’-O-methyl nucleic acids show steric blocking effects and help inhibit gene expression.

Using uw-BASI as the analytical method, neither the mRNA hairpin nor the six antisense sequences need to be labeled, since the detection signals result from the quantity of target bound on the surface instead of from the label tagged on the target. The measurements were taken while the binding reactions happened on the sample stand of the instrumental system, which avoids any interruption from moving the substrate and
demonstrates the ability of our method to study reaction kinetics. The experimental results were shown to be consistent with previous studies\textsuperscript{88} that suggest the importance of adding a spacer in the designed antisense sequences when attaching them on the surface to ensure the mobility and accessibility of the antisense sequences for hybridization.

5.2 Experimental Section

5.2.1 Materials

Nominally undoped silicon wafers were obtained from University Wafer. Methyltrichlorosilane was purchased from Sigma-Aldrich Inc. Carboxyethylsilanetriol (sodium salt, 25% in water) was purchased from Gelest Inc. N-hydroxysuccinimide (NHS, Fluka) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, Sigma) were used as received. Millipore-filtered water was used for all aqueous solutions and rinsing. The 47-mer fragment of mutant Ha-ras mRNA was purchased from Dharmacon (5’ GGU GGU GGU GGG CGC CGU CGG UGU GGG CAA GAG UGC GCU GAC CAU CC 3’). The 2’-O-methyl-RNA probes with 5’ C6 amino linker were purchased from Integrated DNA Technologies Inc. and their sequences are respectively 5’ CAC CAC CAC C (M3270), 5’ GCG CCC ACC A (M3271), 5’ UUG CCC ACA C (M3283), 5’ CAC UCU UGC C (M3284), 5’ CAC ACC GAC G (M3291), and 5’ CGA CGG CGC C (M3292) where we have adopted Freier’s notation for the probes.\textsuperscript{80}
5.2.2 Surface Attachment Chemistry and Hybridization with the mRNA Hairpin

Silicon wafers were diced into pieces of 1.3 cm × 2.8 cm dimensions to be used as substrates. These were cleaned for one hour in boiling piranha solution, freshly prepared by mixing 3 volumes of 30 % hydrogen peroxide and 7 volumes of 98 % sulfuric acid. The substrates were rinsed repeatedly with water and dried under flowing nitrogen gas. Photoresist (Shipley 1805) was micropipetted by hand in a pattern of 2 rows of 3 spots each, with spacing of ~2 mm between the center of adjacent spots. Shown in Figure 5.1 is the schematic structure of the spots on the chip.

![Figure 5.1](image)

Figure 5.1 Three sets (outlined by the boxed) of probe sequences attached on the chips. On each of the chips is an array of 2×3 spots, in 4 of which were attached 2 different probe sequences. They are respectively M3270 (dark green) on (1,1) (2,1) and M3271 (orange) on (1,3) (2,3), M3283 (purple) on (3,1) (4,1) and M3284 (light green) on (3,3) (4,3), M3291 (blue) on (5,1) (6,1) and M3292 (yellow) on (5,3) (6,3).

Volumes of 0.2 μL photoresist were applied to each spot and spread to form ~1mm diameter dots, which were left 30 min for drying at room temperature. The substrate was soaked in a solution of 25 μL methyltrichlorosilane (Gelest Inc.) and 10 mL hexanes for 5 min at room temperature. Subsequently, the substrate was washed three times in 7 mL acetone each time to remove the photoresist and expose the hydrophobic surface with hydrophilic inner wells. Then the substrate was put into another solution of
420 μL carboxyethylsilanetriol sodium salt (25% in water, Gelest Inc.) and 8 mL water, being ultrasonicated for 10 minutes at room temperature. This was followed by water rinsing, nitrogen drying and a 30-min annealing step at 100 °C in the oven. The selected hydrophilic wells (indicated by color) were modified by applying 0.2 μL of a mixture of equal volumes of EDC (31 mM) and NHS (7.8 mM) along with 0.2 μL 40 μM probes in MES (4-morpholine ethane sulfonic acid) buffer solution (pH 6.5) for 8 hours. The substrate was then rinsed with water and dried under flowing nitrogen gas.

The substrate was placed into the flow cell, fixed by the PDMS gasket and the prism on the top. After that, 1 mL mRNA hairpin (2 μM in buffer of 100 mM sodium, 10 mM magnesium, 10mM phosphate, pH 7.0) was injected into the flow cell at room temperature for hybridization. Measurements were taken after 30 minutes and 8 hours.

### 5.2.3 Ellipsometric Measurements

The oxide thicknesses on the silicon substrates were determined before the deposition steps by spectroscopic ellipsometry (JL Woollam M-2000).

### 5.2.4 uw-BASI Measurement

The instrumental set up of uw-BASI was described in Chapter 4. The reflectivities of a spectrally filtered Arc lamp from the functionalized Si substrate were measured before and after adding target solution into the flow cell.
5.3 Results and Discussion

5.3.1 Secondary Structure Map of the mRNA Hairpin

A structure map of the target, which is a 47-mer fragment of mutant Ha-ras mRNA, is shown in Figure 5.2 as calculated using the modeling software mFold.\textsuperscript{89, 90} It is a hairpin structure with a loop and a stem. Labeled by blue color are the three probes M3270, M3271 and M3292, whose bases are complementary to the corresponding sites on the stem, and part of M3292 is also complementary to the corresponding site on the loop. Labeled by red color are the other three probes M3283, M3284 and M3291, whose bases are complementary to the sites on the loop. Presumably, the blue-color probes will not bind the target, because their complementary sequences have already partially or completely formed a double-stranded conformation. However, the red-color probes should show very strong interaction with the target, since all the bases from the counter sites seem to be structurally available for incoming binding.
Figure 5.2 Structure map of the 47-mer Ha-ras mRNA fragment and the sequences of the six probes. (a) Structure map of the 47-mer fragment corresponding to residues 18-64 of mutant Ha-ras mRNA. (b) The sequences of the six designed probes M3270, M3271, M3283, M3284, M3291, and M3292.

5.3.2 The mRNA Hairpin Detection Results

Three 6-spot chips were made by the procedure described in the experimental section in order to evaluate the 2'-O-methyl-RNA probes in parallel experiments. Probes M3270, M3271 and M3292, have sequences complementary to the sites on the stem of the hairpin structure, but the stem has already paired to form a double strand. Shown in Figure 5.3a and right spots in Figure 5.3c are the detection results implying almost no binding after adding the target solution into the flow cell. This is due to the low binding affinity between the target and M3270, M3271, and M3292. In contrast, probes M3283, M3284 and M3291, are all complementary to sites on the loop of the hairpin structure, which presumably means there is no need for the target structure to open up and expose
the bases for hybridization. We therefore expected high binding affinity for all of the three. However, this turned out not to be the case. Except that M3291 showed clear binding with the Has-RNA target (Figure 5.3c, left spots), we did not see specific binding between the target and M3283 or M3284 (Figure 5.3b).

Figure 5.3 Experimental assessment of reflectivity for the arrays of M3270, M3271, M3283, M3284, M3291, M3292 submerged underwater. The figures of the left two columns are reflected images at incidence angle with minimum reflectivity before and after adding the target solution, for (a) M3270 and M3271, (b) M3283 and M3284, (c) M3291 and M3292. The figures of the right column are respectively the intensity profiles of (a) bottom spots, (b) bottom spots and (c) top spots, averaged within the spot’s Y pixel range. Black and red curves are correspondingly before and after adding the target solution.
From the experimental results, only M3291 showed very clear binding with the mRNA hairpin. However, the fact that there was no difference between the results for M3283 and M3284, is very surprising, since M3283 is supposed to bind with the target and M3284 is not. We therefore considered the possibility that steric restrictions on the immobilized molecules make hybridization inefficient. For example, the probes might bend over and stick to the surface due to the small distance between the surface and the probes, so that they are not accessible for hybridization. Considering this, we designed two new probes with the same sequences of M3283 and M3284 but with a 9T spacer between the 5’ linker and the probe sequences, M3283T and M3284T. Using these probes, the results of measurements analogous to those in Figure 5.3 are shown in Figure 5.4.

Figure 5.4 Experimental assessment of reflectivity for the arrays of M3283T and M3284T submerged underwater. The left two figures are reflected images at incidence angle with minimum reflectivity before and after adding the target solution for M3283T and M3284T. The right figure is the intensity profiles of bottom spots averaged within the spot’s Y pixel range. Black and red curves are correspondingly before and after adding the target solution.

These results confirmed our speculation that steric effects suppressed hybridization without the oligothymine spacers and we observed clear binding between
the incoming target and M3283T. M3283T has much higher intensity change, which means a stronger binding than M3284T. M3284T showed very weak binding, compared with M3284 (Figure 5.3b, right image). This is a result we do not understand.

### 5.3.3 Quantitation of Binding Amount by BASI Sensing

Table 5.1 indicates that probe M3291 was specific for binding with the mRNA hairpin, showing an intensity increase of 33%. M3283T and M3284T also showed some binding with the mRNA hairpin, with intensity increasing 19% and 10% respectively, even though the same sequences M3283 and M3284 without TTT TTT TTT spacers showed little binding (6% and 8%). For probes M3270, M3271 and M3292, the intensity changes were relatively small too, 2%, 6% and 4% respectively. Considering the intensity noise (within the range of $\pm 50$), corresponding to about $\pm 2\%$ of our experimental measurement signals ($\sim 3000$), we conclude that there was no binding between the target and M3270, M3271 and M3292, based on the detection limit of signal to noise ratio $\sim 3$. We also concluded that M3283T and M3291 probes immobilized on our substrates are specific for binding with the target.
Table 5.1 Intensity data at the probe sites using the same procedure described in Chapter 4. The intensity changes were calculated after corrections from the control-well intensity changes from before to after, -49.5 ± 35.8, -23.6 ± 33.8, -30.4 ± 24.8 and 12.2 ± 21.1, for M3270 and M3271, M3283 and M3284, M3283T and M3284T, and M3291 and M3292 respectively.

Quantitative calculations (Equation 4.2) can be done to infer the binding amounts of the target on the different probes. The intensity changes for M3270, M3271, and M3292 are comparable with the error, and those for M3283 and M3284 are lower than for M3283T and M3284T, so the calculated results in Table 5.2 are shown for binding amounts of M3283T, M3284T and M3291. For M3291 binding with the target, the intensity change translates to a thickness increase of 7.5-Angstrom for the target above the 10.6-Angstrom probe over 3.87-nm control well. For M3283T and M3284T binding with the target, the intensity increases correspond to 5.3-Angstrom and 2.6-Angstrom target above the 13.6-Angstrom and 9.1-Angstrom probe spots respectively over 4.43-nm control well.
Table 5.2 Quantitative results calculated by Equation 4.2 to infer the surface thicknesses after immobilizing the probes and binding to the target for M3283T, M3284T and M3291.

<table>
<thead>
<tr>
<th>Probe sequence</th>
<th>M 3283T</th>
<th>M 3284T</th>
<th>M 3291</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness at the control well (nm)</td>
<td>4.43 ± 0.01</td>
<td>4.43 ± 0.01</td>
<td>3.87 ± 0.01</td>
</tr>
<tr>
<td>Thickness at the site before (nm)</td>
<td>5.79 ± 0.065</td>
<td>5.34 ± 0.037</td>
<td>4.93 ± 0.002</td>
</tr>
<tr>
<td>Thickness after adding target (nm)</td>
<td>6.32 ± 0.014</td>
<td>5.60 ± 0.007</td>
<td>5.68 ± 0.015</td>
</tr>
</tbody>
</table>

5.4 Conclusions

We have utilized Brewster Angle Straddle Interferometry for in situ secondary structure sensing of a 47-mer fragment of mutant Ha-ras mRNA at chip-based surface in buffer solution. Simple attachment chemistry was developed to immobilize six 2'-O-methyl-RNA probes on polished silicon wafers functionalized with carboxylic silanes via an amino linker. The results showed that adding a 9T spacer in the probe design helped to improve hybridization and detection sensitivity.

The six designed probes were complementary to different sites on the hairpin structure of the target as modeled by mFold software. Some of the probes (3270, 3271, and 3292) were paired with sites on the stem while others (3283, 3284 and 3291) were paired with sites only on the loop. We observed strong binding of the target to two of the loop probes, M3291 and M3283T. Target binding to M3284T was weaker, while no binding for target-M3270, target-M3271 and target-M3292 was observed. These results are consistent with our expectations and previous results, since it is easier for the hairpin structure to hybridize with complementary strands on the loop than on the stem where the
target sequences have already formed a double-stranded structure. Explaining why different binding is observed for M3284 requires further investigation. Thermodynamic studies in the literature\textsuperscript{80} have demonstrated that it is easiest for 3291 to bind the loop, and it is the hardest for 3284, although all of the three target sites on the loop are single-stranded. It suggested that it requires no conformational change or even provide more favorable conformational change to form the hairpin-3291 hybrid. However, in order to bind with 3283 and 3284, the loop structure comes at a thermodynamic cost, and 3284 has even less favorable effect than 3283. This is possibly why in the parallel measurement M3283T shows higher binding signals than M3284T. The binding amount on the surface can be determined quantitatively, since the value of the reflectivity minimum is parabolic with the layer (oxide plus adsorbates) thickness. Based on this analysis, we are able to see increases in reflectivity of 33%, which is due to binding of 7.5 Angstroms of 47-mer fragment of mutant Ha-ras mRNA with specific probe M3291 on the surface. The limit of detection (LOD) is estimated to be \( \sim 1.5 \) angstrom based on signal to noise ratio \( \sim 3 \).

Chapter 6
Summary

6.1 Conclusions

6.1.1 Development of sc-NNRI

We have developed and refined a simple method for measurement of molecular binding at interfaces using Near-Null Reflective Interferometry where only a single-color probe source is required. Sc-NNRI exploits nearly perfect destructive interference and near-zero reflectivity for s-polarized light from an anti-reflection coated surface. s-reflections from the first and the second surface cancel due to the equal magnitude of the reflections at the two interfaces and a half-wavelength difference of the travel optical distances.

Silicon wafers are used as substrates since they are very flat. Silicon has the advantage of being very weakly absorbing for light in the visible below its direct band gap. At the same time, silicon is opaque to visible light so that complications due to backside substrate reflections are avoided. The oxide layer functions as the anti-reflection coating.

Compared with ws-RI, Sc-NNRI uses a monochromatic laser source with better capability of collimation and LBL-ESA to precisely control the coating thickness. It no longer requires a tunable source to provide a good match to the optical thickness of the interference coating. The bulky and expensive implementation with a lamp and
spectrometer is eliminated, at the same time providing a relatively better defined angle of incidence and narrower spectral bandwidth that improves the sensitivity.

Because we use a HeNe laser as the light source, the thickness of the anti-reflection coating is critical when the excitation source is not tunable. Augmenting the thickness using layer-by-layer electrostatic assembly (LBL-ESA) of polyelectrolytes can be done to achieve the nearly ideal coating thickness (142.6 nm) for the given wavelength (632.8 nm). Moreover, poly (acrylic acid) presents useful carboxyl groups that can be used to functionalize the surface with molecularly specific probes such as biotin or, more generally, amino-derived probe molecules, through condensation chemistry.

Reflectivity images are measured before and after biomolecular binding and the reflectivity contrast is obtained by dividing the intensity of the two images. The sensitivity is highly dependent on the ratio of the detected image (after binding) intensity to the background image (before binding) intensity. Three biotin-attached chips with different coating thicknesses were measured for binding streptavidin molecules. The results confirm the theoretical conclusions that the thickness closest to that predicted by theory provides the highest sensitivity, since it enabled near-zero intensity for the background image.

However, we found several critical disadvantages for sc-NNRI. Given a specific probe wavelength, it is necessary to be highly accurate about the coating thickness. Considerable effort must be devoted to the engineering of the coating thickness. Since the inhomogeneity of the oxide and probes is the limit to sensitivity, we only gain marginally by improving collimation and monochromaticity relative to ws-RI. Moreover, the problems introduced by lacking wavelength tunability (i.e. we need to undershoot the best thickness deliberately so that adding layers brings it to destructive interference) are
very difficult. They further compromise sensitivity and, even worse, really lose the ability to be quantitative and get reproducible results from surface to surface. In the end, the LOD of sc-NNRI in terms of thickness was found to be about the same as for ws-RI. In addition, it is not easy to avoid substantial spatial variation of intensity introduced by the interference fringes characteristic of coherent laser sources if detected by an imaging camera. We had to scan the substrates to solve coherence problems which was clumsy and might make some types of measurements that one ultimately wants to do such as binding kinetics impossible. Also the additional functionalization chemistry is not ideal in the sense that the polyelectrolyte thickness/density is dependent on pH and salt in the analyte and somewhat unstable. Another disadvantage that sc-NNRI encounters is the challenge of being implemented under water, due to the very steep incidence angle required at the water/substrate interface.

6.1.2 Detection of Biotin/Streptavidin Binding

The biotin/streptavidin system is of special interest because it has one of the largest free energies of association observed for non-covalent binding of a protein and small ligand in aqueous solution ($K_{\text{assoc}} = \sim 10^{14}$). The complexes are extremely stable over a wide range of temperature and pH. From sc-NNRI detection, the contrast images indicate that when the substrate thickness is close to what theory predicts to be optimal, the reflectivity contrasts $1 + \Delta R/R_o$ for streptavidin-bound regions ($\sim 3$ nm thick) with respect to the background average $76$ over the spots. For a biotin-only spot ($\sim 0.3$ nm thick), the contrast is $17$, and for the nonspecific binding of streptavidin on polyelectrolytes without biotin, a contrast of $5$ was observed. In order to quantitate our experimental results, we need independent measurements of the amount of biotin and
streptavidin bound to the spots and of the surface inhomogeneity. We used ws-RI since thicknesses are easily extracted and found that the average deposition of biotin on their spots was $3.5 \pm 0.5 \text{ Å}$ and streptavidin plus biotin on their spots was $26.5 \pm 0.5 \text{ Å}$. For sc-NNRI measurements, we assumed that the refractive index of the biotin and streptavidin layers is 1.55, typical for organic materials far from resonance. After measuring inhomogeneity and angular divergence and taking them into consideration, we found the theoretical predictions for the intensity contrasts were quite consistent with experimental results extracted from sc-NNRI measurements.

For limit of detection (LOD), we would need reflectivity contrast greater than 1.56 (see Chapter 3) before we could say with confidence that additional adsorption had occurred on the wafer. Dependent on this value, the theory implies minimum detection limits of $1.2 \text{ Å}$ of material. While making some assumptions and estimating the calculations, the sensitivity of sc-NNRI is 0.32 pg/mL. Miller’s group using sc-NNRI also detected a LOD as low as 10 pM for extracellular domain of intimin. Since we prefer making no assumptions about spot geometries or analyte volumes, we still desire to quote the sensitivity in terms of detectable average adsorbate thickness (Angstroms).

### 6.1.3 Development of BASI

We have also successfully developed another simple method for measurement of molecular binding at interfaces using Brewster Angle Straddle Interferometry (BASI), where \textit{in situ} detection was done under water. BASI exploits the same idea of obtaining destructive interference but for p-polarized light instead of s-polarized light. There is a $180^\circ$ phase flip in the reflected electric field for p-polarized light when the angle of incidence is above the Brewster angle and no phase shift for angles below the Brewster
angle. When we still use silicon wafers as the substrates, it is always possible to choose an incident angle above the Brewster angle for the water/oxide interface and below it for the oxide/silicon interface so that the signs of the reflection coefficients are opposite and the magnitudes are nearly equal.

At the same time, there is no need for a difference of the optical path length between these two reflections, so minimum coating thickness or long wavelength is desired to achieve low reflectivity. Achieving near zero coating thickness is impractical, since the native SiO$_2$ will always grow to about 2 nm thick even if removed. Although the non-zero thickness results in imperfect destructive interference, it is a great advantage that no more surface engineering is required to provide precise thickness control. The error built into “incorrect” oxide thickness makes the measurement configuration we use relatively insensitive to probe light bandwidth and collimation so that using incoherent sources does not appreciably compromise efficacy.

BASI provides a large improvement over previous RI methods. It avoids the necessity for monochromatic sources and critical control of the coating thickness. It also accommodates the in situ measurements under water more conveniently than sc-NNRI, due to the moderate angle of incidence at the water/substrate interface. One more advantage of BASI is its capability of quantitating the binding amount on the surface. Since the value of the reflectivity minimum is parabolic with the coating thickness $d$, the bare oxide reflectivity can be used as a reference to infer topology over the entire chip.

6.1.4 Detection of Aptamer/Thrombin Binding
Apatmers are nucleic acid binding species generated by in vitro selection that can bind specific molecular targets. Aptamers can be RNA or DNA and can be used to bind targets ranging from small molecules to entire organisms. The specificity and affinity of aptamers for their ligands are comparable to those of antibodies for antigens. Compared with antibodies, aptamers are resistant to denaturation and degradation, smaller, less complex and easier to manufacture and modify. The binding between aptamer and protein is based on shape/structure recognition. The human-α-thrombin aptamer has a folded and symmetrical structure consisting of a 15-mer consensus sequence, GGT TGG TGT GGT TGG. The structure can form two stacked G-quartets when interacting with human-α-thrombin.

We measured selective binding of human-α-thrombin in solution to DNA aptamers covalently bound to the oxide. The experimental procedure involved an injection of human-α-thrombin solution, allowing binding for 15 minutes, and another injection of water to flush away unbound thrombin. The experimental results showed clear aptamer/thrombin binding with almost no change at the control spots using the images measured by BASI before and after adding thrombin solution. Using the transfer matrix formalism and a refractive index of 1.55 for both aptamer and thrombin, we calculated spot thicknesses after thrombin binding of $12.6 \pm 1.0$ Å and $7.8 \pm 0.8$ Å (thrombin-binding aptamer spots) and $12.0 \pm 1.0$ Å and $6.4 \pm 0.6$ Å (control spots). Combined with the measurements with probe only, $10.8 \pm 0.8$ Å and $6.6 \pm 0.8$ Å (thrombin-binding aptamer spots) and $11.4 \pm 1.0$ Å and $5.6 \pm 0.6$ Å (control spots), we inferred thrombin binding of 1.8 and 1.2 Å on the aptamer spots and 0.6 and 0.8 Å on the control spots. We concluded that we were able to detect 26 nM human-α-thrombin with a
signal-to-noise ratio of ~3 for the specific thrombin-binding aptamer, limited in this case by uncertainty due to nonspecific binding as evidenced by thickening of the control spot.

The limit on the detection sensitivity of thrombin in the experiment was placed by the nonspecific binding on the control spot. This implies sensitivity to changes in thickness as small as 0.06 nm (0.6 Å). For 1 mm spot diameters and material density of 0.7 g/cm³, this corresponds to 32 pg adsorbed (~40 pg/mm²).

### 6.1.5 Detection of Binding between 2’-O-Methyl-RNA and the mRNA Hairpin

Antisense nucleic acids naturally produced by cells interact with complementary mRNA molecules and inhibit their expression. They have been used experimentally and clinically to bind with mRNA and prevent expression of specific genes. We demonstrated in situ sensing of a 47-mer fragment of mutant Ha-ras mRNA by an antisense nucleic acid array immobilized on a BASI chip with high sensitivity.

The six probe sequences immobilized on the surface are paired with different sites on the mRNA hairpin. Three of them (3270, 3271, and 3292) are paired completely or partially with the sites on the stem while others (3283, 3284 and 3291) are paired with the sites only on the loop. The detection results showed strong target-M3291 and target-M3283T binding on the surface. Target-M3284T binding was relatively weaker, while almost no binding for target-M3270, target-M3271 and target-M3292 were observed. These results are consistent with our expectation, since it is easier for the hairpin structure to hybridize with complementary strands on the loop other than on the stem that has already formed a double-stranded structure. More specifically, thermodynamic studies in literature have demonstrated that it is easiest for 3291 to bind the loop, and it
is the hardest for 3284, although the target sites for 3291, 3283 and 3284 are all on the loop and single-stranded. Our results also agree with these studies and prove that BASI could be used as a powerful tool to study the secondary structure of biomolecules, for example, folded RNAs.

6.2 Future Work

There are many directions where BASI could be used for further studies. In order to improve the utility of BASI biosensing, the stability of the biomolecular sensors manufactured by the methods described in previous chapters needs to be further illustrated. High-throughput patterning of the chips and automation of the instrument are other places to enhance the technology platform.

We have demonstrated application of uw-BASI in studying aptamer/thrombin and 2’-O-methyl-RNA/mRNA bindings. Our future studies will be focused on Dynamic Combinatorial Chemistry (DCC), due to its high potential for in vitro detection of metabolites or for sensing of noxious compounds in the environment.

Of course, besides the application of BASI in biosensing fields, we are going to apply uw-BASI to thermodynamics and kinetics studies of various biological systems, which means, watching the reactions at the same time they are happening. We hope to develop a data collection and analysis system superior to imaging SPR that will provide useful thermodynamic and kinetic parameters for research scientists to further investigate the reaction mechanisms.

6.2.1 Stability, High-throughput Patterning and Automation of BASI
We found while attaching nucleic acid molecules as probes, it is very important to design a spacer at the functional end to ensure the structural availability of the sequences. This observation is in agreement with previous literature. When the probe molecules are not nucleic acids, for example proteins, the orientation is much more important than spacing. In particular, we have to understand where the recognition spot is and bind to the substrate far away from it. A further issue related to stability of biomolecular sensors is reusability, which saves cost and time for preparing a new probe chip. What scientists have done is to break the binding between the probes and targets after use and preserve the activity of probes for next cycle of detection. An example would be dehybridization of nucleic acid duplexes to regenerate the monomer. Repeated using of the probe substrate for multiple detection cycles is worth studying for BASI.

The patterning methods we used in the experiments were μCP and “modified photolithography”. For μCP, we used PDMS stamps as templates to cover the surface and to separate hydrophilic spots by applying a hydrophobic region. Those hydrophilic spots were used to attach the probe molecules. “Modified photolithography” involved depositing positive photoresist in a pattern on the surface. Regions unprotected by photoresist were silanized by a hydrophobic silane reagent, so that similarly to patterning with μCP, we generated a hydrophobic region outside the photoresist spots. Later the photoresist was washed off and the bare oxide was exposed for further attachment of probe molecules. The advantages of these two methods are the ability to divide the surface into two domains, hydrophilic regions for attachment and hydrophobic regions that prevent binding solutions from spreading and cross-contaminating each other.

However, all the experiments were done by hand, which means the efficiency would be a big issue for high-throughput measurements, so we are considering using a
high-capacity spot arrayer in the future. For a spot arrayer, different solutions are held in parallel tips on a movable head at the same time, so it is very efficient for the arrayer to spot a large number of different molecules on the slides at a higher rate. More importantly, we could combine the wettability idea with the spot arrayer instrument and design five chambers to fulfill our goal. The No.1 chamber is used for deposition of a positive photoresist array on the slides. Silylation of hydrophobic molecules on the surface happens in organic solvent contained in the No.2 chamber. The No.3 chamber is an oven used for annealing the surface after silylation. Then the photoresist is washed off by the developer in the No.4 chamber to expose an array of hydrophilic spots. Finally the probe molecules are spotted on them in the No.5 chamber. All the chambers are set at the appropriate conditions for the corresponding procedures, including temperature, humidity, time, solution volumes, sonication intensity and time. All of these ideas of high-throughput patterning could be possible subjects of our future plan.

After we are done with high-throughput patterning, we will focus on the automation of the BASI instrument. This involves automating the flow cell system, angle adjusting system and data collection system. The flow cell will be designed to inject target solutions and wash solutions in appropriate volumes and at appropriate flowing rates. The angle of incidence will be adjusted automatically over a broad range until the reflectivity is minimized. Image videos or continuous intensity raw data would be collected and analyzed in real time. If all of these can be done, uw-BASI would be ready for fast and convenient biomolecular sensing.

6.2.2 BASI Studies of DCC
Synthetic receptors have great potential in a variety of applications, including the \textit{in vivo} detection of metabolites (for example, the sensing of glucose in blood of patients suffering from diabetes) or the sensing of noxious compounds in the environment. Molecular recognition leading to the binding of a guest to a receptor involves a complex interplay of subtle non-covalent interactions. The receptor design approach usually involves lengthy and time-consuming syntheses, since computer design of successful receptors is not presently adequate and that additional experimental screening and variation of rationally designed probes is necessary. One promising approach to the screening for new receptors is Dynamic Combinatorial Chemistry (DCC), since the guest selects its preferred receptor and the guest also drives receptor synthesis. Dynamic combinatorial chemistry (DCC) is a newly developed technique for receptor-aided selection of high-affinity ligands from equilibrating combinatorial libraries. DCC makes use of reversible bond-forming reactions to create thermodynamically controlled dynamic combinatorial libraries (DCLs).\textsuperscript{91} In DCC, simple building blocks are linked together using a reversible reaction to form a mixture of compounds (a dynamic combinatorial library, DCL) that interconvert continuously. The composition of a DCL is under thermodynamic control. This characteristic makes it a very powerful tool for the discovery of new receptors.\textsuperscript{92, 93, 94, 95} If a DCL happens to contain a good receptor for a certain guest, addition of this guest to the library will result in host-guest binding, which will stabilize the host and shift the equilibrium towards this species. In other words, addition of the guest results in amplification of the best host in the mixture.

The size of DCLs employed has been somewhat limited thus far due to analytical challenges inherent in the methodology, for example HPLC and MS, since these require a difference in mass for all library members, as well as the ability to separate individual
library members on a chromatography column. The challenge increases as the library size increases. In future work, we hope to adapt uw-BASI as an analytical tool to enable efficient receptor selection and synthesis by DCC. The basic idea is to immobilize the library monomers on the substrate, then add monomers and the target into the flow system. After the system reaches the equilibrium, spots showing the strongest binding with the target would be the final product of the receptor, which is the specific host for the guest. Since BASI could be high-throughput, it does make a difference when the size of DCL is big. We will collaborate with Prof. Ben Miller’s group on this project.

6.2.3 Thermodynamic and Kinetic Studies

Since uw-BASI is capable of real-time measurements, it could be used to study the thermodynamics and kinetics of various biological systems or that of DCC.

One example would be studying how the binding thermodynamics between nucleic acid sequences are affected by immobilization on a microarray. Since BASI is very good at data analysis and quantitation, we are able to watch binding amounts in real time, so it is not hard to measure melting temperatures for the duplex while we change the environment temperatures. The melting temperature, Tm, is the temperature at which 50% of the oligonucleotide and its perfect complement are in duplex. We could also determine the binding affinities. We may measure the thickness changes at equilibrium versus target concentrations for different duplexes and plot the isotherms. These isotherms are used to calculate the binding affinities between the complementary, mismatched or non-complementary duplexes, and furthermore, infer the thermodynamic parameters including $\Delta G$, $\Delta H$ and $\Delta S$. 
Besides the thermodynamic studies, uw-BASI could also be applied to obtain kinetic rate constants from the signal curves versus time, since we are able to take videos or continuous intensity data at the same time the reaction happens. This could be a reliable experimental reference for analyzing reaction mechanisms.

Of course, many other biological systems or DCLs could be the objects of future thermodynamic or kinetic studies and we only mention nucleic acid hybridization as an example. We are looking forward to rapid development of BASI and its wider application in various fields.
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