2007

Biomolecular sensing with light at nanostructured surfaces

Bipin Kumar Singh
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Chemical Engineering Commons

Recommended Citation
Singh, Bipin Kumar, "Biomolecular sensing with light at nanostructured surfaces" (2007). Retrospective Theses and Dissertations. 15515. https://lib.dr.iastate.edu/rtd/15515

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Biomolecular sensing with light at nanostructured surfaces

by

Bipin Kumar Singh

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
Andrew C. Hillier, Major Professor
Sriram Sundararajan
Brent H. Shanks
Aaron R. Clapp
Patricia A. Thiel

Iowa State University
Ames, Iowa

2007

Copyright © Bipin Kumar Singh, 2007. All rights reserved.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Motivation: Label-Free High Throughput Sensing</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Organization of the Dissertation</td>
<td>6</td>
</tr>
<tr>
<td>1.3 References</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 2. BACKGROUND AND LITERATURE REVIEW</td>
<td>8</td>
</tr>
<tr>
<td>2.1 Array-Based Optical Detection Methods</td>
<td>8</td>
</tr>
<tr>
<td>2.1.1 Labeled Detection Methods</td>
<td>9</td>
</tr>
<tr>
<td>2.1.2 Label-Free Detection</td>
<td>10</td>
</tr>
<tr>
<td>2.2 Surface Plasmon Resonance</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1 Prism- and Grating-Coupled Surface Plasmon Resonance</td>
<td>14</td>
</tr>
<tr>
<td>2.2.2 Localized Surface Plasmon Resonance at Metal Nanoparticles</td>
<td>17</td>
</tr>
<tr>
<td>2.3 Surface Plasmon Resonance Sensors</td>
<td>18</td>
</tr>
<tr>
<td>2.3.1 Angle- and Wavelength-Resolved Intensity Measurements</td>
<td>20</td>
</tr>
<tr>
<td>2.3.2 Surface Plasmon Resonance Imaging</td>
<td>22</td>
</tr>
<tr>
<td>2.3.3 Sensitivity and Resolution</td>
<td>23</td>
</tr>
<tr>
<td>2.4 Surface and Coupling Chemistry</td>
<td>25</td>
</tr>
<tr>
<td>2.4.1 Alkanethiolate Self-Assembled Monolayers at Gold Surfaces</td>
<td>26</td>
</tr>
<tr>
<td>2.4.2 Dextran Hydrogel Matrix</td>
<td>27</td>
</tr>
<tr>
<td>2.4.3 Covalent Immobilization Methods for Biomolecules</td>
<td>28</td>
</tr>
<tr>
<td>2.4.4 High Affinity Capture Methods</td>
<td>32</td>
</tr>
<tr>
<td>2.4.5 Strategies for Construction of Microarray Samples</td>
<td>34</td>
</tr>
<tr>
<td>2.5 Grating-Based Optical Sensing Methods</td>
<td>37</td>
</tr>
<tr>
<td>2.6 References</td>
<td>38</td>
</tr>
<tr>
<td>CHAPTER 3. SURFACE PLASMON RESONANCE IMAGING OF BIOMOLECULAR INTERACTIONS ON A GRATING-BASED SENSOR ARRAY</td>
<td>49</td>
</tr>
<tr>
<td>3.1 Summary</td>
<td>49</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>50</td>
</tr>
<tr>
<td>3.3 Experimental Section</td>
<td>52</td>
</tr>
<tr>
<td>3.3.1 Materials and Reagents</td>
<td>52</td>
</tr>
<tr>
<td>3.3.2 Grating Construction</td>
<td>53</td>
</tr>
<tr>
<td>3.3.3 Atomic Force Microscope (AFM) Imaging</td>
<td>55</td>
</tr>
<tr>
<td>3.3.4 Solid Pin Microspotter</td>
<td>55</td>
</tr>
<tr>
<td>3.3.5 Imaging Surface Plasmon Resonance</td>
<td>57</td>
</tr>
<tr>
<td>3.3.6 Ellipsometry and Variable-Angle Surface Plasmon Resonance</td>
<td>58</td>
</tr>
<tr>
<td>3.4 Results and Discussion</td>
<td>58</td>
</tr>
<tr>
<td>3.5 Conclusions</td>
<td>81</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>6.3.4</td>
<td>Formation of Thin Organic Films</td>
</tr>
<tr>
<td>6.3.5</td>
<td>Infrared Reflection Absorption Spectroscopy</td>
</tr>
<tr>
<td>6.3.6</td>
<td>Ellipsometry</td>
</tr>
<tr>
<td>6.3.7</td>
<td>Simulation of the Grating Response</td>
</tr>
<tr>
<td>6.4</td>
<td>Results and Discussion</td>
</tr>
<tr>
<td>6.5</td>
<td>Conclusions</td>
</tr>
<tr>
<td>6.6</td>
<td>References</td>
</tr>
</tbody>
</table>

**CHAPTER 7. GENERAL CONCLUSIONS** 181
This dissertation describes the development of label-free methods for biomolecular detection based on surface plasmon resonance and optical diffraction. The ability of topographically modulated metal gratings to excite surface plasmons under direct illumination conditions is exploited in this research. Sensing surfaces are constructed by molecular self-assembly and well-established selective amine-coupling methods. The sensing surfaces are manipulated by the inclusion of specific functional groups, which modify the chemical characteristics of the surface. Microarray sensor chips are created using contact and non-contact printing methods, which allow parallel sensing for high throughput detection. The proof-of-concept sensors have been used for immunoassay applications and for the detection of submonolayer thicknesses of protein films. These efforts primarily relate to the development of techniques for fabrication of sensitive yet flexible biosensors that have a variety of applications in drug discovery, proteomics as well as biological and chemical detection.
CHAPTER 1. INTRODUCTION

This dissertation is primarily concerned with the development of sensor platforms that can be used to detect biomolecular interactions in a high-throughput and label-free format. Such sensing techniques are especially useful in the field of proteomics and drug discovery where it is crucial to understand the strength of the affinity interactions between biomolecules. Two optical methods based on nanostructured surfaces, namely, surface plasmon resonance and diffraction are the primary signal transduction methods used in this research. The construction of proof-of-concept sensors that can detect interactions between proteins, antibodies, and molecular films is described. Contact and non-contact printing methods have been used to create microarray sensor chips that are capable of detecting multiple biomolecular interaction events simultaneously. We have used these sensor platforms to detect covalent immobilization of proteins and immunoreactions of antibodies with the attached proteins.

Figure 1.1 outlines the general methodology that is followed in the development of prototypes of the sensor platforms that are described in this dissertation. A good design of sensor platform requires that optimal decisions are made about the four major elements of this task – signal transduction method, surface chemistry, multiplexing, and signal-readout. Signal transduction method is the mechanism by which the information about molecular binding events is translated into a more readily measured physical quantity such as electrical voltage or intensity of light. The signal transduction methods used in this research are based on surface plasmon resonance and diffraction. A direct observation of molecular binding events is difficult due to the small size of individual molecules. However, if the biomolecular
Figure 1.1  Typical steps followed in the sensor development.
interactions take place at surfaces that support surface plasmon resonance or diffraction, the information about the interactions can be inferred by observing the characteristics of light that is transmitted or reflected from these surfaces. The physical signal transduction mechanisms cannot distinguish between specific and non-specific interactions taking place at the sensor surface. Appropriate surface chemistry should be chosen to maximize the efficiency of the sensor and minimize the likelihood of false results. The surface chemistry can also be used to create matrices with thicknesses of few tens of nanometer which can be used to increase the sensitivity of the sensor. For example, mesh-like dextran matrices are often preferred over planar molecular films in commercial sensors due to the increased sensitivities that they offer.

Multiplexing is important in developing cost-effective sensors. Simultaneous detection of many analytes using microarray chips can reduce the time, effort and amount of reagents needed for the assay. The concept of doing reactions in miniaturized formats has resulted in technological advances in such areas as lab-on-chip devices and DNA microarray-based genomics. In this work, we have developed a pin-contact printing method to create protein microarrays and used a non-contact inkjet-printing method to deposit exact amounts of proteins in a microarray format that can be interrogated with surface plasmon resonance methods. Several methods exist for interrogation of samples. While some methods might be more suited when the measurements need to be made on a single sample, other methods can be suitable for microarray based samples. For example, the imaging mode in surface plasmon resonance techniques has the capability to interrogate numerous interaction events simultaneously. Thus, the design of signal readout methods also constitutes an important step in the development of sensor platforms.
1.1 Motivation: Label-Free High Throughput Sensing

In the proteomics and drug research, the knowledge about the specific interactions between biomolecules such as receptor-ligand and drug-target pairs is vital for the discovery and design of better drugs and elucidation of the cause of diseases. Most of the conventional assays for measuring the binding interactions require labeling of molecules with fluorophores and radioactive isotopes. Indeed, high sensitivity has made labeled assays such as enzyme-linked immunosorbent assay (ELISA) and radio immunoassays (RIA) as standard detection methods in laboratories. However, as with all systems, these assays are also not without drawbacks. Fluorescence interference and quenching can cause false results. In addition, a label attached to a molecule (especially proteins) can change its structure and modify the binding characteristics. As an example, it has been reported that the binding of antibodies to fluorophore-labeled protein is weaker than that to unlabeled protein.[1] Also, high costs of labeling reagents and their disposal make these assays expensive.

Label-free sensing refers to those methods that do not require labeling of biomolecules with reporter molecules such as radioactive isotopes and fluorophores for their detection. These methods rely on a physical transduction mechanism for inference of the occurrence of interaction between molecules. Such methods provide an alternative to the fluorescence and radiolabeling based assays. Surface plasmon resonance methods are the most well-known in the field of label-free detection. Useful information about the kinetics of the interaction between immobilized molecules and analytes can be derived from these methods. However, most of the label-free sensing techniques are low in throughput. Addition of parallel and high-throughput capabilities to biochemical assays is considered a significant improvement.
because it reduces the testing time and cost per sample. Indeed, most of the assays are done in industry-standard multiwell formats that use small quantities of reagents. An idea of doing high-throughput biology using DNA microarrays has created a niche market since it was first reported in 1995.[2] Simple in concept and essentially a miniaturization of a familiar technology, this high throughput tool gave biologists the first opportunity to explore genome function across biological samples and cellular states in a way that was impossible otherwise. On the same note, high throughput and parallel combinatorial techniques are now considered as new paradigm for addressing complex physical problems.[3]

An application of microarray based label-free detection methods is in the development of miniaturized sensor chips that detect multiple biological agents in environment, water and food samples. For example, numerous agents can be simultaneously detected by using immunosensor chips that have respective antibodies arranged in a microarray format. This methodology is especially helpful for quick “fingerprinting” of test samples as portable detection systems and in field-applications. Other significant advantages of such a high throughput label-free detection method are that problems associated with secondary detection or auxiliary reagents are eliminated, assay development steps and times are shorter, kinetic parameters for the binding of unmodified biomolecules are measured, and the same sensor platform can be used for many kinds of biomolecular interactions by designing appropriately responsive surfaces. In this dissertation we describe our attempts in building label-free sensor platforms that enable biomolecular interaction analysis in microarray format that allows higher throughputs.
1.2 Organization of the Dissertation

This thesis is a collection of research efforts involving the development and prototyping of four sensing platforms that detect local changes in the refractive index near sensor surface and use this phenomenon as a signal-transduction mechanism for detection of molecules binding to the sensor surface. Chapter 2 includes a review of the relevant literature and provides a knowledge base that will aid in understanding the material presented in subsequent chapters. In Chapter 3, the development of a grating-based SPR sensor that utilizes an imaging method to detect protein binding at the surface in an array format is described. Chapter 4 will deal with a new type of multicolor SPR sensor that is used to image a protein microarray constructed by chemical inkjet printing. Chapter 5 will include the description of a sensor based on surface plasmon enhanced transmission of light that is used as an immunosensor. In Chapter 6, grating coupler assisted infrared reflection absorption spectroscopy for the characterization of organic thin films is described. Final remarks and future directions are included as Chapter 7.

The phenomenon of surface plasmon resonance forms the centerpiece of the work described in this dissertation. Although the first observation of surface plasmon resonance dates back to more than a century, recent advances in nanofabrication and computational tools have rekindled the interest of scientific community in this phenomenon and its applications to the fields of biosensing and nanophotonics. The ability to tune the interaction between light and nanostructured materials have resulted in the emerging field, often known as nanoplasmonics, that has the potential of contributing to the next generation of sensors, chips, and optical computers. This dissertation represents an original contribution to the
scientific literature and highlights the development and control of nanostructured surface plasmon resonance biosensors.

The recent developments in SPR imaging methods combine the advantages of traditional SPR (kinetics and affinity analysis) and with high throughput capabilities which allows simultaneous monitoring of numerous biomolecular interactions. When combined with protein microarrays, this technique has the potential to become an invaluable tool for applications that require high-throughput analysis of biomolecular interactions such as in the fields of proteomics and drug-discovery.

1.3 References


CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

This chapter presents background on array-based optical detection methods including surface plasmon resonance and diffraction based optical techniques. This will be useful for the reader to understand current state of the art in these areas. A framework surrounding methods to build sensor chips and advances in the technology that directly relate to the work presented in the dissertation is also developed in this chapter.

2.1 Array-Based Optical Detection Methods

Array-based detection methods such as DNA and protein microarrays have revolutionized modern biochemistry methods.[3-5] DNA and protein microarrays are increasingly finding applications in numerous areas of genomics, proteomics, pharmacology, and toxicology.[5, 10-17] Microarrays are dense collections of “spots” of biologically relevant moieties on a planar surface arranged in a convenient spatially addressable way, typically a two-dimensional matrix.[5] These moieties can be DNA, proteins, carbohydrates or oligonucleotides and are attached to a solid support via covalent interactions or specific interaction chemistries (e.g., avidin-biotin). High densities of spots are achieved by utilizing advanced techniques of patterning such as photolithography, contact, and non-contact printing methods.[5, 15] The interaction of biomolecules with microarray spots is deciphered in terms of an easily observed variable such as the intensity of fluorescence from molecules but alternative labeling methods and label-free detection methods are also being developed.[23]
The detection of interaction between surface-bound species and the target analyte is possible by using a number of optical techniques. The primary differences in the techniques are from the methods that are used to generate, visualized and quantify signals. The most commonly used technique for reading microarray data is the optical scanning technique in which the samples are illuminated with light and fluorescent signals generated by different fluorescent dyes are measured. Various optical filters are used for wavelength selection while photo multiplier tubes and charge-coupled devices are used as the detectors.[4] More sophisticated evanescent fluorescence techniques have also been developed.[29-32] The primary advantage of this technique is that the background fluorescence is suppressed since only the fluorophores that are close to the surface (~hundreds of nm) are excited. Newer detection platforms utilize integrated electro-optic systems for reading microarray based samples.[33]

2.1.1 Labeled Detection Methods

The most well-established bioassay methods utilize biomolecules that are decorated with reporter or “labeling” molecules.[34, 35] The most common labeling strategies utilize fluorescent tags due to the commercial availability of a large number of fluorescent dyes that have narrow emission spectra and are compatible with established microscopy techniques.[36, 37] In addition, fluorescence-based detection methods are generally the preferred detection method because they are simple, sensitive, and exhibit very high spatial resolution.[1, 38] Quantitative information about the biomolecular interactions can be extracted from the fluorescence signals in the bioassays. Fluorescent dyes such as Cy3 and Cy5 are very commonly used as they can be efficiently incorporated within biomolecules
enzymatically or via chemical reactions.[37, 39, 40] One of the critical disadvantages of organic fluorescent dyes is that bleaching or quenching can significantly decrease the detection ability.[39]

An alternative to organic dyes are semiconductor quantum dots which have emerged as very promising for fluorescence-based detection methods since they are at least twenty times as bright and hundred times as stable as conventional dyes.[41] An interesting property of the quantum dots is that they exhibit a wide absorption band and a tunable narrow emission peak. This makes them suitable for multiplexed assay applications.[42-45]

Other strategies that are currently used are enzymatic labeling strategies which utilize enzymes such as horseradish peroxidase and alkaline phosphatase as well as radiolabeling methods.[39, 46, 47]

2.1.2 Label-Free Detection

Label-based detection platforms work well in the detection of binding events but also increase the assay costs and may affect the activity of the compounds under investigation. For this reason, various label-free detection schemes have emerged for monitoring molecular interactions. Some of these techniques are acoustic detection,[48] surface plasmon resonance(SPR) methods, and ellipsometry.[49, 50]

Ellipsometry utilizes the change in polarization of light reflected from a sample on a surface. This technique is based on the fact that polarization state of reflected light is changed as molecules adsorb on a surface. These changes are dependent on the thickness of the adsorbed layer on the surface and can be converted to surface concentration of molecules.
Ellipsometry can also be used in an imaging mode to visualize a large number of biomolecular interactions on a microarray.[51]

Several other optical methods such as colorimetric resonant reflection[52, 53] and reflectrometric interference spectroscopy[54-56] have also been developed that allow detection of molecular interactions in label-free high-throughput format. Table 2.1 lists some common commercial optical label-free platforms. Clearly, surface plasmon resonance (SPR) based techniques are the most common in label-free sensing due to their excellent sensitivity.

### 2.2 Surface Plasmon Resonance

Surface plasmons (SPs) are quanta of electromagnetic surface waves that are associated with a collective oscillation of electrons at a metal-dielectric interface (Figure 2.1). These oscillations can occur in optical region for such metals as gold, silver, and copper. The excitation of SPs with light is usually termed as surface plasmon resonance (SPR). While the complete derivation of mathematical equations describing the physics of SPR can be found elsewhere,[57] we will summarize only the fundamental characteristics and applications of SPR in this section.

The first observation of SPR was reported by Wood as anomalous peaks in the spectra from a continuous source of light when reflected from a ruled metallic grating.[58] The “Wood’s anomalies” were first explained as surface waves by Fano[59] and later Hessel[60] developed a theory on the same idea.

The optical excitation of SPs requires matching of the energy as well as momentum of photons to that of plasmons. Since the momentum and wavevector of light are related by a scalar constant, the resonance condition is equivalently stated as matching of the wavevector.
<table>
<thead>
<tr>
<th>Company</th>
<th>Technology</th>
<th>Product</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axela</td>
<td>Diffractive optics technology</td>
<td>DOT™</td>
<td>axelabiosensors.com</td>
</tr>
<tr>
<td>Bioanalytica Jena</td>
<td>SPR</td>
<td>BIAffinity®</td>
<td>analytik-jena.de</td>
</tr>
<tr>
<td>Biacore</td>
<td>SPR</td>
<td>A100, T100, X100, 3000, Flexchip</td>
<td>biacore.com</td>
</tr>
<tr>
<td>BioRad</td>
<td>SPR</td>
<td>ProteOn™ XPR36</td>
<td>bio-rad.com/proteininteraction</td>
</tr>
<tr>
<td>CSEM</td>
<td>Waveguide grating evanescence</td>
<td>EVASENS WIOS</td>
<td>csem.ch</td>
</tr>
<tr>
<td>Corning</td>
<td>Resonant waveguide grating</td>
<td>Epic™</td>
<td>corning.com</td>
</tr>
<tr>
<td>EcoChemie</td>
<td>SPR</td>
<td>AutoLab Espirit</td>
<td>ecochemie.nl</td>
</tr>
<tr>
<td>Farfield Sensors</td>
<td>Dual polarization interferometry</td>
<td>AnaLight®</td>
<td>farfield-sensors.com</td>
</tr>
<tr>
<td>ForteBio</td>
<td>Biolayer interferometry</td>
<td>Octet™</td>
<td>fortebio.com</td>
</tr>
<tr>
<td>GWC Technologies</td>
<td>SPR</td>
<td>SPRimager®II, FT-SPRi200</td>
<td>gwctechnologies.com</td>
</tr>
<tr>
<td>IBIS</td>
<td>SPR</td>
<td>IBIS-1, IBIS-2, IBIS-iSPR</td>
<td>ibis-spr.nl</td>
</tr>
<tr>
<td>Johnson &amp; Johnson</td>
<td>ThermoFluor®</td>
<td>Thermofluor®</td>
<td>jnjpharmarnd.com</td>
</tr>
<tr>
<td>Reichert</td>
<td>SPR</td>
<td>SR7000</td>
<td>reichertai.com/spr</td>
</tr>
<tr>
<td>Solus Biosystems</td>
<td>Isoelectric focusing/IR</td>
<td>Solus100™</td>
<td>solusbiosystems.com</td>
</tr>
<tr>
<td>SRU Biosystems</td>
<td>Colorimetric Resonant reflection</td>
<td>BIND™</td>
<td>srubiosystems.com</td>
</tr>
</tbody>
</table>
Figure 2.1 A representation of excitation of surface plasmons at a metal-dielectric interface.
The wavevector of light in a medium with dielectric constant $\varepsilon_D$ is always shorter than the real part of the wavevector of the SPs at the interface of the metal and the dielectric. This means that a momentum-enhancing configuration is needed to achieve SPR. As an example, Figure 2.2A shows the calculated relationship between energy and wavevector of surface plasmons and gold at planar gold surface in air interface as well as the light-line in air. The light line is to the left of the SPR dispersion curves at all frequencies which simply illustrates the inability of light to excite SPs at planar metal surface via direct illumination. At short wavelengths, the energy of the surface plasmons asymptotes to a value that is equal to $(1/\sqrt{2})$ times the energy of the bulk plasma oscillations inside the metal.[57]

An important characteristic of SPs is their transverse magnetic nature which means that only $p$-polarized light can participate in SPR. $p$ and $s$ are two eigen polarizations of light that have transverse magnetic and transverse electric nature. $p$-polarized light has the electric field in the plane of incidence and perpendicular to the propagation direction while $s$-polarized light has the electric field perpendicular to the plane of incidence (Figure 2.2B).

Two devices that are commonly used to enhance the momentum of light are gratings and prisms. Gratings take advantage of the diffracted orders while prisms utilize the fact that the wavevector of light increases while traveling through an optically dense medium.

2.2.1 Prism- and Grating-Coupled Surface Plasmon Resonance

The idea behind prism coupling is that the wavevector of light is increased by a factor of $n$ when it passes through a medium that has a refractive index value of $n$. The refractive index is numerically equal to the square root of the dielectric constant. The prism-coupling
Figure 2.2  (A) Calculated dispersion of surface plasmons at gold-air interface. (B) Definition of $p$- and $s$-polarized light.
configuration that is the most useful is named after Kretschmann.[57] In this setup, a gold-coated glass slide is brought into optical contact with a high refractive index prism and light is made to illuminate the gold film from the back side. It also turns out that the angle of incidence at which SPR occurs at gold surface in air is higher than the critical angle for total internal reflection at the prism-water or prism-air interface. This means that SPR is realized under the conditions of total internal reflection when evanescent waves are produced at the prism-gold interface. These waves decay exponentially within the thickness of the film and excite the SPs at the other side of the gold film. More details about the physics of prism-coupled SPR can be found in Section 4.4.

The evanescent waves generated under the conditions of total internal reflection form the basis of some other analytical techniques such as total internal reflection fluorescence. The evanescent fields of light can excite the fluorescent molecules just as normal light. However, since the evanescent field has a penetration depth of a few hundred nanometers, only surface-bound fluorophores are excited.[35, 61] The main advantage of this technique is associated with the surface confinement of the fluorescence excitation. This results in significant reduction of the background noise and elimination of the wash steps since unbound molecules don’t fluoresce.[31]

The field-enhancement associated with SPR has been used advantageously in conjugation with fluorescence spectroscopy and has resulted in a technique called surface plasmon enhanced fluorescence spectroscopy.[62, 63] This technique has been used for studies of cell and lipid membranes,[64, 65] detection of membrane protein expression,[66] sterol,[67] green fluorescent protein,[68] ribonuclease activity,[69] primer extension reactions,[70] immunoassays,[71, 72] and DNA hybridization.[42, 73, 74]
The principle behind grating-coupled SPR is that the interaction of light with a periodically modulated surface or grating results in the scattering of light waves in particular directions and these diffracted orders can provide for the deficit between the light wavevector and the wavevector of the SPs. More details about the physics of grating-coupled SPR can be found in Section 3.4.

In addition to the grating-coupled SPR, many other techniques have been developed for analytical sensing based on diffraction methods. Diffraction-based methodologies are widely used to interrogate structure and ordering in materials at various length scales.[75] Diffraction based methods are particularly useful for surface-sensitive applications since they readily respond to changes in refractive index near the grating surface. More details about these methods are presented in Section 2.5.

### 2.2.2 Localized Surface Plasmon Resonance at Metal Nanoparticles

Surface plasmons can be also excited at metallic nanoparticles. The electrons are unable to move freely when the size of metal structures is decreased to nano-scale (<100 nm). As a result, metallic nanostructures exhibit a size-dependent extinction in ultraviolet-visible region when the incident light resonantly interacts with the electrons in the conduction band and is known as localized surface plasmon resonance (LSPR). This behavior of metallic nanoparticles was first explained by Mie.[76, 77] The strong dependence of extinction peaks have made this technique attractive for the development of colorimetric sensors[78-80] and bioassays.[81]
2.3 Surface Plasmon Resonance Sensors

SPR sensors are based on the principle that the conditions of light coupling to the plasmons are very sensitive to the local refractive index near the metal surface. Thus, any event that causes a change in the dielectric environment at the sensor surface can be detected by SPR sensors. The high surface sensitivity of the SPR sensing techniques is due to the confinement of electromagnetic field near the surface. The magnitude of the electromagnetic field associated with the SPR is the maximum at the surface and decays exponentially with the distance away from the surface. This enables SPR techniques to detect weakly bound species at the sensor surface in the presence of excess solution species. Molecular adsorption events near the SPR sensor typically result in an increase in the index of refraction (related to the dielectric environment) which modifies the characteristics of the surface plasmons. This subsequently results in a change in such characteristics of the interacting light as incidence angle, wavelength, intensity, and phase. A measurement of any one of these characteristics, in principle, yields information about the molecular adsorption events. Figure 2.3 schematically represents the principle of operation of SPR based sensing platforms.

We have used prism-coupled and grating-coupled SPR have been used in this research work. A notable advantage of the prism-coupled SPR over grating-coupled SPR is that the light never passes through the sample since it illuminates the gold film from the back side and not from the side where plasmons are excited. The significance of this is that measurements can be made on turbid and opaque sample solutions without interference from light absorption or scattering by the sample.
Figure 2.3  Principle of operation of SPR based sensor platforms.

- Molecular Recognition
  - Binding of molecules near metal surface
  - Change in the refractive index
  - Modification of the surface plasmon wavevector
  - Change in the characteristics of light coupling to the surface plasmons

- Signal Transduction

- Output Response
  - Angle of incidence
  - Phase
  - Wavelength
  - Intensity
The possibility of monitoring the angle, wavelength or intensity of light coupling to the plasmons has resulted in angle-resolved intensity measuring techniques, SPR spectroscopy and SPR imaging, respectively. Explanation of each of these techniques is given in the following sections.

2.3.1 Angle- and Wavelength-Resolved Intensity Measurements

The angle resolved intensity measurements are typically done using a high-accuracy goniometer that allows a precise angular positioning of the illuminating light and the detector. A monochromatic source of light is typically used to illuminate the sample and the reflectance is recorded by a photodetector connected to a computer as a function the angle of incidence. Figure 2.4A shows an example SPR curve that was captured using a computer-controlled goniometer at a gold grating surface. A more robust configuration for angle-resolved measurements utilizes a fixed optical train of lenses that generates a converging wedge of monochromatic light. This wedge of light illuminates the sample at a narrow rectilinear region and the reflected wedge of light is detected by a precisely calibrated linear photodetector. Figure 2.4B shows an SPR curve that was captured using this technique. The SPR curves are similar to those in Figures 2.4A and 2.4B when the variable parameter is wavelength (SPR spectrometry) instead of the incidence angle. The SPR curves exhibit a shifting to higher angles or wavelength when molecules adsorb at the sensor surface. This is schematically represented in Figure 2.4C. The extent of the shift is directly proportional to
Figure 2.4  (A) SPR curve recorded on a gold grating and (B) on a BK7 prism. (C) A schematic illustrating shifts in SPR curve after binding of molecules at the sensor surface.
the mass of the adsorbed film. Typical SPR instruments detect the position of the minimum of this curve and relate it to the mass coverage.

There have been some attempts to perform SPR spectrometry on array samples in a spatially resolving manner. Typically the array samples are moved in a plane using computer-controlled stages and the minimum wavelength is recorded at each point. This generates a pseudo-image that distinguishes between different extents of surface reactions occurring at each sensor spot.[82-85] A more convenient method of performing spatially resolved SPR measurements on microarray-based samples is SPR imaging.

2.3.2 Surface Plasmon Resonance Imaging

The technique of SPR imaging combines the advantages of spatial imaging techniques and high surface sensitivity of SPR detection schemes. The SPR sensors that record the entire SPR curve as a function of angle or wavelength are very accurate but offer limited throughput. SPR imaging technique[86] offers simultaneous measurement of hundreds of biomolecular interactions. A typical SPR imaging apparatus consists of a collimated monochromatic light source whose polarization states can be switched between p and s by using a linear polarizer. The angle of incidence is typically set to a value that is slightly less than the SPR minimum angle. The reflected light from the sensor surface is imaged by a CCD (charge-coupled device) camera. The brightness of the sensing surface is then directly proportional to the amount of material bound at the surface.[87, 88]

SPR imaging technique has since been used for many surface systems, including self-assembled monolayers,[89, 90] mono- and multilayer Langmuir Blodgett films,[91, 92] and multilayer films built by alternate polyelectrolyte layer deposition.[93, 94] Array-based SPR
imaging methods have been used to detect DNA and RNA hybridization,[95-98] protein-DNA interactions,[99-102] Protein interactions using protein arrays,[103] and antibody-antigen epitope mapping and studies of enzymatic reactions using peptide microarrays.[99, 104] Angle resolved SPR imaging have been used to image DNA-DNA and DNA-drug interaction kinetics.[105]

2.3.3 Sensitivity and Resolution

The response of SPR sensors is proportional to the refractive index change occurring within the field of SPs. Since the electromagnetic field is largely localized near the surface region, the SPR sensors are especially sensitive to changes in the dielectric properties of this region. An obvious issue that arises due to the high sensitivity of SPR sensor to refractive index is that they are affected by changes occurring in the refractive index due to reasons other than the adsorption of molecules at the sensor surface. The refractive index changes can occur due to bulk refractive index changes (due to a variation in buffers) as well as temperature variations. Several methods have been used to account for the background refractive index interferences. Multi-channel SPR sensors utilize reference channels that are used to provide correction for the background refractive index due to change in buffer conditions.[106-109] Interpretation of the data recorded by the SPR sensors requires conversion of the sensor response to the thickness of the film. This is typically done by calibration of the instrument or theoretical modeling. Knowledge of the refractive index of the film is crucial in order to extract information about its thickness from SPR measurements since the sensor response is proportional to its optical density (= refractive index × thickness). Some of the methods that have been developed to deconvolute the effects due to film thickness and those due to
refractive index changes include SPR measurements using buffers with varying refractive indices,[110] theoretical analysis of SPR dip shape,[111, 112] two-color SPR,[113] multimode waveguide coupled SPs,[114, 115] and SPR on gratings whose profile has multiple harmonic components.[116]

Sensitivity of SPR sensors is often reported in terms of refractive index unit (RIU) changes. Some commercial instruments measure the sensitivities in terms of resonance units (RUs). One RU change is equivalent to a change of $1 \times 10^{-6}$ RIU. The sensitivity of a SPR platform depends on the excitation wavelength since it is related to the sampling-depth of the electromagnetic field of the SPs. In general, the sensitivities are better with longer excitation wavelengths.[57] As an example, at 600 nm wavelength, the sampling depth is about 31 nm on gold side and 280 nm on air side. The relationship between angular shifts and surface protein coverage has been found to be independent of the size of adsorbed protein which provides a direct way of calibrating an SPR sensor. For example, a surface protein concentration of 1 pg/mm$^2$ induces a shift in SPR response that is equivalent to a refractive index change of $1 \times 10^{-6}$ RIU.[117] Current SPR technologies can detect lower than $1 \times 10^{-5}$ RIU changes.[118] The sensitivity of SPR imaging technique have been reported to be about 10 fmol for 18-mer single-stranded DNA hybridized to a DNA array[96] and 1 fmol for specific antibody adsorption onto a peptide array.[119]

For SPR imaging experiments, the spatial resolving capabilities are also important in addition to the sensitivity. The spatial resolution of SPR imaging sensors is determined by the propagation length of the surface plasmons.[120] The propagation length is defined as the lateral distance at which the intensity of the electromagnetic field falls to $1/e$ of its original value. In general, better spatial resolutions are achieved using shorter excitation wavelengths
which in turn result in shorter propagation lengths.\cite{57} Since, as noted before, shorter wavelengths result in compromised sensitivities, optimal choice of wavelength needs to be made for SPR imaging experiments. As an example, the propagation length at gold surface is 14 $\mu$m and 0.5 $\mu$m for wavelengths of 676 nm and 531 nm, respectively. Furthermore, at long wavelengths, the SPR curve is narrow which means that a higher contrast is produced in the imaging mode\cite{120} where the sensitivity is directly related to the contrast in the captured images.

### 2.4 Surface and Coupling Chemistry

SPR is a signal transduction tool as it translates the information associated with the molecular binding events into a more readily measured variable such as angle of incidence or wavelength of reflected light. The SPR sensors, by themselves, do not have any ability to discriminate between specific binding or non-specific interactions between molecules and the sensor surface. Thus, the sensor surface is typically modified so that it interacts specifically with an analyte of interest and resists non-specific interaction with other molecules. A recent review describes various surface modification strategies that are used in the production of DNA and protein microarrays.\cite{121}

Most of the SPR sensors utilize gold as the metal at the surface of which plasmons are excited. The low reactivity of gold towards ordinary molecules is advantageous as it provides stability to the sensor in air as well as physiological buffer conditions however it also limits the flexibility in chemical modification of a gold surface. Functionalized alkanethiols constitute a class of molecules that that show strong specific interaction with gold and are almost exclusively used to build the foundation for introducing chemical functionalities at the
gold surface. Thus the gold surface provides both the physical conditions necessary to generate SPR signals and the surface at which the interaction being studied occurs.

2.4.1 Alkanethiolate Self-assembled Monolayers at Gold Surfaces

A convenient and robust method for building chemically functionalized surfaces on gold is the self-assembly of \( \omega \)-functionalized \( n \)-alkanethiols on gold surfaces. A variety of surface functionalities can be fabricated on gold surfaces by tailoring the headgroup, \( X \), of \( \text{HS–(CH}_2)_n–X \) and allowing the molecules to form self-assembled monolayers (SAMs). The S–H bond cleaves spontaneously and a strong coordinate covalent bond is formed between gold surface and the sulfur atom. The strength of Au–S bond has been measured to be as high as 44 kcal/mol.[122] It is generally understood that the thiolate molecules in a well-formed monolayer stand upright and adopt a \( \sqrt{3} \times \sqrt{3} \text{ R30}^\circ \) overlayer structure at the threefold hollow sites with \( \sim 30^\circ \) tilt from the surface normal.[123]

The organization of alkanethiol SAMs on gold is a first step in the functionalization of gold. Once thiols are anchored to the surface with \( \omega \)-functional group exposed, the full tool chest of organic chemistry can be brought into action to transform the SAM surface into different molecular entities which can then be used to tether biomolecular recognition elements at the surface. For example, reactive amine and carboxylic acid surfaces can be made on gold by using SAMs with \( X = \text{NH}_2 \) and \( \text{COOH} \), respectively.

After formation of functionalized SAMs at gold surface, many coupling chemistries can be performed to attach appropriate molecules. As outlined earlier, the specificity to the SPR analysis is determined by the nature and properties of the molecules attached to the sensor surface. For example, if the interaction between molecules A and B is to be studied then A
(or B) is attached to the sensor surface and SPR signals are monitored as the sensor is exposed to a solution of B (or A). In general, biomolecules are attached to the surface of the sensor chip by methods of covalent immobilization or high affinity capture. In covalent immobilization methods the biomolecules are directly attached to the surface through formation of covalent chemical bonds. For example, ligands with pendant NH$_2$ groups can be covalently linked to a COOH terminated SAM surface via NHS/EDC coupling chemistry. High affinity capture methods are those in which the biomolecules are attached by non-covalent interactions with another molecule which is bound very strongly to the surface.

2.4.2 Dextran Hydrogel Matrix

Sometimes a “3-dimensional” hydrogel matrix of carboxymethylated dextran is used in commercial SPR sensors (for example, Biacore SPR chips). Dextran is a linear unbranched polymer based on 1,6-linked glucose units and is covalently attached to an epoxide terminated alkanethiol SAM on gold. Since the field of evanescent wave penetrates only several hundreds of nanometers (~300 nm at 760 nm wavelength) into the sampling region, the thickness of the hydrogel matrix is kept very thin (25–100 nm) in these sensors. The polymer chains contain numerous carboxylic acid groups which gives it a high capacity to attach ligand as compared to a planar surfaces.[124] The dextran matrix provides a hydrophilic environment that is favorable to most solution-based biomolecular interactions. The layer also provides an effective barrier between the bulk solution and the gold surface and minimizes non-specific binding. Carboxylic acid (COOH) groups can be generated along the poly-sugar backbone by treating it with bromoacetic acid.[125] The COOH groups in the
matrix can then be further modified by amine, thiol or aldehyde coupling chemistries[126, 127] to immobilize various classes of molecules.

2.4.3 Covalent Immobilization Methods for Biomolecules

Amine coupling chemistry is one of the most commonly used method for attaching biomolecules covalently to the sensor surface. The COOH groups at the sensor surface are first activated with a mixture of \( N \)-hydroxysuccinimide (NHS) and \( N-(3\text{-dimethylaminopropyl})-N'\text{-ethylcarbodiimide hydrochloride} \) (EDC) to generate reactive succinimide esters. These esters react spontaneously with the primary amine groups in the ligands to link them covalently to the COOH surface (Figure 2.5).[126] This strategy has been used to print microarrays of BSA, poly-L-lysine, casein, and lactate dehydrogenase.[128]

Thiol coupling is a method that makes use of exchange reactions between thiol and active disulfide groups. The active disulfide species may be introduced either at the surface (to exchange with a thiol group on the ligand, referred to as the ligand thiol approach) or on the ligand molecule (to exchange with a thiol group introduced at the sensor surface, referred to as the surface thiol approach). Active disulfide groups are introduced using the reagent \( 2-(2\text{-pyridinyl}dithio) \) ethaneamine (PDEA).[127] An advantage of the thiol coupling method is that it can help to immobilize ligands in a defined orientation since the number of potential attachment sites is often less than with amine coupling. The two different thiol coupling approaches are illustrated in Figure 2.6 and Figure 2.7.

Aldehyde coupling is useful for ligands containing aldehyde groups. Molecules that have \textit{cis}-diols can be also oxidized to produce aldehyde groups. In this coupling chemistry, the
Figure 2.5 The amine coupling method using NHS/EDC.
Figure 2.6 The ligand thiol coupling chemistry.
Figure 2.7 The surface thiol coupling chemistry.
COOH surface is first activated with hydrazine. Aldehyde coupling is especially useful for immobilizing glycoproteins and other glycolconjugates.[127] The chemistry of aldehyde coupling is summarized in Figure 2.8.

In addition, thiol-containing DNA, peptide, carbohydrate and other capture molecules can be covalently attached using sulfosuccinimidyl 4-(Nmaleimidomethyl) cyclohexane-1-carboxylate[95, 129, 130](SSMCC) and N-succinimidyl S-acetylthiopropionate[119, 131](SATP) bifunctional linker molecules. Some molecules such as 9-fluorenylmethoxycarbonyl (Fmoc) are used as protective moieties for amine groups in multi-step surface modification procedures.[129] Some other specialized surface reaction such as the carbonyldiimidazole surface reaction can be used to attach antibodies on modified gold surfaces.[132]

2.4.4 High Affinity Capture Methods

The interaction between streptavidin and biotin is a very common example of a very routinely used non-covalent interaction that is utilized to form essentially irreversible and specific linkage between biomolecules and/or surfaces.[127, 133] Streptavidin can bind up to four biotin molecules with unusually high affinity (dissociation constant = \(10^{-15}\) M) and thus can act as a linker for two biotinylated molecules.[134] This approach is particularly useful when the ligands are nucleic acids[135] but is also applicable for attachment of molecules such as liposomes[136] and heparin.[137]

Histidine tagged proteins can be conveniently attached to nitrilotriacetic acid (NTA) molecules that are covalently attached to a COOH surface. This surface can be activated by a pulse of NiCl\(_2\) which forms a chelating complex with NTA that binds polyhistidine peptides.
Figure 2.8 The aldehyde coupling chemistry.
The surface is regenerated and the ligands are released by a pulse of ethylenediaminetetraacetic acid (EDTA).[138]

2.4.5 Strategies for Construction of Microarray Samples

The ability of an experimental technique to interrogate multiple samples simultaneously is a significant advantage as it lowers the cost and time per sample as well as allows high throughputs. Parallel methods have obvious time advantage over serial methods. An idea of doing high-throughput gene expression studies, DNA microarrays, has created a niche market since it was first reported in 1995.[1] Simple in concept and essentially miniaturization of the established fluorescence technology, this high throughput tool gave researchers the first opportunity to explore genome function across biological samples and cellular states in a way that was previously impossible. Indeed combinatorial methods are now considered as new paradigm for addressing complex physical problems.[139]

Microarrays that have functional molecules such as DNA, proteins, cells, and tissues attached on solid substrates are important tools for research in fields of genomics, proteomics and cell analysis. Microarrays are basically a collection of miniaturized functional regions that are arranged in a regular pattern, usually a two dimensional matrix array, at a solid surface. The features of these microarrays are kept sufficiently small (~10–500 μm) to allow construction of dense microarrays. The methods that are used to create these arrays can be broadly classified as contact or non-contact methods. The methods can also be classified as serial or parallel methods (Table 2.2).

The simplest method to print microarrays is solid pin contact printing in which a solid pin is first dipped in a reservoir to load the sample at its tip and then touched to the surface of
Table 2.2 Printing Methods for Construction of Microarray Samples

<table>
<thead>
<tr>
<th></th>
<th>Contact</th>
<th>Non-contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serial</td>
<td>Contact pins,[1, 2] AFM grafting[5, 6]</td>
<td>Inkjet printing,[5, 7, 8] laser writing[5, 9]</td>
</tr>
<tr>
<td>Parallel</td>
<td>Multiple contact pins, microcontact printing[18-20]</td>
<td>Electroprinting,[21, 22] photochemical patterning,[24-26] electrospray deposition[27, 28]</td>
</tr>
</tbody>
</table>
substrate to deposit a small droplet. The solvent evaporates leaving behind the dissolved material. Microcontact printing is a technique that allows “stamping” of a large number of identical spots on the substrate. In this method, a stamp with micron sized features is created from elastomeric materials or hydrogels using photolithography. The sample is adsorbed on the surface of the stamp which is then transferred to the surface of the substrate by means of physical contact with the stamp. Protein nanoarrays can be created by dip pen lithography and AFM grafting methods. Reviews concerning with the methods for protein printing are available.

Primary methods for non-contact printing are based on the commercial inkjet printing technology. Piezoelectric inkjet printing is a technique of choice for heat-sensitive biological samples as heating of sample is not required. In this technique a small quantity of solution containing material of interest is introduced into a glass capillary whose walls are surrounded by a piezoelectric. The glass walls of the capillary contract when a voltage pulse is applied to the piezo and a small droplet (volume ~ nL) of the sample is ejected towards the substrate. The solvent evaporates leaving behind the material on the substrate.

The microarrays for SPR applications have an additional restriction that the surface of the substrate is covered with a thin film of gold. Typically, the gold surfaces are modified with thiol chemistry and then the molecules of choice are anchored to the thiol film. Upon UV-irradiation in air, alkanethiolates on gold are photooxidized to corresponding alkanesulfonates. Moreover, these alkanesulfonates can be readily displaced when immersed in a second alkanethiolate solution. This strategy can be used to form arrays of functionalized alkanethiol surfaces in a background of second type of alkanethiol. Multi-step chemical modification process based on this technique are used to create RNA
aptamer microarrays,[144] and DNA microarrays.[129] Contact pin-printing methods have also been used at gold surfaces to attach thiol-linked molecules,[145] and proteins.[128] Microcontact printing has also been used to directly print proteins on gold surfaces for performing SPR imaging.[146]

Sometimes microfluidic flow cells made from elastomeric materials (such as PDMS) are used for patterning surfaces with proteins.[103] In this process elastomeric flow cells with microchannels are placed against a substrate and used as capillaries. Protein loaded solutions are passed through the capillaries from which the proteins spontaneously adsorb at the gold surface. The flow cells can now be rotated by 90° and test solutions of antibodies can be passed in a perpendicular direction.[103]

2.5 Grating-Based Optical Sensing Methods

As mentioned in Section 2.2.2, the behavior of light at gratings is strongly dependent on the dielectric conditions at the surface. The pitch of the grating can affect the number and angular positions of diffracted orders as determined by Equation 2.2. However, a change in the dielectric properties (or refractive index) at the grating surface changes the intensity of diffracted orders.[147] These properties of gratings are advantageously utilized in the development of sensors for various applications. For example, grating based sensors have been used for simultaneous measurement of current and temperature using a magnetostrictive alloy,[148] gasoline leakage detection,[149] temperature and strain,[150-153] evanescent wave sensing,[154] pesticide sensing,[155], DNA hybridization,[156] proteins[157-160] and whole cells.[161, 162]
Diffraction methods are widely used to interrogate structure and ordering in materials at various length scales. Grating-based diffraction sensor strategies have generated renewed interest due to their surface-sensitive optical properties. Examples include two-photon fluorescence enhancement and light reflection spectroscopy, which have been used for the characterization of colloids and nanoparticles as well as the determination of bulk refractive indices. The detection of volatile organic compounds by chemoselective polymeric gratings has recently been demonstrated as well as “tunable” diffraction gratings constructed from electroactive materials. The ability of metal gratings to excite surface plasmons has also been used in surface-enhanced Raman spectroscopy in traditional grating-based surface plasmon resonance sensors and for developing new geometries for biosensing such as surface plasmon-enhanced diffraction.

2.6 References


CHAPTER 3. SURFACE PLASMON RESONANCE IMAGING OF BIOMOLECULAR INTERACTIONS ON A GRATING-BASED SENSOR ARRAY


Bipin K. Singh and Andrew C. Hillier

3.1 Summary

A surface plasmon resonance sensor array based upon a grating substrate was developed for the detection of biomolecular interactions. The substrate consisted of a gold grating prepared by wet chemical treatment of a commercial recordable compact disk. A custom-built floating pin microspotter was constructed to deliver solutions containing ω-functionalized linear alkanethiols to the grating surface and produce an array of sensor elements with different exposed functional end groups. This array platform can be used to study biomolecular interactions in a label-free, sensitive, and high-throughput format. To illustrate the performance of this device, a test protein (bovine serum albumin) was exposed to sensor elements containing an array of functionalized alkanethiols possessing either activated carboxylic acid-, amine-, or hydroxyl-terminated regions. Local changes in plasmon resonance were monitored in a fixed-angle imaging configuration. Plasmon images clearly distinguish the degree of protein attachment at the various surfaces. The molecular binding events on the grating were also confirmed by ellipsometry. This grating-based SPR imaging
platform represents a simple and robust method for performing label-free, high-sensitivity, and high-throughput detection of biomolecular interactions.

### 3.2 Introduction

Surface plasmon resonance (SPR) sensing has emerged as a powerful tool to measure the binding of analytes to functionalized surfaces and immobilized (bio)molecules and has found utility in the development of immunoarrays,[1-3] proteomics,[4, 5] drug discovery,[6, 7] immunogenicity studies,[8, 9] and detection of environmental pollutants[10] and food contaminants.[11] It has also been used to monitor such events as DNA hybridization[12, 13] and protein-DNA interactions.[14, 15] Several reviews are available in the literature regarding surface plasmon resonance sensing.[14, 16-19] The advantages that SPR sensing provides over competing techniques for measuring biomolecular interactions such as radioimmunoassay and enzyme-linked immunosorbent assay include that it requires very low reagent quantities, obviates the need for labeling or complex reaction/washing steps, and can be performed in real time with high precision and specificity of the assay.[20] In addition, the ability to perform SPR imaging makes this technique a promising high-throughput screening tool.

To achieve high-throughput SPR sensing, one can increase the number of sensor elements per unit area by miniaturization or by the creation of sensor arrays. The use of microfluidic channels has been demonstrated for the study of antibody/antigen interactions at varying concentration levels as a one-dimensional array.[21] Two-dimensional array-based SPR sensing platforms have also been reported for a range of biological analytes.[22-29] A variety of schemes have been employed to excite surface plasmons, including the use of
prisms and gratings,[18, 19, 28] as well as the recent discovery of surface plasmons excited by subwavelength features.[30, 31] In the majority of SPR applications, the prism based Kretschmann configuration is used for optical excitation of surface plasmons in a thin metal film under conditions of attenuated total internal reflection.[18] In this arrangement, light travels through the prism to excite plasmons in a thin metal film from the backside of the test sample. Thus, attenuation and optical artifacts due to the sample environment are avoided. However, only films of a precise and limited range of thickness may be used. An alternative form of excitation involves placing a sample directly onto a topographically modulated surface such as a grating and exciting surface plasmons by direct illumination.[32] The advantages of grating-based SPR sensing include the fact that a prism is not necessary to excite surface plasmons and the optical quality of the substrate is not crucial. In addition, the narrow window of thicknesses allowed for the metal film in the Kretschmann configuration is obviated with grating excitation. The use of external reflection with a grating substrate allows macroscopically thick metal films to be interrogated. Another appealing feature of grating-based SPR is that inexpensive and disposable plastic gratings can be used as substrates. For example, commercially available compact disks (CDs) and digital versatile disks (DVDs), which are mass produced by injection-molding techniques, can serve as inexpensive grating substrates. The presence of a pregroove (vide infra) in CD-Rs has motivated their use in SPR sensing. Indeed, the use of a gold grating from a commercial CD-R for excitation of surface plasmons was recently reported.[33] In addition, silver and gold CD-Rs have been used as a source of planar electrodes[34-36] and as a platform for the construction of alkanethiolate monolayers.[37] One limitation of grating-based SPR sensing is that, for in situ SPR studies, the solution should be transparent and interact minimally with
the incident light. However, there is no such restriction if SPR detection is done in air, which makes it suitable for portable field testing applications and as a label-free tool for readout of DNA and protein microarrays.[38]

In this report, we describe the development of a grating-based sensor array for surface plasmon resonance imaging of biomolecular adsorption. A commercial CD-R is modified by chemical treatment and subsequent spot printing to create an array of carboxylic acid-, amine-, and hydroxyl-terminated self-assembled monolayer regions on gold. The binding of bovine serum albumin (BSA) is then interrogated by SPR imaging. Images depicting changes in SPR intensity at a fixed angle of incidence were captured to illustrate variations in BSA coverage versus position. Differences in BSA binding to carboxylic acid-, amine-, and hydroxyl-terminated regions are observed as well as significant nonspecific adsorption onto unmodified gold. This work demonstrates the utility and flexibility of this sensing technique as well as a discussion of some of the design issues associated with the grating geometry and sensor assembly.

3.3 Experimental Section

3.3.1 Materials and Reagents

Absolute ethanol, 11-mercaptopoundecanoic acid (MUA), 11-merapto-1-undecanol (MUL), 11-amino-1-undecanethiol hydrochloride (MUN), BSA, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Glycerol and sodium chloride were acquired from Fisher Scientific (Fairlawn, VA), and nitric acid was from J. T. Baker (Phillipsburg,
NJ). All chemicals and reagents were used as received. Recordable compact disks (MAM-A Gold CD-R) were purchased from Inkjet Art Solutions (Salt Lake City, UT). Gold (99.999%) was purchased from Ernest Fullam (Latham, NY). HEPES-buffered saline (HBS, 10 mM HEPES, 150 mM sodium chloride) was prepared with the pH adjusted to 7.4 using 10 mM NaOH and stored at 4 °C. All buffers and solutions were prepared with 18 MΩ deionized water (NANOPure, Barnstead, Dubuque, IA). Solutions containing 10 mM MUA, MUN and MUL were made in glycerol and sonicated for 1 h prior to use.

3.3.2 Grating Construction

The CD-Rs used in this work consisted of sequential layers of polycarbonate, dye, gold, protective lacquer, and polymer coatings (Figure 3.1). In commercial CD-Rs, the polycarbonate layer has a spiraling groove (called a pregroove) formed during the injection molding process that assists in laser tracking during writing and recovery of data. A photosensitive dye is coated on top of this substrate, and it is this layer that is “burned” in the process of writing data on a CD-R. A thin (50-100 nm) layer of metal (e.g., gold) is sputtered on top of this dye followed by protective coats of lacquer and a polymer to prevent the metal film from damage. We took advantage of the fact that the pitch of the pregroove in CD-Rs is sufficient to excite surface plasmons on gold in air.

Further preparation of the CD-Rs involved removing the protective polymer and lacquer layers on the topside of the gold layer by a straightforward wet chemical treatment.[36] Briefly, pieces from CD-R were cut to size and then immersed in concentrated nitric acid for 4 min. The protective polymer and lacquer layers spontaneously delaminated to expose the gold grating, which was then washed several times with water and ethanol followed by
Figure 3.1  Diagram illustrating various layers of a commercial CD-R. The pregroove is stamped onto the polycarbonate substrate and coated with sequential layers of dye, gold, lacquer, polymer and label.
drying with a nitrogen stream. The polycarbonate and gold surfaces were not damaged by this treatment. The gold surface was then cleaned in an oxygen plasma for 1 min to remove any residual organic impurities (plasma cleaner PDC-32G, Harrick Scientific, Ossining, NY). Freshly prepared gratings were typically used for each experiment.

### 3.3.3 Atomic Force Microscope (AFM) Imaging

AFM images of the various grating surfaces were acquired with a Dimension 3100 scanning probe microscope and Nanoscope IV controller (Veeco Metrology, LLC, Santa Barbara, CA). Imaging was performed in tapping mode using silicon TESP7 AFM tips (Veeco Metrology, LLC) with a spring constant of ~70 N m$^{-1}$ and a resonance frequency of ~280 kHz.

### 3.3.4 Solid Pin Microspotter

A custom-built, floating pin microspotter was developed for construction of sensor arrays. The microspotter consisted of a floating solid pin attached to a computer-controlled positioning system. Samples were placed beneath the floating pin near to a multiwell plate, which was used for reagent storage and sampling. The spotting pin consisted of a 229-μm diameter stainless steel solid pin (V&P Scientific) attached to a mount that allowed the pin to float during contact with the surface to be printed. The floating feature allowed a constant force to be applied during printing, as dictated by the weight of the pin. The positioning system consisted of a motor driver (UNIDRIV6000, Newport Corp., Irvine, CA) connected to three integrated stepper motor/linear translation stages (ILS Series, Newport Corp.) assembled in an orthogonal fashion to provide three independent directions of motion. The
motor driver was interfaced via a motion controller card (ESP6000, Newport Corp.) to a personal computer. Spotter positioning was controlled with a custom-designed printing program written in LabView (National Instruments, Austin, TX). The sample platform consisted of an optical table (Newport Corp.) with a tilt stage. A combination of a sample mount and a holder for a multiwell plate was attached to the tilt stage. The multiwell plate served as a storage container for the various reagents used in deposition and rinsing of the floating pint. Printing solutions typically contained a 10 mM concentration of the molecule of interest dissolved in glycerol. During formation of a single printed spot, the pin was immersed into the printing solution to a depth of ~100 μm and then positioned over the desired sample location. The pin was brought into contact with the sample surface for a predetermined period of time and then removed. This process delivered a well-controlled droplet of glycerol containing the molecule of interest. Arrays of various chemistries were created by subsequently cleaning the pin by dipping into a rinse solution, moving to a new reagent well, dipping of the pin to collect this solution, and then repeating the spotting procedure on a new location of the sample. This entire procedure was automated with the various parameters, including dipping time, spot location, and surface pattern, input using a custom LabView program. In the examples contained herein, spots of MUA, MUN, and MUL were created in array patterns consisting of alternating spots or quadrants of spots with different chemistries. More complicated patterns with additional chemistries could be easily fabricated.

Following delivery of the glycerol droplets, the solution was allowed to react with the surface for a period of ~15 min. This reaction resulted in the formation of a self-assembled monolayer of the specific alkanethiol at the spot location. Spots were subsequently washed
with copious amounts of ethanol and water and dried in a stream of nitrogen. Activation of carboxylic acid-terminated monolayers was achieved by exposing the sample to an aqueous solution containing 150 mM EDC and 30 mM NHS for 30 min. This reaction resulted in the formation of NHS-activated MUA regions, while it showed little effect on other surface regions. Subsequent attachment of BSA to the array surface was achieved by exposing the surface for 90 min to a 0.8 mg mL\(^{-1}\) solution of BSA in HBS. The sample was then washed with HBS and dried under nitrogen. All reactions were carried out in covered 3-5-mL Teflon vessels.

3.3.5 Imaging Surface Plasmon Resonance

A custom-built device was used to collect SPR imaging data. The apparatus consisted of a white light source (LS-1 Tungsten Halogen Light Source, Ocean Optics, Dunedin, FL) coupled to a narrow band-pass interference filter with a central wavelength of 632 nm and full width half-maximum (fwhm) of 10 nm (Newport Corp.). The emerging light was collimated with a convex lens having a focal length of 150 mm (Newport Corp.). The resulting beam passed through a linear polarizer before illuminating the grating sensor. The sample was mounted on a rotating tilt stage for manual alignment and rotation. Reflected light was focused onto a high-sensitivity monochrome CCD camera (EHDkamPro02, EHD Imaging GmbH) using a variable zoom lens (Zoom7000, Navitar). Images were captured with a frame-grabber card (Pinnacle Systems, Inc., Mountain View, CA) using commercially available software (Studio 8, Pinnacle Systems, Inc.). Typically, images were captured using both p-polarized and s-polarized light with the sample rotation near the minimum in surface plasmon intensity. The polarization state of light was switched between p- and s-states by
simply rotating the linear polarizer. To calculate absolute intensities, the response of CCD was calibrated using several neutral density filters. A linear response resulted with ~18 pixel values giving a 10% change in intensity.

3.3.6 Ellipsometry and Variable-Angle Surface Plasmon Resonance

Measurement of ellipsometry or variable-angle SPR was achieved using an automated, multifunctional optical system (Multiskop, Optrel GbR). Ellipsometric data were acquired at a single wavelength (632.8 nm) with a beam diameter of ~0.6 mm in the PCSA configuration at 70° angle of incidence. Values of ellipsometric angles, $\Delta$ and $\Psi$, were translated into equivalent optical thicknesses using a three-medium model. Variable-angle surface plasmon resonance measurements were performed using the same instrument in variable-angle mode after removing the compensator. The laser and detector arms were rotated using a two-arm motorized goniometer with an angular resolution of 0.001°. A rotatable Glan-Thompson polarizer with an extinction ratio of $10^{-8}$ (Halle) was used to control the polarization state of the laser. Angle scans were captured using both $p$- and $s$-polarized light reflected from the sample into a photodiode detector. The ratio of reflected $p$- and $s$-polarized light ($R_p/R_s$) was then calculated from these results.

3.4 Results and Discussion

Samples were prepared by cutting pieces of the CD-R to size and then subjecting them to the wet chemical treatment (vide supra). Soaking a piece of CD-R in a concentrated nitric acid solution for 3-5 min was sufficient to remove the protective layers of lacquer and polymer from the surface of the gold layer. Optical transmission measurements indicated that the
thickness of the gold layer was ~70 nm. Atomic force microscopy images revealed a modulated structure (Figure 3.2A). Roughness analysis indicated a reasonably smooth gold surface with typical root-mean-squared roughness of ~1.4 nm μm⁻². A cross-sectional profile perpendicular to the grating (Figure 3.2B) shows the longer-range topology of the surface with a period of 1560 nm and an amplitude of 115 nm. The shape of this surface profile approximates that of a trapezium with rounded corners.

Optical excitation of surface plasmons (SPs) at a metaldielectric interface requires that the momentum of the incident light matches that of SPs in the metal. This occurs when the in-plane wavevector of incident light (k||) matches that of the SPs. The SPs have a wavevector (k_sp), the real part of which is described by the following dispersion relation

\[ k'_p = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon'_M \varepsilon'_D}{\varepsilon'_M + \varepsilon'_D}} \]  

where \( \lambda \) is the wavelength of excitation while \( \varepsilon_M = \left( \varepsilon'_M + i\varepsilon''_M \right) \) and \( \varepsilon_D \) are the dielectric constants of the metal and dielectric layers. Since the momentum of incident light in air is lower than that given by eq 3.1, a coupling device is needed. The most commonly used coupling involves use of a prism in the Kretschmann configuration. In this configuration, the momentum of light is increased due to the prism having a higher dielectric constant than air. Under conditions of total internal reflection, an evanescent wave is generated at the prism-metal interface, which then propagates and couples with SPs at the metal-dielectric surface.

Momentum can also be matched using metal diffraction gratings.[32] For a periodically modulated interface between a metal and a dielectric with a period \( \Lambda \) (Figure 3.3), the surface component of the wavevector of incident light can increase (or decrease) by integral
Figure 3.2  (A) AFM image (4 μm x 4 μm) of exposed gold layer on CD-R. (B) Cross-sectional profile (solid line) from the AFM scan and trapezoidal grating profile (dashed line) used for the simulations.
Figure 3.3  Schematic of surface plasmon resonance on gold grating.
multiples of the grating wavevector. If this increase matches that of SPs in the metal, it can couple to and excite them. Mathematically, this relationship can be expressed by

\[ k_{sp} = \frac{2\pi}{\lambda} \sqrt{\epsilon_M \epsilon_D} = \frac{2\pi}{\lambda} \sqrt{\epsilon_D} \sin \theta + m \frac{2\pi}{\lambda} = k_{\parallel} \]

(3.2)

where \( \theta \) is angle of incidence and \( m \) is an integer (0, ±1, ±2, ...) indicating the diffracted order. For surface plasmons that propagate in the forward direction, the diffracted order of \( m = \pm 1 \) will result in the strongest coupling.[39] When this coupling occurs, a sharp resonance dip is observed in the reflectance of p-polarized light.

To compare the SPR response of the commercial gratings used here to theoretical predictions, modeling was done using a commercial diffraction grating solver (PCGrate, International Intellectual Group, Inc., Penfield, NY). This solver uses an integral method to numerically solve the electromagnetism equations.[40] Results for several model surfaces along with an experimentally measured reflectivity profile for the gold-coated grating are shown in Figure 3.4. These curves depict relative reflectivity \( (R_p/R_s) \) versus angle of incidence for light at a wavelength of 632 nm. The experimental profile for the commercial CD-R grating shows a resonance dip with a minimum at 39.37°. Simulated curves are provided for gold surfaces having the same amplitude and period as measured from the CD-R grating, but with three different surface profiles: trapezoidal, triangular, and sinusoidal. In these simulations, the electric permittivity of air and gold was taken as 1 and \(-11.84 + i1.36\).[41] The general shape of all the curves is similar, but in all cases, the simulated results overestimate the measured angle of resonance minimum. The trapezoidal profile most closely captures the shape of the experimental curve. The difference between the simulated and experimental curves could arise from several factors. The biggest effect on the
Figure 3.4  SPR curves as measured on grating at 632.8 nm (solid line) and as simulated with trapezoidal (bold solid line), triangular (dashed line) and sinusoidal (dash-dot line) grating profiles.
position of the resonance minimum is the pitch of the grating. For example, a decrease in the
pitch by 25 nm would lower the predicted resonance minimum by 0.5°, which is enough to
account for the differences between the simulated and experimental curves. The values input
for the optical constants of gold in the simulation could also vary slightly from the true
values exhibited by the gold-coated grating, but a sensitivity analysis indicated that these
parameters did not have a significant effect on the value of the resonance minimum in the
simulation. The thickness of the gold layer in these studies (~50 nm) is such that it allows
some light to pass through the grating, which could influence the response. Although there
was some concern about the impact of the underlying phthalocyanine-based dyes used in
these CD-Rs, they do not absorb light at the wavelength used for analysis.[42] Notably, prior
work has shown that the gold surface prepared by a similar protocol behaves comparably to
commercial gold electrodes in their electrochemical behavior[36, 43] and also allows
formation of self-assembled thiolate monolayers indistinguishable from those formed on
evaporated gold.[37]

The gold gratings were subsequently modified to create a sensor array. Preparation of the
sensing surface was achieved by functionalization of the gold using alkanethiols. A variety of
surface functionalities can be readily fabricated on gold surfaces by tailoring the headgroup,
X, of an ω-functionalized n-alkanethiol, HS-(CH₂)ₙ-X and allowing it to self-assemble on
gold.[44, 45] Similar self-assembly techniques have also been used to construct protein
microarrays.[46] We chose MUA, MUL, and MUN to construct modified gold surfaces.
MUA is a convenient choice for covalent coupling to proteins since NHS activation can be
used to create a surface that will readily react with pendant primary amines.[47] MUL was
chosen as a similarly sized control that presents a hydroxyl-terminated surface on gold, but is
unaffected by the NHS treatment and, thus, should not covalently bind with proteins. MUN was also chosen as a similarly sized molecule that is not influenced by the NHS treatment but would provide a surface chemistry different from that of MUL. Two different sample types were used in the studies described here – patterned and unpatterned. Unpatterned samples were used for characterization with ellipsometry and variable-angle SPR scans, while the patterned samples were constructed as sensor arrays to be analyzed with SPR imaging.

The unpatterned samples were created by immersing the gold grating in a 10 mM solution of MUA, MUL, or MUN in glycerol for 15 min. After sequentially washing with ethanol and water and then drying with nitrogen, the samples were immersed in an aqueous NHS/EDC solution for 30 min. After a brief wash, the samples were blown dry with nitrogen and then immersed in a 0.5 mg mL\(^{-1}\) solution of BSA in HBS for 90 min. The samples were then rinsed with water, dried under nitrogen, and then immediately characterized.

Unpatterned samples were characterized at various stages of fabrication by variable angle SPR and ellipsometry. Angle scans of the reflectivity of \(p\)- and \(s\)-polarized light as well as ellipsometry measurements at a fixed angle of incidence were performed at the same spot on each sample. The samples were aligned so that the orientation of the gratings was perpendicular to the plane of incidence formed by the arms of the goniometer. This was achieved by ensuring that the diffracted reflections fell in the incidence plane of the laser arm. Reflectivity scans were recorded as a function of angle of incidence for \(p\)- and \(s\)-polarized light such that normalized reflectivity \((R_p/R_s)\) could be plotted (Figure 3.4A). After completion of each reflectivity scan, a compensator was added to the instrument such that a null-ellipsometry measurement could be performed without disturbing the sample. Ellipsometric parameters were measured at 70° angle of incidence.
Figure 3.5A depicts SPR angle scans of the bare gold surface, after assembly of the MUA monolayer, following NHS ester formation, and after attachment of BSA to MUA. The three surface modification steps caused the SPR minimum to increase in intensity and shift toward progressively higher angles by 0.13°, 0.2°, and 0.4° with respect to the clean gold surface (Table 3.1). Ellipsometry measurements provided total film thickness values of 1.61, 1.76, and 5.54 nm for the MUA, MUA/NHS, and MUA/NHS/BSA layers, respectively. Alternatively, the individual film thicknesses for MUA, NHS, and BSA layers are 1.61, 0.15, and 3.78 nm. To calculate film thickness from ellipsometric parameters, a three-layer ambient-film-substrate model was used with a refractive index of 1.45 for the film.[48] Fitting the SPR curves to a thin-film model was not done due to computational limitations of the grating solver. Although modeling the results from a typical Kretschmann configuration is straightforward, solving the grating equations for a surface/film/ambient interface is much more complex and beyond the scope of this work. Therefore, ellipsometry was used as a calibration for the shifts in the SPR angle or intensity. Comparison of numerous angle shift and thickness measurements gives a calibration factor of ~14.5 nm deg⁻¹.

In addition to the MUA layers, the various other surface combinations were measured and the results tabulated in Table 3.1. The three different monolayers exhibited comparable thicknesses of 1.61, 1.68, and 1.58 nm for MUA, MUL and MUN on gold. These thicknesses are similar to those reported in the literature for densely packed thiolate monolayers formed out of ethanolic solutions.[48, 49] Treatment of the MUA surface with NHS produced a small thickness change of 0.15 nm. Similar treatment of the MUL, MUN, and bare gold surfaces produced no distinguishable change in the film thicknesses. Exposure of the various surfaces to BSA resulted in a range of responses. The largest change was observed on NHS-
Figure 3.5  (A) SPR curves recorded for clean gold grating (solid line) and after subsequent surface modifications viz. MUA monolayer (dashed line), NHS esterification of MUA (dash-dot line), and covalent attachment of BSA (bold solid line). (B) Differential increase in reflectivity after MUA SAM formation (solid line) and covalent attachment of BSA (dashed line) with respect to clean gold grating.
Table 3.1: SPR and Ellipsometric Results for Various Films.

<table>
<thead>
<tr>
<th>Surface</th>
<th>SPR shift in minimum angle versus Au (degrees)</th>
<th>Differential SPR shifts for topmost layer (degrees)</th>
<th>Total Film thickness from ellipsometry (nm)</th>
<th>Thickness of topmost layer from ellipsometry (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au/MUA</td>
<td>0.13</td>
<td>0.13</td>
<td>1.61</td>
<td>1.61</td>
</tr>
<tr>
<td>Au/MUL</td>
<td>0.14</td>
<td>0.14</td>
<td>1.68</td>
<td>1.68</td>
</tr>
<tr>
<td>Au/MUN</td>
<td>0.15</td>
<td>0.15</td>
<td>1.58</td>
<td>1.58</td>
</tr>
<tr>
<td>Au/MUA/NHS</td>
<td>0.20</td>
<td>0.07</td>
<td>1.76</td>
<td>0.15</td>
</tr>
<tr>
<td>Au/BSA*</td>
<td>0.28</td>
<td>0.28</td>
<td>3.13</td>
<td>3.13</td>
</tr>
<tr>
<td>Au/MUL/BSA*</td>
<td>0.18</td>
<td>0.04</td>
<td>2.14</td>
<td>0.46</td>
</tr>
<tr>
<td>Au/MUN/BSA*</td>
<td>0.24</td>
<td>0.09</td>
<td>2.59</td>
<td>1.01</td>
</tr>
<tr>
<td>Au/MUA/NHS/BSA</td>
<td>0.40</td>
<td>0.20</td>
<td>5.54</td>
<td>3.78</td>
</tr>
<tr>
<td>Au/MUA/BSA</td>
<td>0.16</td>
<td>0.02</td>
<td>2.06</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Results for Au, MUL and MUN surfaces exposed to the NHS/EDC treatment are indistinguishable from those reported here.
activated MUA with a measured film thickness of 3.78 nm after BSA exposure. This thickness is similar to those reported in the literature\cite{50,51} and agrees well with the $14 \times 4 \times 4$ nm ellipsoidal shape of BSA.\cite{52} This result is also consistent with the idea that carboxylic acid groups that have been “activated” via NHS treatment will covalently bind amine moieties in the BSA structure. Notably, in the absence of NHS treatment, the BSA film thickness reached a value of only 0.27 nm on MUA. The thickness of BSA on the other surfaces was less than that on NHS-activated MUA. The bare gold surface showed large amounts nonspecific adsorption with a measured film thickness of 3.13 nm. Nonspecific adsorption of BSA was significantly lower on MUN and MUL surfaces (Table 3.1) with film thicknesses of 1.01 and 0.46 nm. It should be noted that submonolayer ellipsometric film thicknesses can be interpreted as incomplete film formation on the surface. Our results are consistent with literature reports describing reduced adhesive forces between BSA and hydrophilic (OH, NH$_2$, COOH) thiolate SAMs\cite{53} and, as a result, lower nonspecific interactions.\cite{54} This reflects the ability of BSA to readily participate in nonspecific adsorption onto various surfaces. In variable-angle SPR, the resonance minimum is readily extracted and can be interpreted in terms of a thickness change. In contrast, SPR imaging typically records changes in intensity at a fixed angle of incidence. To choose the optimum angle for SPR imaging, relative changes in intensity were evaluated as a function of angle of incidence (Figure 3.5B). The maximum change in intensity for both the attachment of MUA on gold and further modification with BSA occurs at $\sim 38.5^\circ$. This angle roughly corresponds to the inflection point in the descent of the reflectivity curve as the minimum is approached from lower angles. SPR imaging at this angle should provide the highest degree of contrast with changing thickness, which should optimize the sensitivity of film thickness
measurements. The measured values of SPR angular shifts due to nonspecific adsorption of BSA on bare gold, MUA, MUL, and MUN surfaces are summarized in Figure 3.6 and Table 3.2 summarizes along with the ellipsometric thicknesses for various films.

Samples for SPR imaging consisted of an array of spots constructed from MUA, MUL, or MUN by printing onto the grating surface (Figure 3.7). Printing was achieved by “inking” a solid floating pin with a glycerol solution containing the molecule of interest and contacting the surface at a specified location (Figure 3.7A). Variables that influenced the size and quality of the printed spot included the pin size, the immersion depth, and the time in contact with the surface. An example array is depicted in Figure 3.7B, which consists of a $10 \times 10$ array of 100 glycerol droplets containing MUA with a row and column spacing of 0.8 mm. Generally, the printed droplets showed slight spreading and elongation in a direction parallel to ridges on the grating. Arrays of various sizes and density could be readily prepared by modification of the printing routine. After a contact time of ~15 min, the glycerol droplets were then rinsed from the surface to leave behind an array of spots containing the covalently linked alkanethiol monolayers.

Glycerol was used as a solvent for printing for several reasons. The high viscosity and low evaporation rate compared to ethanol, which is typically used to prepare self-assembled monolayers, made it a desirable solvent for printing. These properties minimized droplet spreading and also prevented the droplet from evaporating before a dense monolayer had formed. The low amount of spreading allowed dense arrays to be fabricated. In addition, glycerol provided sufficient solubility to the alkanethiols of interest to allow delivery to the surface in a small droplet. The rapid kinetics of alkanethiolate monolayer formation[55] also encouraged us to try glycerol. Although the formation rate appears slower in glycerol than in
Figure 3.6 (A) SPR curves for bare gold grating and non-specifically adsorbed BSA on gold. (B) SPR curves for bare gold grating, MUA and non-specifically adsorbed BSA on MUA. (C) SPR curves for bare gold grating, MUL and non-specifically adsorbed BSA on MUL. (D) SPR curves for bare gold grating, MUN and non-specifically adsorbed BSA on MUN.
Table 3.2: SPR Curve Minimum Angles and Ellipsometric Parameters

<table>
<thead>
<tr>
<th>Surface</th>
<th>SPR minimum (degrees)</th>
<th>Ellipsometric parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ</td>
<td>Ψ</td>
</tr>
<tr>
<td>Au</td>
<td>39.23</td>
<td>101.00</td>
</tr>
<tr>
<td>Au + BSA</td>
<td>39.51</td>
<td>98.16</td>
</tr>
<tr>
<td>Au + MUL</td>
<td>39.37</td>
<td>99.45</td>
</tr>
<tr>
<td>Au + MUL + BSA</td>
<td>39.41</td>
<td>99.04</td>
</tr>
<tr>
<td>Au + MUN</td>
<td>39.38</td>
<td>99.54</td>
</tr>
<tr>
<td>Au + MUN + BSA</td>
<td>39.47</td>
<td>98.63</td>
</tr>
<tr>
<td>Au</td>
<td>39.37</td>
<td>101.91</td>
</tr>
<tr>
<td>Au + MUA</td>
<td>39.50</td>
<td>100.42</td>
</tr>
<tr>
<td>Au + MUA + NHS</td>
<td>39.57</td>
<td>100.27</td>
</tr>
<tr>
<td>Au + MUA + NHS + BSA</td>
<td>39.77</td>
<td>96.83</td>
</tr>
<tr>
<td>Au</td>
<td>39.45</td>
<td>101.78</td>
</tr>
<tr>
<td>Au + MUA</td>
<td>39.59</td>
<td>100.42</td>
</tr>
<tr>
<td>Au + MUA + BSA</td>
<td>39.61</td>
<td>99.87</td>
</tr>
</tbody>
</table>
Figure 3.7: (A) A schematic showing the printing of MUA containing glycerol droplets. (B) Optical image showing $10 \times 10$ array of glycerol droplets containing thiol molecules printed in 7 mm $\times$ 7mm region of gold. The separation between the droplets is 0.8 mm along the rows and columns.
ethanol, a fairly high SAM coverage occurred after a relatively short (~15 min) exposure time. Notably, a somewhat lower coverage and less well-packed monolayer might be desirable for biosensing due to the reduced steric hindrance. Indeed, mixed SAMs containing long and short alkanethiols have proven most effective for protein immobilization.[47] A short adsorption time can also help minimize multilayer formation onto the gold surface.[56]

SPR images of the grating arrays were acquired by collecting the reflected light intensity ($R_p/R_s$) over the sample surface at a fixed angle of incidence and a single wavelength with the grating orientation perpendicular to the light path. The angle of incidence was chosen to be slightly smaller than the minimum angle for the substrate (vide supra) in order to provide maximum sensitivity for thickness changes.[8] The size of the imaging region was small enough (~7×7 mm) that the influence of grating curvature on the optical response was minimal. A white light source combined with a narrow band-pass interference filter having a central wavelength of 632 nm and a fwhm of 10 nm was chosen for imaging. Notably, the use of a white light source with a bandpass filter helps eliminate the interference fringes commonly observed in SPR images obtained with a laser light source without significant loss of sensitivity when compared to the monochromatic source.[8] Indeed, it has been shown that using a 820-nm light source with a 12-nm fwhm does not significantly degrade the quality of SPR images in prism-based sensing platforms compared to a monochromatic light source.[57]

Figure 3.8A depicts an SPR image of a 5×7 array of alternating MUA and MUL spots on a gold grating at a 38.5° angle of incidence. This image indicates the successful arraying of the gold surface with MUA and MUL spots. The spot sizes are ~0.3 mm² and the spacing between spots is ~1 mm. Images were captured using both $p$- and $s$-polarized light and the
Figure 3.8  (A) FFT-corrected SPR intensity image of a sensor chip containing 5×7 array of alternating MUA and MUL spots. MUA is the first spot in the upper left and all subsequent spots down the rows and columns alternating between MUL and MUA at a spacing of ~1 mm. The outlined region in the lower right of the figure depicts the interference fringes present in the raw data. (B) Line profile of reflected intensity ($R_p/R_s$) along topmost row of spots. (C) Schematic showing alternating regions of MUA and MUL on gold-coated grating.
pixel values were divided to produce normalized ($R_p/R_s$) intensity images. The intensity change across a given row of spots shows a variation between gold and monolayer regions (Figure 3.8B). The MUA and MUL spots are indistinguishable in the image. The monolayer regions show an increase in intensity of $R_p/R_s \sim 0.07$ over the gold regions. This intensity change is consistent with that observed in variable-angle SPR (Figure 3.5B) and with the fact that the MUA and MUL layers are of comparable heights (Figure 3.8C). The detection limit of this imaging scheme can be surmised from the noise level depicted in Figure 3.8B at $R_p/R_s \sim 0.01$, which corresponds to a height change of $\sim 0.25$ nm. On the other extreme, saturation of the CCD imaging camera would occur at a film thickness (assuming a linear response) of $\sim 10$ nm. However, these numbers could potentially be improved with better optics and a higher quality detector. The spots in Figure 3.8A also present a somewhat distorted oval shape. This shape is partly due to droplet spreading along the grating ridges (down the columns). Also, since the images were acquired at an angle of $38.5^\circ$, the widths of the spots in the horizontal direction appear shorter by a factor of $\sin 38.5^\circ$.

All raw images captured using p-polarized light showed a light and dark band artifact (similar to interference fringes) as depicted in the inset of Figure 3.8A. These bands appear parallel to the grating ridges, but their origin is unclear. To reduce the impact of these bands upon the analyzed data, a fast-Fourier transform (FFT) was performed and the frequencies associated with the bands were removed. An inverse transform was then applied to yield the final images used for analysis. The filtering procedure did not degrade the image quality in any other way.

The ability of this array to adsorb the protein BSA was subsequently interrogated. The surface was first modified with NHS in order to activate the carboxylic acid groups on MUA
to give it a high affinity for bonding to the amine groups in BSA. Although the entire array surface was exposed to the NHS solution, only the carboxylic acid-terminated MUA regions were modified. Figure 3.9B shows the SPR intensity image of the sensor chip after activation with NHS/EDC and immersion in BSA solution. The raw SPR image was subtracted from Figure 3.8A to present a difference image, where dark regions reflect little change while light regions reflect a large increase in intensity, which is presumably due to BSA binding. A schematic of this surface that identifies the various surface regions is shown in Figure 3.9A. The SPR image clearly shows that BSA interacts differently with gold, the OH-terminated surface of MUL, and the NHS-activated surface of MUA. In Figure 3.9B, the bright spots correspond to BSA covalently bound to MUA. The dark spots are MUL and their low intensity reflects minimal adsorption of BSA. The background is intermediate in brightness, which suggests substantial nonspecific adsorption of BSA on the gold surface, although not as much as that seen on MUA. A line profile of topmost row of spots (Figure 3.9D) shows the variation in reflected intensity along the gold substrate and alternating spots of MUA and MUL. The increase in intensity for MUL is ~0.05, for gold it is ~0.2, while for MUA is ranges between 0.26 and 0.29. A schematic of the proposed surface adsorption is given in Figure 3.9C. The large change in reflected intensity on MUA regions is considered to be due to the strong binding between the amine groups on BSA and the activated carboxylic acid sites. Notably, binding of BSA on MUA that was not modified by NHS treatment was very limited (Table 3.1). A reduced intensity is observed for BSA binding on gold, but it shows a sufficient magnitude to indicate a strong interaction between BSA and the substrate. It is unlikely that gold undergoes covalent binding with BSA, but the nonspecific interactions are large enough to create a fairly dense layer. The small change in SPR intensity at the MUL
Figure 3.9  (A) Schematic of sensor chip depicting alternating spots of MUA and MUL on gold grating. (B) FFT-corrected SPR difference image showing reflected intensity after immobilization of BSA. The dark spots represent MUL and the light spots are MUA after exposure the BSA. (C) Schematic depicting immobilization of BSA at various surfaces regions. (D) Line profile of reflected intensity ($R_p/R_s$) along topmost row of spots.
regions reflects the ability of this hydroxyl-terminated surface to substantially reduce nonspecific adsorption of BSA.

A further example of the utility of this printing method and SPR imaging on functionalized grating surfaces is illustrated in Figure 3.10. In this sample, a 6×6 array of spots containing MUA and MUN was created in a pattern in which the upper right and lower left quadrants of spots consist of MUA while the upper left and lower right are MUN (Figure 3.10A). MUL is then used to fill the regions between the spots to provide a background that inhibits BSA binding as compared to unmodified gold. Activation of the MUA regions is again accomplished using NHS treatment. This is followed by exposure of the surface to BSA. A SPR difference image of the resulting surface is shown in Figure 3.10B. The image shows three levels of contrast. The background displays a low reflected intensity, consistent with limited BSA adsorption onto the MUL-coated regions. The remaining spots show two distinct levels of contrast. The upper left and lower right quadrants, which contain MUN spots, display an intermediate brightness while the MUA spots in the upper right and lower left are much brighter. An intensity profile across the uppermost row of spots (Figure 3.10D) shows the quantitative intensity differences. The SPR intensity on MUA increases by ~0.23, MUN by ~0.8, and MUL by 0.03. These differences are interpreted by the schematic in Figure 3.10C, which shows a high BSA coverage on MUA, intermediate adsorption at MUN and essentially no adsorption on MUL. These results are consistent with what was observed in the variable-angle SPR and ellipsometry results, which indicated weak BSA binding at MUL, strong binding at MUA, and intermediate binding at MUN.

Figures 3.9 and 3.10 represent just two examples of the range of surface patterns and chemistries that can be prepared and evaluated in this manner. In principle, one could
Figure 3.10  (A) Schematic of sensor chip depicting spots of MUA and MUN on MUL-coated gold grating. The upper left and lower right quadrants contain MUN spots while the upper right and lower left contain MUA. (B) FFT-corrected SPR difference image showing reflected intensity after immobilization of BSA. (C) Schematic depicting immobilization of BSA on MUA, MUN and MUL regions. (D) Line profile of reflected intensity \( \frac{R_p}{R_s} \) along topmost row of spots.
substantially increase the number of spots in these printed arrays, with the primary limitations being the size of the imaging window and the minimum spacing between spots. Our current SPR imaging system allows a maximum image window of 1-in. diameter due to the size of the imaging optics. This image window could be increased by using larger lenses. At present, our printing system allows a minimum droplet spacing of ~0.7 mm to avoid merging of the printing droplets. This spacing would allow an array of ~225 spots cm$^{-2}$ to be constructed. In principle, the chemistry of each spot could also be different or consisting of various chemistries with numerous replicas.

3.5 Conclusions

We have described a simple and inexpensive, yet robust and flexible sensor platform based upon surface plasmon resonance imaging of a grating surface. The results indicate a highly sensitive detection scheme that is highly quantitative to film formation down to fractions of a monolayer. It has been demonstrated that prism and grating-based SPR coupling methods give comparable theoretical sensitivities for angular interrogation modes.[58] However, there are several advantages of using grating-based SPR sensing. There is no need for expensive glass components (prisms, slides) or index-matching fluid. Minimal alignment of optics is required, which imparts robustness to grating-based sensing. In addition, the sensor chip is mass-producible using the same inexpensive injection molding technology that is the state of the art for production of compact disks. Although commercially available CD-Rs provide a somewhat limited selection of grating periods and amplitudes, one could readily fabricate other sizes and shapes using standard photolithography and etching techniques.
In this work, we have focused on the development of a sensor platform that utilizes ex situ detection in air after exposure to a solution of interest. This format simplifies the optics required for readout of the detector response. However, one could readily adapt a grating substrate for in situ detection by fabricating a cell with optical windows at the appropriate viewing angles. The primary disadvantages of this approach would be the added complexity and the attenuation experienced by the light as it travels through the solvent.

The ability to construct arrays via pin printing of various surface chemistries allows complex sensor platforms to be easily created. The pin printing we have described allows a range of chemistries and array structures to be constructed with high reproducibility. Although this type of contact printing has some limitations when compared to jetted printing, its robustness and simplicity provides clear advantages. Indeed, pin printing systems are commonly used in the manufacture of protein microarrays and numerous printing tools are commercially available.[59-62] In addition, the pin printing described here could be easily performed on any variety of surfaces and is not limited to the gratings substrates we used.

We have demonstrated the performance and utility of this method using a selection of functionalized self-assembled monolayers for the adsorption of BSA. This technique could be easily expanded and adapted to a variety of more complex surface chemistries as well as being readily applied to additional proteins and complex biomolecules.

### 3.6 References


CHAPTER 4. MULTICOLOR SURFACE PLASMON RESONANCE IMAGING OF INKJET-PRINTED PROTEIN MICROARRAYS

In preparation for submission to *Langmuir*

Bipin K. Singh and Andrew C. Hillier

4.1 Summary

We report on a method that utilizes the surface plasmon resonance (SPR) dispersion as a mechanism to provide color-contrast in the imaging of molecular thin films. The applicability of this method in the development of simple hand-held diagnostic array-based microdevices for portable chemical and biological sensing is also described. Upon illumination with $p$-polarized white light in Kretschmann configuration, gold surface exhibits distinct colors due to excitation of surface plasmons and selective absorption of specific wavelengths. The color of the surface is sensitive to the formation of thin molecular films ($<1$ nm thick) which represents a simple method for detection of film-formation events such as those that occur when biomolecules deposit at surfaces. As an example, this method is used to image a custom-made protein microarray that is printed using a commercial chemical inkjet printer. Sub-monolayer films of a test protein (bovine serum albumin) were readily detected by this method. Higher detection sensitivity was achieved at angles where longer wavelengths coupled to the plasmons however a better contrast and spatial resolution was obtained when the angle of incidence was such that the shorter wavelengths coupled to the plasmons. The
reported work demonstrates the applicability of this technique towards the development of a rapid and versatile high-throughput microsensor for label-free detection using natural broadband light sources.

4.2 Introduction

Surface plasmon resonance (SPR) sensors are now well-accepted as a label-free detection platform for applications in immunoassaying,[1-3] proteomics,[4, 5] drug discovery[6, 7] and detection of environmental pollutants[8] and contaminants.[9] Its utility has also been found in monitoring and detecting biologically relevant events such as DNA hybridization[10, 11] and protein-DNA interactions.[12, 13] Advantages of using SPR-based methods include that minute quantities of reagents are needed, labeling reactions are not required and the results can be obtained in real-time.[14] Details of various SPR based platforms and their applications can be found in recent reviews.[15-18]

Prior reported works in SPR imaging were single-color experiments in which the changes in intensity of reflected light are monitored at a fixed angle of incidence near the reflectivity minimum.[19] A two-color SPR sensing method has been developed to determine the refractive index and thickness of adsorbed films.[11, 20] However, the sensing capabilities of multicolor SPR experiments have not been reported in detail except for a report in which changes in color due to surface plasmons (SPs) were used to image a patterned substrate in the visible spectrum.[21] The basis for multicolor SPR imaging is the absorption of specific wavelengths from white light due to coupling to the SPs.[22] This changes the color of reflected light from white to a characteristic color depending on the absorbed wavelengths. A change in the refractive index near the surface of the SPR sensor (which can occur due to
binding of molecules to the surface, for example) causes red-shift of the absorbed wavelengths which, in turn, changes the color of the reflected light. Thus complicated changes in the spectrum of reflected light are recognized as simple color changes. The color changes can easily be perceived by a human operator or can be analyzed digitally using methods similar to those reported for lithium ion sensing optodes[23, 24] and oxygen sensors.[25]

SPR imaging experiments are mostly used in conjunction in a high-throughput format with array-based strategies. Multi-channel[26] and two-dimensional array-based[19, 27, 28] sensing platforms allow for simultaneous interrogation of large number of samples and include reference surfaces on the sensor itself. Proteins are the true functional components of a cell and the need for understanding their structure-property and cross-functional relationships in a high-throughput manner has resulted in the field of protein microarrays.[29-32] SPR imaging of protein microarrays[33] is now being used to decipher complex interactions of proteins with DNA[34-36] and other proteins.[27, 37, 38] A high degree of correlation has been found between traditional biochemistry methods such as ELISA and SPR imaging based methodologies[38, 39] which proves that SPR imaging based protein-detection is emerging as a disruptive biotechnique.

In this work, we describe the development of the color SPR imaging method for imaging a protein microarray surface. A customized microarray of bovine serum albumin (BSA) was constructed by a commercial chemical inkjet printer and interrogated by the developed multicolor SPR imaging method. Color SPR images of the microarray depict changes in color at a fixed angle of incidence and clearly distinguish between unmodified gold
background and BSA-film covered regions. This work demonstrates the utility, flexibility and various other aspects of the multicolor SPR imaging method.

4.3 Experimental Section

4.3.1 Materials and Reagents

Bovine serum albumin (Sigma-Aldrich, St. Louis, MO), nitric acid (J.T. Baker, Phillipsburg, NJ), (3-mercaptopropyl)trimethoxysilane (MPTS) (Aldrich, Milwaukee, WI), gold (99.999%, Ernest Fullam, Latham, NY), hydrogen peroxide, sulfuric acid and toluene (Fisher Chemical Company, Fair Lawn, NJ) were used as received. All solutions were prepared with 18 MΩ deionized water (NANOPure, Barnstead, Dubuque, IA).

4.3.2 SPR Sensor Chip Preparation

1×3 inch glass slides (Fisher Scientific, PA) were cut to desired sizes and cleaned in 2% detergent (Neutrad, Decon Laboratories, PA) solution and immersed in piranha solution (H₂SO₄/H₂O₂ 30%, 75:25 v/v, Caution: piranha solution reacts violently with organic compounds and must be handled with extreme care) at 50 °C for 30 min followed by copious rinsing with water and drying in nitrogen. A thiol surface was formed by incubating cleaned glass slides in a 5 mM solution of MPTS in toluene for 6 hours. The slides were rinsed in toluene to remove unbound MPTS and dried in nitrogen. The modified slides were subsequently placed in a vacuum chamber for deposition of gold by resistive heating (Model Bench Top Turbo III, Denton Vacuum, Moorestown, NJ) and 50 nm of gold was deposited at a rate of 0.1 Å s⁻¹.
4.3.3 Protein Microarray Construction

A fresh solution of BSA in water was prepared to a concentration of 0.25 mg mL\(^{-1}\). BSA solution with a concentration of 0.025 mg mL\(^{-1}\) was prepared by making 1:10 dilution of the 0.25 mg mL\(^{-1}\) BSA solution. The solutions were filtered through 0.2 \(\mu\)m syringe filters (Varian, Inc., Palo Alto, CA) prior to use to avoid clogging of the microjet devices. The protein microarray used in this study was printed using a chemical inkjet printer that works on the principle of piezoelectric dispensing (JetlabII, Microfab Technologies, Inc., Plano, TX).[40] Microarray was constructed by printing drops at predetermined locations on SPR sensor chip in a 6×10 array format using the printing software accompanying the printer. After rapid evaporation of water from the dispensed drops, a thin film of BSA was formed on the SPR sensor chip. The top three rows of the array were printed using 0.25 mg mL\(^{-1}\) BSA solution while the bottom three rows were printed using 0.025 mg mL\(^{-1}\) BSA solution. The first spot in each row was formed by printing a single drop and the number of drops dispensed at each successive spot was increased by one. Thus, the last (10th) spot in each row was made by printing 10 drops at its location. Since the dispensed drops dried in \(~3\) seconds, a time gap of 10 seconds was kept between printing of consecutive drops at the same location to ensure drying of the previously printed spot. The row and column spacing of the spots was set as 200 \(\mu\)m. Same nozzle was used for all the printing to avoid any variation in the size and shape of drops due to changes in the microjet device.
4.3.4 Estimation of Camera Response

The sensitivities of red, green and blue light detection elements of the camera (Canon EOS Digital Rebel XT) were estimated. Collimated beam from a tungsten halogen light source passed through a slit and the resulting sheet of light was incident at an angle on one face of a BK7 equilateral prism (Edmund Optics, Barrington, NJ). The white light was dispersed and created a spectrum of constituent wavelengths due to wavelength-dependent refraction in prism which was subsequently imaged by the camera. Neutral density filters (Newport) were employed to avoid saturation of the sensor. Position of specific wavelengths in the spectrum was identified by using a set of interference filters. This yielded the calibration of red, green and blue pixel values with the wavelength of light being imaged. The linear response of the camera was also confirmed by using the neutral density filters.

4.3.5 Imaging of Surface Plasmon Dispersion

A custom-built set-up was used to image the relationship between surface plasmon resonance with the wavelength of light and its angle of incidence. A tungsten halogen lamp (LS-1, Ocean Optics, Dunedin, FL) was used as the white light source (360–2500 nm) in the apparatus. The light from the lamp was collimated with a biconvex lens (focal length = 100 mm) (Newport Corporation, Irvine, CA) and a rotating linear polarizer (Edmund Optics, Barrington, NJ) was placed next to lens to allow for switching between p- and s- polarization states. A custom made slit with a dimension of 25 mm × 0.5 mm was placed horizontally in the light path to shape the light beam as a 0.5 mm thin collimated sheet. The sheet of light was made convergent by passing through a plano-convex lens (focal length = 75 mm) and it
illuminated the BK7 hemicylindrical prism coupler (focal length = 25 mm). A glass slide coated with 50 nm gold film was kept in optical contact with the flat side of the prism using an index-matching liquid (Index Matching Liquid 150, Norland Products Inc, Cranbury, NJ). The reflected diverging sheet of light was re-collimated by using another identical plano-convex lens and passed through a transmission diffraction grating (Edmund Optics, Barrington, NJ) with the grooves aligned parallel to the sheet of light. The sheet of light was diffracted while passing through the grating and the first order dispersion was captured by a digital camera equipped with a high-sensitivity and high-resolution CMOS sensor (Canon EOS Digital Rebel XT). Identification of spatial location of specific wavelengths was done using a series of interference filters (Edmund Optics, Barrington, NJ) with wavelengths centered at 488, 532, 632 and 671 nm.

4.3.6 Surface Plasmon Resonance Imaging

A custom-built device was used to collect SPR imaging data. A right angled BK7 prism (Edmund Optics, Barrington, NJ) was mounted on a rotating tilt-stage for alignment and rotation to desired angle of incidence. Manual rotation of linear polarizer allowed switching of polarization states of the incident light between $p$ and $s$. 632 nm band-pass filter was used for monochromatic imaging. The hypotenuse face of the prism was brought into optical contact with the sample gold slide using a thin film of index-matching liquid. The collimated beam of white light was made to illuminate the sample slide from backside. The reflected image was magnified by means of a 10X objective (Mitutoyo, Japan) and was imaged by the camera.
4.3.7 Theoretical SPR Response

N-phase Fresnel calculations were utilized to model SPR reflectivity curves as a function of wavelength and a published procedure.[41] Scripts were written in MATLAB® (The Mathworks, Inc., NJ) to calculate normalized reflectivities as a function of angle of incidence and wavelength of light for 3 layer (BK7/gold/air) or 4 layer (BK7/gold/film/air) models. Wavelength dependent refractive indices of BK7 glass and gold[42] were used in the calculations. The refractive index for the film was assumed to be constant at a reasonable value of 1.45. The dispersion of BK7 glass was estimated using Sellmeier equation:[43]

$$\lambda^2 = C_1 + \frac{B_1 \lambda^2}{\lambda^2 - C_1} + \frac{B_2 \lambda^2}{\lambda^2 - C_2} + \frac{B_3 \lambda^2}{\lambda^2 - C_3}$$  \hspace{1cm} (4.1)

where $n$ is the refractive index of BK7, $\lambda$ is the wavelength of light in μm and the values of the constants were taken from Schott glass: $B_1 = 1.03961212$, $B_2 = 2.31792344 \times 10^{-1}$, $B_3 = 1.01046945$, $C_1 = 6.00069867 \times 10^{-2}$ μm$^2$, $C_2 = 2.00179144 \times 10^{-2}$ μm$^2$, $C_3 = 1.03560653 \times 10^2$ μm$^2$.

4.3.8 Image Analysis

All images were analyzed using ImageJ (NIH).

4.4 Results and Discussion

We started by investigating the sensitivities of the red (R), green (G) and blue (B) pixels of the digital camera. A collimated beam of white light was passed through a ~500 μm slit and the resulting sheet of light was incident on the face of an equilateral BK7 prism. The
refracted spectrum of transmitted light was imaged by the camera. Figure 4.1 shows the response of the detection elements as a function of wavelength of light. The minimum and maximum possible value for red (R), green (G) and blue (B) pixels in this camera is 0 and 255, respectively. Note that the pixel value is a measure of intensity while pixel location refers to spatial location in an image. The sensitivity of the camera clearly spans across the visible spectrum (400–700 nm) with the sensitivity curves for red, green, and blue pixels peaking at 620, 548 and 472 nm, respectively. This measured sensitivity of the camera has been used in this work to emulate the response of the camera and it also provides an effective way of converting information from wavelength space to the RGB color space. Indeed, the convenient method of converting optical spectrum to RGB color space has been previously used for easy visualization in the chemical analysis of metal surfaces by scanning tunneling microscopy.[44]

In the present studies, we employed prism-coupler based Kretschmann configuration for excitation of surface plasmons due to a superior sensitivity of this method among all SPR based techniques.[45] In Kretschmann configuration, light travels through the prism to excite plasmons in a thin metal film from the back side of the test sample under conditions of attenuated total internal reflection. The sample consists of a gold coated glass slide that is brought into optical contact with the prism using an index matching fluid. Optical excitation of surface plasmons (SPs) at the metal-air interface requires that the momentum of the incident light matches that of the SPs which is ensured by the use of a prism. When light illuminates the prism-slide assembly under the conditions of total internal reflection, the
Figure 4.1  Sensitivity calibration curves for the red, green and blue light detection elements of the digital camera.
momentum of light is increased by a factor of \( \sqrt{\varepsilon_{\text{prism}}} \) and at appropriate conditions, it can excite the plasmons:\[46\]

\[
k'_{sp} = \frac{2\pi}{\lambda} \sqrt{\varepsilon'_{\text{Gold}} \varepsilon'_{\text{Air}}} = \sqrt{\varepsilon'_{\text{prism}}} \frac{2\pi}{\lambda} \sin \theta_i = k_{||}
\]

(4.2)

where \( \lambda \) is the excitation wavelength, \( k_{||} \) is the surface-parallel component of light wavevector, \( k'_{sp} \) is the real part of the surface plasmon wavevector, \( \theta_i \) is the angle of incidence, \( \varepsilon'_{\text{Gold}} = \varepsilon'_{\text{Gold}} + i\varepsilon'_{\text{Gold}} \), \( \varepsilon'_{\text{Air}} \), and \( \varepsilon'_{\text{prism}} \) are the dielectric functions of gold, air, and prism, respectively. The optical excitation of plasmons is observed as a minimum in the (totally) reflected light intensity which is a function of wavelength and angle of incidence of the light. Using a prism-coupler method ensured that a large shift in absorbed wavelength occurred for a given change in refractive index at the surface of the SPR sensor which resulted in a large color contrast due to the high sensitivity of this method.\[45\]

We chose gold as the metal to support surface plasmons because it shows a sharp plasmon resonance and due to its non-reactive and stable nature.\[46\] Gold does not adhere well to glass surface which necessitates use an adhesion-promoting metal (e.g. Cr) underlayer in the construction of SPR sensor chips.\[47\] A disadvantage using such adhesion layers is the diffusion of metals via grain boundaries which, in turn, can affect the surface and electronic properties of gold.\[48\] We circumvented this problem by depositing gold on an adhesive organic monolayer on glass. Gold coated slides were prepared by evaporating \( \sim 50 \) nm gold directly on a thiol surface that was formed on glass slides by a spontaneous reaction of silane moieties of MPTS with the glass through siloxane bonds. The sensor chips thus formed offered excellent adhesion between gold and glass.\[48\]
An attempt was made to directly image the surface plasmon resonance on gold slides as a function of angle of incidence and wavelength of light in a way that is similar to that reported previously.[49] Figure 4.2 illustrates the setup used in imaging the dispersion of the SPs (see Experimental Section). Briefly, a thin sheet of p-polarized white light was shaped into the form of a wedge which posed a range of incidence angles at a clean gold slide. The reflected wedge of light was shaped back into a sheet of light and was passed through a transmission diffraction grating which separated its constituent wavelengths in an orthogonal direction. The resulting image was focused and imaged by the camera and is shown in Figure 4.3A. The dark region passing across the image depicts the absorption of light as a function of wavelength and angle of incidence due to coupling to SPs. The broadening of SPR resonance at shorter wavelengths can be noticed. To compare the SPR response to theoretical predictions, the reflectivity was calculated using a reported algorithm.[41] The calculated reflectivity was multiplied by the measured camera sensitivity (Figure 1) to yield Figure 3B. The striking similarity between Figure 4.3A and 4.3B verifies the high quality of SPR observed on the gold slides prepared by using MPTS as a molecular adhesive for gold.

Whole spectrum of colors assumed by SPR images of thin film of gold in air was visualized by removing the diffraction grating from the setup illustrated in Figure 4.2 and replacing it with the camera. A wedge of light that illuminates the prism with varying angle of incidences captures reflectivity-angle information which would otherwise have to be extracted by recording images at numerous fixed angles. To directly compare, we also captured similar images using monochromatic light by placing a 632 nm filter in the optical path. Figures 4.4A and 4.4B show the featureless images recorded by the camera when s-polarized white light and 632 nm light were used. However, a multicolored image was
Figure 4.2 Plan view and profile view of the experimental setup used for imaging the surface plasmon dispersion of BK7/gold/air system.
Figure 4.3  (A) Image of surface plasmon dispersion captured by the digital camera. (B) An estimation of surface plasmon dispersion using calculated reflectivity values and the calibration curves of the camera.
Figure 4.4  
(A) $s$-image captured by the camera using converging beam of white light and (B) 633 nm light. (C) $p$-image captured by the camera using converging beam of white light and (D) 633 nm light. (E) Intensity profile from C showing the response of red, green and blue detection elements of the camera at the conditions of surface plasmon resonance. (F) Reflectivity profile of the ($p/s$) image captured using 633 nm light.
obtained with $p$-polarized white light (Figure 4.4C) and an image containing a dark band was obtained in $p$-polarized 632 nm light (Figure 4.4D). Depending on the angle of incidence that various rays of light comprising the illumination wedge present at the sample, various quantities of specific wavelengths are absorbed which generates a varying hue across the sample (Figure 4.4C). A yellowish-white hue corresponding to the natural reflectance of gold was observed for angles of incidence that were less than 43°. The exposure time of camera was kept low to prevent the pixels from saturating. For angles between 43° and 44.5°, the color sharply changes to green due to increased absorbance of red light. The hues gradually change to magenta for values of incidence angles greater than 44.5°. A line profile of pixel values across Figure 4.4C is plotted in Figure 4.4E. The yellow-green color transition is marked by increased absorption of red colors while the green-magenta color transition is marked by gradual recovery of red values and a drop in green values. Note that the blue channel is relatively featureless and it only adds a background to the images. Figure 4F shows the normalized reflectivity of the monochromatic image of Figure 4.4D. The sharp SPR curve clearly demonstrates that narrow bandwidth light sources can be used to replace a strictly monochromatic light source (e.g. lasers) without any degrade in the quality of the SPR data.[50] The minimum of reflectivity in Figure 4.4F occurs at an angle that is closer to the theoretical value of 43.8° than the red channel of Figure 4.4E due to the fact that Figure 4.4E is actually a product of the sensitivity of the camera and the true SPR response of the gold film.

As a next step, we performed sensitivity studies of the multicolor SPR imaging method and determined the optimum angle for imaging experiments. Determination of optimal imaging angle at which maximum contrast in images will be produced for a given shift in
SPR conditions is fairly straightforward when imaging is done using monochromatic sources. Typically, the angle of incidence is chosen where the difference in reflectivity before and after film formation is the highest.[19] However, for multicolor SPR experiments the, the product of camera sensitivity and the SPR response has to be optimized in order to get maximum contrast in the SPR images. We calculated the shifts in SPR due to formation of a 5 nm thick film on gold. The two curves in Figure 4.5A are loci of SPR minima before and after formation of a 5 nm film. For the multicolor SPR imaging, we define an angle to be optimum when the sum of the absolute changes in the red and green channel values is maximized. This is achieved by looking for angles of incidence at which

\[ f = (R_1 - R_2) - (G_1 - G_2) \]

will be at an extremum where \( R_1, G_1 \) and \( R_2, G_2 \) are the red and green values for the images captured after and before formation of the film, respectively. To compute the optimal imaging conditions, we calculated the SPR response before and after formation of 5 nm film and multiplied it with the sensitivity of the camera to emulate the camera response. The background of Figure 4.5A shows the calculated function \( f \) as a function of wavelength and angle of incidence. A local minimum and a maximum of the function exist between 43° and 44° and are marked in the figure as region 1. The meaning of these extrema can be elucidated by reading the plot parallel to the ordinate for a fixed wavelength. For example, Figure 4.5B shows the pixel values for 632 nm wavelength. For 632 nm curve, the maximum occurs at an angle that is slightly less than the angle at which SPR minimum of bare gold occurs (43.8°). This is usually chosen as the angle at which imaging is carried out with a monochromatic light source.[19] Similarly, a minimum also occurs in the 632 nm SPR curve which corresponds to another optimal position for imaging, the only difference being that a decrease in the intensity of light will be observed after the
Figure 4.5  (A) An overlaid illustration showing the loci of the reflectivity minima for bare gold and 5 nm film-covered region as well as the calculated function $f = (R_1 - R_2) - (G_1 - G_2)$ where $R_1$, $G_1$ and $R_2$, $G_2$ are the red and green values for the images captured after and before formation of the film, respectively. (B) Values attained by the function $f$ for wavelengths of 632 nm and 559 nm.
formation of film since this angle corresponds to the rising portion of SPR curve. In Figure 4.5A, another local minimum occurs between 44.5° and 46° and is marked as region. The values in red channel are rising while those in green channel are falling (Figure 4E) at this position. Nevertheless, an extremum indicates that a high color contrast can be observed between bare gold and the regions covered with film since values in both red and green channels are changing (Figure 4.4E). To test our optimization method, we chose 45° as a second angle for performing SPR imaging. A sensitivity curve extracted from Figure 4.5A shows that if we were performing imaging with source wavelength of 559 nm (green color), then 45° angle of incidence would have been the optimum. Following this rationale, we chose to perform the SPR imaging at 43.8° and 45°.

In order to test the efficacy of the multicolor SPR imaging method, a customized microarray of BSA was created. A commercial inkjet printer was used to print the protein microarray. Inkjet drop-on-demand printers have proved to be extremely versatile technology to deliver extremely minute quantities of material to a surface. Indeed, inkjet-delivery of viable cells,[51] proteins[52] and many other materials[53] has already been reported. The printing nozzle of the chemical inkjet printer that we used consisted of a glass capillary fused with a piezoelectric crystal. A droplet of ink was dispensed from the nozzle when a voltage pulse was applied to the crystal. Figure 4.6A shows image of the device ejecting aqueous droplet containing 0.025 % BSA by weight at a rate of 500 Hz. The diameter of the droplet is $48 \pm 2 \mu m$ which is very close to the inside diameter of the glass capillary (50 $\mu m$). This diameter corresponds to a drop volume of $58 \pm 7 \ pL$ and $1.45 \pm 0.175 \ pg$ BSA. Larger or smaller droplets can be generated by using microjet devices with larger or smaller diameters of capillaries. We used two solutions of BSA in water to print the protein micrarray: 0.25 mg
Figure 4.6  (A) An image captured by a CCD camera showing the glass capillary microjet device dispensing aqueous drops containing BSA at a concentration of 0.25 mg mL$^{-1}$. The drops were constantly being dispensed at 500 Hz but appear stationary in the image due to a temporal aliasing method (stroboscopic effect). (B) A diagram showing the location and number of drops dispensed on the gold slide by the microjet device using 0.025 mg mL$^{-1}$ and 0.25 mg mL$^{-1}$ BSA for the construction of the protein microarray.
mL$^{-1}$ and 0.025 mg mL$^{-1}$. A schematic of the printing methodology is shown in Figure 6B which shows a protein microarray with $6 \times 10$ spots on a gold slide. The top 3 rows were printed using 0.25 mg mL$^{-1}$ solution while the bottom 3 rows were printed using 0.025 mg mL$^{-1}$ solution on a gold-coated glass slide. The first column in the sample was printed using a single drop of solution and the number of printed drops was increased by one for each subsequent column. This resulted in a microarray in which precise multiples of 1.45 or 14.5 pg BSA were deposited.

After printing of BSA microarray, the gold slide was imaged at 43.8° and 45° with p- and s-polarized light. A neutral density filter (OD = 0.1) was placed in the light path to prevent the pixels from saturation while capturing s-images. At 43.8°, images were also captured using a 632 nm band pass filter to directly compare the sensitivities of the color SPR method with monochromatic SPR imaging. With s-polarized light, no features of the microarray were discernible, however, the p-images clearly showed the features of the protein microarray which are shown in Figure 4.7. All the spots of the microarray appear oval in shape due to the oblique angles of incidence used in the experiments. Figure 4.7A was captured at 43.8° using 632 nm light which is very close to the SPR minimum angle. The incident light is completely absorbed in exciting SPs at bare gold regions at this angle of incidence which results in a dark background. At locations where protein is deposited, the SPR angle is shifter away from 43.8° which results in an increase in the light reflectivity. Figure 4.7B shows the image when the 632 nm filter was removed and the microarray was illuminated by white light. Yellow spots on green background can be clearly seen in the image. Green color of the background is a result of absorption of red wavelengths. However, the yellow color of the protein spots is counter-intuitive due to the fact that in varying-angle or spectrometric SPR
Figure 4.7  $p$-images of the protein microarray on being illuminated by (A) 632 nm light at an angle of incidence of 43.8°, (B) white light at 43.8°, and (C) white light at 45°. Zoomed regions of the microarray show the morphology of the protein spots created by printing 9 successive drops of 0.025 mg mL$^{-1}$ and 0.25 mg mL$^{-1}$ BSA solutions.
measurements, film formation is associated with shifts of SPR angle or absorbed wavelengths to larger values which is associated with a magenta hue (Figure 4.4C). However, the apparent contradiction is easily resolved on noticing that for the regions covered with protein film, the color palette is translated towards higher angles. Thus, film-covered regions will appear yellow or the color associated with lower wavelengths at that particular angle of incidence. Another multicolor SPR image was captured at an angle of incidence of 45° which is shown in Figure 4.7C. In this image, green spots on magenta background are visibly more noticeable and the colors of the spots and background can be explained in a similar way.

The zoomed-in spots are also shown in Figure 4.7 to illustrate the spatial resolution capability of the SPR imaging method. Each pixel in the image corresponds to ~3 μm and is solely limited by the optics and the size of the camera sensor chip. The spatial resolution of an SPR imaging method is directly related to the propagation length $L_x$ of the SPs which is defined as the lateral distance in the direction of propagation at which the intensity of the SPs is reduced by $1/e$ times its value at the point of excitation and its value is given by the following relationship:[22]

$$L_x = \left( \frac{2\pi}{\lambda} \left( \frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \right)^{\frac{3}{2}} \frac{\varepsilon_1^*}{(\varepsilon_1^2)} \right)^{-1}$$  \hspace{1cm} (4.3)

where $L_x$ is the propagation length of the SPs and other symbols have been defined in Equation 4.2. In contrast to propagation length, the spatial extension of the electromagnetic field of the SPs is measured in terms of skin depth, which is defined as depth at which the magnitude of the field falls $(1/e)$ times. A larger skin depth indicates increased sensitivity of the SPR detection method. A list of calculated propagation lengths and associated skin depths
for SPs at gold-air interface is presented in Table 4.1. A monotonically increasing relationship can be established between the wavelength of source light and the propagation lengths of the SPs as well as the skin depths. For enhanced sensitivity larger wavelengths are desirable while shorter wavelengths are preferable for spatially resolving imaging methods.[54] The increased spatial resolution when lower wavelengths are coupling with the SPs can be clearly seen from the morphology of the zoomed-in spots of Figure 4.7. Each of those spots was created by evaporation of 9 successive solution drops. The protein spots formed from 0.025 mg mL\(^{-1}\) BSA solution appear very homogenous in surface concentration in contrast to the spots formed from 0.25 mg mL\(^{-1}\) BSA solution. The spots formed with 0.25 mg mL\(^{-1}\) BSA solution demonstrate the well-known phenomenon of increased concentration of material at the periphery of a drying drop which is attributed to a capillary flow of solvent to replenish the drying pinned end of the drop.[55] In addition, 6-8 sub-spots can be noticed near the inside of the ring and the overall deposition pattern is very similar to Figure 6(a) of an article by Deegan.[56] Note that the structure of the ring as well as the non-homogenous structure miniature spots is very clearly resolved in Figure 4.7C and not in Figure 4.7A or 4.7B due to smaller propagation lengths of the SPs. The diameter of sub-spots in the bigger protein spot is ~ 5 μm in Figure 4.7C.

Another advantage of imaging at 45° for this sample is the increased “dynamic range” or an expansion in the range of colors expressed by the SPR image. At any angle, the only colors expressed by an SPR image are those corresponding to lower angles as explained earlier. So, at 43.8° the colors that can be expressed in an SPR image are green and yellow (Figure 4.4C). Thus, both the center and the border of the spot appear yellow in Figure 7B despite the presence of a greater quantity of BSA at the peripheral ring. At 45°, the SPR
Table 4.1  Calculated propagation lengths and skin depths associated with surface plasmon resonance at air-gold interface.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Propagation length (µm)</th>
<th>Skin depth (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>559</td>
<td>1.6</td>
<td>212</td>
</tr>
<tr>
<td>632</td>
<td>10.0</td>
<td>328</td>
</tr>
<tr>
<td>700</td>
<td>25.2</td>
<td>439</td>
</tr>
</tbody>
</table>
images become capable of expressing magenta, green and yellow colors (Figure 4.4C). Thus, the center of the spot is green surrounded by a yellow ring in zoomed-in spots of Figure 4.7C. Alternatively, the enhancement in number of colors due to SPR in a single image can be explained by Figure 4.4E. The magnitude of the slope of only the red profile is large near 43.8° but both red and green profile curves are very steep at 45°.

To further characterize the developed SPR imaging technique, we investigated the sensitivity and limit of detection of this method. Figure 4.8A shows a plot of the profile along the fourth row of Figure 4.8A. The three rows at the top of the microarray were not used for the sensitivity studies because the spots in these rows are relatively non-uniform in protein coverage and their reflectivity was clearly not limited by the lower limits of detection of this technique. On an average, an increment of 4.2% in reflectivity is observed between each consecutive spot. Since each of the dispensed drops had deposited 1.45 pg of BSA on these spots, average surface concentration of BSA ($\Gamma_{\text{BSA}}$) was calculated for each of the spots after measuring their diameters. An upper bound on the values of film-thickness and $\Gamma_{\text{BSA}}$ can be estimated by taking into account the $14 \times 4 \times 4$ nm ellipsoidal structure of a BSA molecule.[57] Assuming that BSA has been deposited with the longest axis parallel to the surface, the surface covered by one BSA molecule is $14 \times 4$ nm$^2$. This gives the value for $\Gamma_{\text{BSA}}$ as 1.97 ng mm$^{-2}$. Note that actual thickness as well as $\Gamma_{\text{BSA}}$ will be slightly less than 4 nm since neither any voids nor any protein unfolding has been accounted for in the calculations. Indeed, the thickness of a BSA monolayer has generally been measured to be slightly less than 4 nm.[58, 59] A plot of reflectivity versus concentration of BSA on the surface is shown in Figure 4.8B. For comparison, theoretical increase in reflectivity with increasing film thickness is also shown in the same plot. The plot indicates that the first nine
Figure 4.8  (A) A plot of reflectivity along the fourth row of the protein microarray revealing the rise in reflectivity with increments in mass of deposited protein in the row. (B) A plot of average reflectivity as a function of surface concentration of BSA on the spots.
spots in the row have sub-monolayer films however they appear continuous because the film is homogenous over length scales of the resolution limit of the SPR imaging method. A straight line through the first five points with low BSA surface coverage gave a limit of detection of 15 pg mm\(^{-2}\) or \(~0.1\) Å film. This corresponds to an effective refractive index (\(n_{\text{eff}}\)) unit (RIU) change of \(3\times10^{-5}\) RIU assuming a skin depth (\(l_{\text{skin}}\)) of 300 nm in air and using the following equation to estimate effective refractive index:[60]

\[
n_{\text{eff}} = \frac{2}{l_{\text{skin}}} \int_{0}^{\infty} \ln(z) \exp\left(2z/l_{\text{skin}}\right) dz
\]

This limit of detection is equivalent to \(1.35\times10^{-5}\) refractive index unit (RIU) based on the conversion factor of \(0.9\times10^{-6}\) RIU mm\(^2\) pg\(^{-1}\).[61] However the noise floor in the acquired images restricted the actual limit of detection to 68 pg mm\(^{-2}\) which is equivalent to \(1.36\times10^{-4}\) RIU and \(6.12\times10^{-5}\) RIU using method 1,[60] and method 2,[61] respectively.

Figures 4.9A and 4.9B show the red and green intensity profiles for the fourth row of Figure 4.7B and 4.7C, respectively. At 43.8°, the information about SPR is primarily indicated by the red values because at 43.8° only red wavelengths couple with SPs. Note that the limit of detection of the imaging system has worsened to \(~150\) pg mm\(^{-2}\) (method 1: \(3\times10^{-4}\) RIU, method 2: \(1.35\times10^{-4}\)) due to removal of the bandpass filter. However at 45°, both red and green wavelengths excite the SPs (Figure 4.4E). At 45°, the slope of red profile is positive in Figure 4.4E which explains the inverse relationship between \(\Gamma_{\text{BSA}}\) and the intensity of reflected red light. Conversely, the reflectivity of green light increases with increased BSA concentration due to a negative slope of green profile in Figure 4.4E. The limit of detection at this angle of incidence has worsened to \(~400\) pg mm\(^{-2}\) (method
Figure 4.9  (A) Line profile of red and green pixel values along the fourth row of the images shown in Figure 4.7B and (B) Figure 4.7C.
1: $8 \times 10^{-4}$, method 2: $3.6 \times 10^{-4}$ RIU) at the expense of higher spatial resolution but is still sufficient to enable simple visual detection of a fourth of a BSA monolayer.

### 4.5 Conclusions

We have described a method that utilizes a white light source to generate SPR and utilizes the corresponding changes in the color of the reflected light to differentiate between surfaces that are covered with films of varying thickness. The spatially resolving capabilities and the limits of detection that were demonstrated by this method make it suitable for applications in SPR microscopy and in hand-held miniaturized SPR sensors. The lesser number of optical components in the reported SPR device is especially advantageous for microsensing applications. Moreover, the optical readout of the developed device is in the form of color changes which is much easier to detect than the subtle changes in the light intensities which is the most common readout format for microarray-based sensors.

The reported method of multicolor SPR imaging can also be used for a simultaneous determination of the refractive index and thickness of adsorbed films in a manner that is analogous to the reported two-color SPR method.[11, 20] The ability of this platform to couple any wavelength of light in red and green regions of spectrum to SPs also makes this technique more flexible and convenient for this application. The limit of detection of this method was determined to be as good as 150 pg/mm$^2$ or $3 \times 10^{-4}$ RIU using a custom-made microarray with spots containing measured quantities of BSA. With this limit of detection, this technique is capable of sensing less than a monolayer of proteins while providing a simple color-readout method for the interpretation of surface film formation.
4.6 References


35. Shumaker-Parry, J.S., R. Aebersold, and C.T. Campbell, *Parallel, quantitative measurement of protein binding to a 120-element double-stranded DNA array in real


CHAPTER 5. SURFACE PLASMON RESONANCE ENHANCED TRANSMISSION OF LIGHT THROUGH PERIODICALLY MODULATED GOLD FILMS

In preparation for submission to *Langmuir*

Bipin K. Singh and Andrew C. Hillier

5.1 Summary

Narrow peaks are observed in the transmission spectra of *p*-polarized light through one-dimensional diffraction gratings covered with a thin translucent gold film in air at wavelengths that correspond to excitation of surface plasmon resonance at the gold-air interface. The spectral position of the peaks can be tuned to the desired wavelength by a rotation of the grating and without any modification to the illumination and detection optics. The transmission peaks exhibit red-shifts of wavelength when thin films are adsorbed on the grating surface. The utility of this platform in immunosensing is demonstrated by using it to detect a model immunoreaction occurring between bovine serum albumin (BSA) and anti-BSA. A monolayer of 11-mercaptoundecanoic acid is self-assembled on gold surface and an amine coupling chemistry is used to covalently attach BSA. The BSA film thus formed is subsequently exposed to antibody solutions. The formation of a film of antigen-antibody complexes on the sensor surface is clearly visualized by measuring the shifts in the wavelength of the transmission peak and is confirmed using ellipsometry. The formation of the immunocomplexes on the sensor surface is related to the concentration of antibodies in
the solution and closely follows Langmuir-type binding isotherm. This grating-based transmission SPR device represents a simple and sensitive platform which can be modified to study any kind of biomolecular interactions.

### 5.2 Introduction

Sensors based on surface plasmon resonance (SPR) are emerging as sensitive label-free tools to measure the binding strength of analytes to functionalized surfaces and immobilized biomolecules. The utility of SPR sensors is in the development of immunoassays,[1-3] proteomics,[4, 5] drug-discovery,[6, 7] monitoring DNA hybridization[8, 9], and protein-DNA interactions.[10, 11] Reviews of SPR based sensing techniques are available elsewhere.[11-15] Elimination of complex labeling/conjugation steps and usage of very low amounts of solutions give SPR sensing an edge over competing techniques which utilize fluorescently labeled molecules in detection assays.[16]

Surface plasmons (SPs) are coherent oscillations of charge density at a metal surface and can be optically excited using prisms or nanostructured surfaces.[17] In typical SPR sensing experiments, one of the molecules of the interacting pair is immobilized near the metal surface using appropriate surface chemistry. When the complementary molecule binds with the immobilized molecule, it increases the refractive index near the metal surface. This induces changes in the properties of surface plasmons and hence the characteristics of the interacting light. Most of the SPR sensors detect the changes in the angle of incidence, wavelength or intensity of light interacting with SPs.[14, 15]

The large field-enhancement associated with SPR is the basis for many surface analytical techniques such as surface-enhanced raman scattering,[18] surface-enhanced
fluorescence,[19] surface plasmon-enhanced diffraction[20, 21] and surface-enhanced infrared absorption spectroscopy.[22, 23] Monitoring of light in reflection[14] or transmission[24] geometries is a common method to observe SPR. At appropriate conditions, a reduction in the intensity for wavelengths of light that satisfy the condition for SPR is observed for most cases. However, contradicting this assumption, it has been found recently that the transmission of specific wavelengths of light can be actually enhanced by SPR at subwavelength hole and slit arrays[25]. This phenomenon is termed as the extraordinary optical transmission because the observed intensity of the emitted light is much higher than the calculated values based on the classical aperture theory. The nanoaperture arrays serve to convert photons into plasmons on one side of the film which then tunnel through the metal film to the other side where they are converted back into photons at the nanoapertures. The optical tunneling effects have been theoretically explored[26] and form the basis for emerging photonic devices such as resonant optical antennas.[27]

Due to the large role of surface plasmons in the transmission of light through nanohole arrays, the transmission peak of nanohole arrays is sensitive to refractive index changes near the array surface and can be used to detect molecular binding events at the sensor surface. For example, sensors have been developed based on the transmission peaks observed at a single nanohole[28] or nanohole[29-32] and nanoslit[33] arrays. These sensors have two distinct advantages over conventional SPR sensing schemes. The sensors in which a dip in the reflection intensity is measured can be strongly affected by noise. The advantage of configuration in which a signal peak is measured is analogous to the ease of determining small shifts in the position of a bright spot on a dark background than vice versa. Second, the transmission measurements can be performed in a simpler collinear geometry where the light
source, sample and detector can be aligned to form a straight line instead of precise angular alignment that is required by conventional SPR sensors. A significant limitation that has prevented usage of the sensors based on nanohole arrays is the need for expensive machining processes to create the subwavelength hole or slit arrays.

In this work, the development of a new platform that exhibits transmission peaks similar to that at nanohole arrays is described. Peaks at wavelengths corresponding to SPR at the gold-air interface are observed when a plastic diffraction grating is covered with a thin gold film and illuminated with \( p \)-polarized white light. Indeed, it has been recently proved that perforations are not necessary and peak in the transmission of light can be observed through continuous metal films.[30, 34-36] The transmission peaks can be used to detect the formation of thin molecular films on the sensor surface. The utility of this SPR device is shown in a model immunosensing application where the formation of immunocomplexes between BSA and anti-BSA is observed. This work demonstrates the utility and versatility of this grating-based SPR sensing technique.

5.3 Experimental Section

5.3.1 Materials and Reagents

Absolute ethanol, 11-mercaptopoundecanoic acid (MUA), hexanethiol (HT), decanethiol (DT), octadecanethiol (ODT), BSA, polyclonal rabbit anti-BSA, \( N \)-hydroxysuccinimide (NHS), \( N \)-(3-dimethylaminopropyl)-\( N' \)-ethylenediamine hydrochloride (EDC), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich (St. Louis, MO). All chemicals and reagents were used as received. Recordable
digital versatile discs (DVD-R, 4.7GB) were purchased from Inkjet Art Solutions (Salt Lake City, UT). Gold (99.999%) was purchased from Ernest Fullam (Latham, NY). HEPES-buffered saline (HBS) was prepared with the pH adjusted to 7.4 using 10 mM NaOH and stored at 4 °C. All buffers and solutions were prepared with 18 MΩ.cm deionized water (NANOPure, Barnstead, Dubuque, IA).

5.3.2 Grating Construction

The DVD-Rs used in this work are single-sided and single-layered with a storage capacity of 4.7 GB. The DVD-Rs consist of two 0.6 mm circular polycarbonate pieces that sandwich a reflective metal and a dye layer between them (Figure 5.1). One of the polycarbonate pieces has a continuous circular spiral groove formed during the injection molding process that assists in laser tracking during writing and recovery of data. This polycarbonate piece forms the clear face of the DVD-R. A photosensitive dye is coated on top of the pregroove which is “burned” in the process of writing data on a DVD-R. A thin (50-100 nm) layer of reflective metal is sputtered on top of the dye layer followed by another protective 0.6 mm polycarbonate cover piece to prevent physical damage to metal and dye layers. We utilized the grooved polycarbonate layer in a DVD-R as an inexpensive source of high quality plastic diffraction gratings. We have previously described the construction of sensitive grating-based SPR sensing chips derived from recordable compact discs (CD-Rs).[37]

For preparation of the gratings, the DVD-R is manually split into two constituent polycarbonate pieces at the center-plane. The grooved polycarbonate piece is easily
1. Split

2. Wash with ethanol

3. Deposit gold

Figure 5.1  Scheme depicting the construction of gold gratings.
distinguished from the unstructured polycarbonate piece as it exhibits a “rainbow” of diffracted light and has a blue tint due to the dye stuck to it. The dye is readily removed from the piece by washing in ethanol followed by drying in a stream of nitrogen. Dried grating is then placed in a vacuum chamber for deposition of additional gold (Denton Vacuum Turbo III, Morrestown, NJ). Gold is deposited onto the gratings at a rate of 1–2 Å s$^{-1}$ and a pressure of $7 \times 10^{-5}$ Torr under nitrogen atmosphere.

5.3.3 Atomic Force Microscope (AFM) Imaging

AFM images of the sample surfaces were acquired with a Dimension 3100 scanning probe microscope and Nanoscope IV controller (Veeco Metrology, LLC, Santa Barbara, CA). Imaging was performed in tapping mode using silicon TESP7 AFM tips (Veeco Metrology, LLC, Santa Barbara, CA) with a spring constant of $\sim 70$ N m$^{-1}$ and a resonance frequency of $\sim 280$ kHz.

5.3.4 Formation of Thin Films

Gold gratings were immersed in 2 mM ethanolic solutions of MUA, HT, DT or ODT for 24 h. The gratings were subsequently rinsed with ethanol to remove unbound thiol and dried in nitrogen. Activation of carboxylic acid terminated surfaces of MUA was achieved by exposing the sample to an aqueous solution containing 150 mM EDC and 30 mM NHS for 45 min. This reaction resulted in the formation of reactive succinimide esters on the surface which has the ability react with primary amine groups of a protein molecule. Attachment of BSA to the modified MUA surface was achieved by exposing the surface to 0.5 mg mL$^{-1}$
solution of BSA in HBS for 2 h. The sample was then rinsed briefly with HBS to remove unbound BSA and dried.

5.3.5 Formation of Antigen-Antibody Complexes

Gratings with covalently attached BSA films were successively immersed for 3 h in each of the solutions containing 1, 5, 20, 50, 100, 200 and 500 μg mL⁻¹ (or equivalently 7 nM, 33 nM, 0.13 μM, 0.33 μM, 0.67 μM, 1.33 μM, and 3.3 μM) of anti-BSA in HBS. The sample was gently removed and dried between each incubation step and ellipsometry and transmission measurements were done on the sample. The non-specific adsorption of BSA antibodies was tested on MUA covered gold gratings by immersing it in the 500 μg mL⁻¹ solution of anti-BSA for 3 h.

5.3.6 Ellipsometry

Film thicknesses were measured using ellipsometry. A motorized variable-angle null ellipsometer (Multiskop, Optrel GbR) operating in the PCSA configuration utilizing a single wavelength (632.8 nm) at 70° angle of incidence, was used to measure the ellipsometric parameters Δ and Ψ. The values of Δ and Ψ were translated into equivalent optical thicknesses using a three-medium model. The optical constants of the gold substrate (refractive index n and the absorption coefficient k) were first determined using a two-phase model (air/substrate). The thickness of the adsorbed film was then found using a three-phase model (air/film/substrate) with a value of 1.45 for the refractive index of the SAMs[38] as well as protein films.[39]
5.3.7 Light Transmission Spectroscopy

All optical transmission measurements were carried out in a collinear geometry. White light from a halogen source (Model LS1, Ocean Optics, Dunedin, FL) was collimated using a convex lens with focal length of 150 mm (Newport Corp.). The resulting beam passed through a linear polarizer before illuminating the grating sample through a 2 mm diameter aperture. The sample was mounted on a rotating sample holder for manual alignment. The transmitted light was collected by a 600 μm optical fiber and recorded by an Ocean Optics spectrometer. The signal to noise ratio of the spectra was increased by averaging 100 individual spectra. The raw spectrum is further smoothened in MATLAB using the built-in RLOESS smoothing function that is resistant to outlier points.[40] The processed spectrum was checked against raw data to confirm the absence of broadening of peaks and generation of new spectral features.

5.4 Results and Discussion

The gold gratings used in this study were constructed by depositing 60 nm gold film onto commercial gratings derived from DVD-Rs (Figure 5.1). Figure 5.2A shows the topography of the gold gratings prepared by this method. The cross sectional profile indicates a period of 743 nm and amplitude of 165 nm (Figure 5.2B). Roughness analysis reveals fairly smooth gold surface with a typical root mean squared roughness of 2.1 nm over an area of 1 µm².

The transmission spectra were recorded for the gold gratings. Figure 5.3 shows a schematic of the experimental setup that was used to record the transmission spectra. A collimated beam of white light illuminated the sample at an area of ~3 mm² after passing
Figure 5.2  (A) AFM image (6.4 μm × 6.4 μm) of the topography of gold grating used in the experiments. (B) Cross-sectional profile from the AFM scan.
through a linear polarizer that allowed the polarization state of light to be switched between $p$ and $s$. The light transmitted from the grating was collected by a 600 μm optical fiber and analyzed by a computer with an on-board spectrometer. The grating was mounted on a rotation stage to allow for transmission measurements of the 0th order beam at different angles of incidence, $\theta$, while keeping the light and collection optics stationary in a collinear geometry. Figures 5.4A and 5.4B show the transmission spectra for $s$- and $p$-polarized white light for $\theta = 0^\circ$ to $80^\circ$ at increments of $5^\circ$. The transmission spectra are relatively featureless for $s$-polarized light irrespective of the angle of incidence. However, the transmission measurements with $p$-polarized light clearly show development of peaks whose spectral position and height are a function of $\theta$. At $\theta = 5^\circ$ a peak starts developing at 700 nm which shifts to smaller wavelengths as $\theta$ is increased progressively. In addition to the spectral movement of the peak, the strength of the transmission feature is also a function of $\theta$. The height of the peak reaches a maximum at $15^\circ$ and eventually vanishes at $\sim 40^\circ$ angle of incidence as $\theta$ is increased. A further increase in $\theta$ results in the appearance of a new transmission peak at $\sim 45^\circ$. This peak exhibits different relationship with $\theta$ as it moves to higher wavelengths with an increase in the value of $\theta$. Similar results have been observed on subwavelength hole arrays where the spectral location of the peaks is dependent on the angle of incidence.[25]

To understand the nature of the peaks in the transmission spectra, we investigated the possible role of plasmons. The peaks observed in the spectra change in intensity and move in
Figure 5.3  A schematic illustrating the experimental setup used for measuring the transmission spectra of the grating samples.
Figure 5.4  (A) Transmission of s- and (B) p-polarized light through 45 nm gold film on grating for angle of incidence $\theta = 0^\circ, 5^\circ, 10^\circ, \ldots, 80^\circ$. 
opposite directions which is very similar to the results reported for coupling of light to SPs on gratings. [41-44] SPs are coherent oscillations of charge density at the surface of a metal. Optical excitation of surface plasmons (SPs) at a metal-dielectric interface requires matching of the momentum of the incident light and the SPs. Since momentum and wavevector differ by a constant multiplying factor, an equivalent condition is to say that photons can couple to SPs when the surface-parallel component of the wavevector of incident light ($k_{||}$) matches that of the SPs. The SPs have a complex wavevector $k_{sp}$, the real part of which is described by the following dispersion relationship[17]:

$$k'_{sp} = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon'_M \varepsilon_D}{\varepsilon'_M + \varepsilon_D}}$$

(5.1)

where $\lambda$ is the wavelength of excitation while $\varepsilon'_M = (\varepsilon'_M + i\varepsilon''_M)$ and $\varepsilon_D$ are the dielectric constants of the metal and dielectric layers. Since the momentum of incident light in air is lower than that given by eq 5.1, a coupling device is needed. [17] For a periodically modulated interface between a metal and a dielectric with a period $\Lambda$, the surface component of the wavevector of incident light can be increased (or decreased) by integral multiples of the grating wavevector to an effective wavevector. If the effective changed wavevector matches that of SPs in the metal, it can couple to and excite them. Mathematically, this relationship is expressed by

$$k'_{sp} = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon'_M \varepsilon_D}{\varepsilon'_M + \varepsilon_D}} = \frac{2\pi}{\lambda} \sqrt{\varepsilon_D} \sin \theta + m \frac{2\pi}{\Lambda} = k_{||}$$

(5.2)

where $\theta$ is angle of incidence and $m$ is an integer (0, ±1, ±2, ...) indicating the diffracted order. A reasonable approximation that went into Equation 5.2 is that the dispersion relationship of the SPs on a modulated metal surface is same as that on a flat metal
surface.[17] A more correct modeling should take into account the squared dependence of
the angular red-shift in the SPR minimum on the amplitude of grating modulation. For the
gratings used in this study this shift is < 0.1° and is ignored.[45]

A graphical solution for Equation 5.2 is shown in Figures 5.5A and 5.5B. In these figures,
the curves represent the dispersion relation of SPs or the relationship between the energy and
in-plane component of the wavevector ($k_\parallel$). The straight lines intersecting with the curves
represent $k_\parallel$ for $m = 0, \pm 1, \pm 2$. As shown in Figure 5.4A, the intersection points of the
straight lines with the curve represent possible solutions to Equation 5.2 for $\theta = 0°$. As $\theta$
is increased, $k_\parallel$ increases by an amount that is proportional to $(\sin \theta/\lambda)$. Graphically, this is
equivalent to tilting of the various straight lines. The limiting case of $\theta = 90°$ is depicted in
Figure 5.4B. The graphical solution also indicates that the wavelength coupling with the SPs
decreases for $m > 0$ or increases for $m < 0$ as $\theta$ is increased which is similar to the behavior
observed in the transmission experiments through the gratings (Figure 5.4B). Figure 5.5C
shows a plot of the spectral location of the peaks as a function of $\theta$ along with the theoretical
solution given by the points of intersection of straight lines corresponding to $m = -2$ and +1.
The close proximity of the peak positions to theoretical curve hints that SPs play a dominant
role in the in enhancement of light transmission at certain wavelengths. Indeed, similar peaks
in the transmission spectra were observed by others for wavelengths that coupled to the
SPs.[46] Theoretical investigations have revealed that enhanced transmission can occur
through modulated metal films.[47] Moreover, the SPs are transverse magnetic in nature
which means that they can be excited by $p$-polarized light which has transverse magnetic
characteristics while $s$-polarized light can not couple to the SPs. This polarization dependent
Figure 5.5  (A) A graphical solution to the Equation 2, at $\theta = 0^\circ$ and $\Lambda = 740$ nm, is governed by the intersection of the plasmon dispersion curve (- - -) and $k_\parallel$ (––) that varies with the order of diffraction ($n = -3, -2, -1, 0, 1, 2$) and $\theta$. (B) A physically limiting situation at grazing angle of incidence $\theta = 90^\circ$ is shown. (C) Theoretical SPR wavelength is shown as a function of angle of incidence for $n = -2$ and +1 along with measured peaks in the spectra observed for the transmission of $p$-polarized light.
transmission behavior is clearly shown by Figures 5.4A and 5.4B which further reinforces the hypothesis regarding the dominant role of SPR in the light transmission.

SPs are known to convert into photons at gratings and rough metal surfaces where diffraction and scattering events serve as a conversion channel.[17, 48-51] Re-radiation of photons from nanostructured surfaces is also the mechanism of the extraordinary transmission of light through subwavelength hole arrays.[48, 52] This phenomenon can also be used to develop devices with high optical efficiencies. For example, experimental and theoretical studies have indicated that increased light output can be obtained from organic light emitting diodes (OLEDs) via surface plasmon enhanced transmission.[52, 53] We further investigated the effect of the thickness of gold film on the transmission peaks. Figures 5.6A, 5.6B and 5.6C show the transmission of p-polarized light through several samples that had 35, 30 and 20 nm gold films, respectively. Clearly, the peaks became shorter and merged with the background as the film thickness was decreased. The broad feature between 500 and 600 nm is due to the wavelength dependence of the refractive index of gold and is also observed for flat gold films. The samples with 60 nm gold films exhibited the most well-defined peaks and thus were preferred to be used as sensor platforms. The film thickness of 60 nm was also sufficiently thin to allow collection of data in reasonable time period.

We further chose to test the sensitivity of the measured peak position to thickness of thin molecular films formed on the grating surface due to the large role of SPR in the transmission. SPR sensors are very sensitive to the refractive index close to the metal surface and readily detect the mass changes that are involved with the formation of ultrathin films. As a first step, we utilized functionalized alkanethiols to chemically modify the gold grating surface. ω-functionalized n-alkanethiols (HS–(CH$_2$)$_n$–X) fall into an important class of
Figure 5.6  The dependence of height of the peaks in the transmission spectra is dependent on the thickness of the gold film that is deposited on plastic grating. The transmission spectra using p-polarized light for samples with (A) 35 nm, (B) 30 nm, and (C) 20 nm thick gold film is presented.
molecules that readily self-assemble at gold surfaces to form monolayer films. These molecules attach themselves from the sulfur terminal in an upright position in a tightly packed manner on gold surfaces that are exposed to a solution containing these molecules. Availability of alkanethiols with various functional groups (for example, $X = \text{CH}_3$, OH, NH$_2$, COOH) makes them attractive for imparting surface functionalization to gold as well as making ultrathin films of well-defined thickness.[54]

Self-assembled monolayers of HT ($n = 5$, $X = \text{CH}_3$), DT ($n = 9$, $X = \text{CH}_3$) and ODT ($n = 17$, $X = \text{CH}_3$) were subsequently prepared on the gold gratings. The position of the transmission peak was measured for the gratings with HT, DT, and ODT films. The transmission was measured at $\theta = 15^\circ$ and $50^\circ$. These two angles were chosen as they corresponded to positions at which the peaks due to $m = +1$ and $-2$ diffraction orders were the tallest. The objective of doing the measurements for $m = +1$ and $-2$ peaks was to investigate if the sensitivity of the grating sensors utilizing one peak was greater than the other. The spectra obtained with $s$-polarized light were featureless for all of the samples. However, when $p$-polarized light was used, it was noticed that the peaks were shifted to higher wavelengths when compared to that of bare gold surfaces. Although modeling the results from typical Kretschmann configuration SPR sensor is straightforward, solving the Maxwell’s equations for a surface/film/ambient interface at gratings is much more complex and beyond the scope of this work. Therefore, ellipsometry was used as a technique for calibrating the shifts in peak with the thickness of molecular films. Figure 5.7 depicts the shifts in the position of peaks as a function of film thicknesses. The shifts are nearly linear with film thickness with $m = +1$ peak being more sensitive than $m = -2$ peak. For 1-nm increase in the film thickness, the wavelength shifts were 1.46 nm and 0.65 nm at $\theta = 15^\circ$ (m
Figure 5.7 A plot of experimentally measured wavelength shifts in the transmission spectra at $\theta = 15^\circ$ and $50^\circ$ and ellipsometrically measured film thicknesses for self assembled monolayers of hexanethiol (HT), decanethiol (DT) and octadecanethiol (ODT).
= +1) and 50° (m = –2), respectively. The HT, DT, and ODT films on gold grating were 0.60, 1.04, and 2.14 nm thick (Table 5.1). These thicknesses are comparable to those reported in the literature for densely packed thiolate monolayers formed out of ethanolic solutions.[38, 55]

Once the response of grating sensors to the thickness of molecular films was established, we explored the utility of the device as an immunosensing platform. BSA and anti-BSA was chosen as a model antigen-antibody system. This model system has routinely been used to demonstrate the performance of SP based sensing platforms such as differential phase measurement SPR,[56] SPR spectroscopy,[57] and localized SPR.[58] A first step in the construction of the immunosensor was the modification of gold grating surface by forming a self-assembled monolayer of MUA (n = 10, X = COOH). MUA is a convenient choice for covalently immobilization of proteins using an amine coupling method.[59] The COOH surface of MUA can be activated to form reactive NHS esters which readily react with pendant primary amine groups that are commonly found in proteins.[60] Clean gold gratings were incubated in 2 mM MUA solution for 24 h after which they were washed with ethanol and dried in nitrogen. The samples were then immersed in aqueous NHS/EDC solution for 45 mins. After a brief wash with water, the samples were immersed in a 0.5 mg mL$^{-1}$ solution of BSA in HBS for 2 h. This coupling step attached BSA to the SAM on gold via covalent amide bonds. Covalently attached BSA molecules have been shown to retain their antigenicity upon their immobilization at a solid surface.[58] Conversely, covalently attached antibodies have been shown to retain their activity towards binding with antigens.[61] The samples with immobilized BSA films were rinsed and incubated for 3 hours sequentially in solutions containing 1, 5, 20, 50, 100, 200 and 500 μg mL$^{-1}$ of anti-BSA at room temperature...
Table 5.1 List of thicknesses and shifts in transmission peak for HT, DT and ODT self-assembled monolayers on gold gratings.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Red Shift in peak location with respect to bare gold at 15° (nm)</th>
<th>Red Shift in peak location with respect to bare gold at 50° (nm)</th>
<th>Ellipsometric Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au/HT</td>
<td>0.91</td>
<td>0.62</td>
<td>0.60</td>
</tr>
<tr>
<td>Au/DT</td>
<td>1.62</td>
<td>0.65</td>
<td>1.04</td>
</tr>
<tr>
<td>Au/ODT</td>
<td>3.07</td>
<td>1.34</td>
<td>2.14</td>
</tr>
</tbody>
</table>
of 21 °C. The samples were dried after each surface modification step and transmission and ellipsometry measurements were carried out (Table 5.2). A set of control MUA-covered gratings were immersed in the 500 μg mL⁻¹ solution of anti-BSA for 3 h to measure the non-specific adsorption of anti-BSA at the grating surface. The film thickness corresponded to 0.86 nm of adsorbed film which was much less than the increments in film thickness when BSA surface was exposed to anti-BSA solution.

Figure 5.8A shows the observed red-shifts in the peak wavelength as a function of the concentration of the antibodies in the solution at angles of incidence of 15° and 50°. These shifts were fitted to a one-to-one antigen-antibody binding model and the resulting best-fit curves are also shown in the Figure 5.8A. The one-to-one interaction between the antibodies and BSA molecules can be described by Langmuir type kinetics[62]

\[
BSA + antiBSA \xleftrightarrow[k_1]{k_{-1}} BSA - antiBSA
\]

(5.5)

where \( k_1 \) and \( k_{-1} \) are the forward (association) and reverse (dissociation) rate constants, respectively. If \( \Gamma \) is the surface coverage of the antigen-antibody complex and \( \Gamma_{\text{max}} \) is the total surface available to the antibodies, then the relative surface coverage \( \theta = \Gamma/\Gamma_{\text{max}} \) at equilibrium is given by the Langmuir adsorption isotherm:

\[
\theta = \frac{Kc}{1 + Kc}
\]

(5.6)

where \( c \) is the antibody concentration and \( K \) (association constant) is the ratio \( k_1/k_{-1} \) and is a measure of the affinity of the antibody to the antigen. This simple model has been successfully used to describe the kinetics of immunoreactions as studied by the technique of surface plasmon resonance[63, 64] and other optical techniques.[16, 65] Since the sensor
Table 5.2  Measured film thicknesses and shifts in transmission peak during formation of MUA self-assembled monolayer, covalent immobilization of BSA and formation of antigen-antibodies complexes on the grating surface upon exposure to anti-BSA solutions.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Red Shift in peak location with respect to bare gold at 15° (nm)</th>
<th>Red Shift in peak location with respect to bare gold at 50° (nm)</th>
<th>Ellipsometric Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au/MUA</td>
<td>2.21</td>
<td>1.03</td>
<td>1.34</td>
</tr>
<tr>
<td>Au/MUA/BSA</td>
<td>6.57</td>
<td>3.55</td>
<td>4.88</td>
</tr>
<tr>
<td>Au/MUA/BSA + 1 µg/ml anti-BSA</td>
<td>8.11</td>
<td>4.11</td>
<td>5.82</td>
</tr>
<tr>
<td>Au/MUA/BSA + 5 µg/ml anti-BSA</td>
<td>10.92</td>
<td>5.83</td>
<td>7.73</td>
</tr>
<tr>
<td>Au/MUA/BSA + 20 µg/ml anti-BSA</td>
<td>13.36</td>
<td>7.12</td>
<td>9.69</td>
</tr>
<tr>
<td>Au/MUA/BSA + 50 µg/ml anti-BSA</td>
<td>14.27</td>
<td>7.58</td>
<td>10.4</td>
</tr>
<tr>
<td>Au/MUA/BSA + 100 µg/ml anti-BSA</td>
<td>15.05</td>
<td>8.02</td>
<td>10.75</td>
</tr>
<tr>
<td>Au/MUA/BSA + 200 µg/ml anti-BSA</td>
<td>15.36</td>
<td>7.99</td>
<td>10.92</td>
</tr>
<tr>
<td>Au/MUA/BSA + 500 µg/ml anti-BSA</td>
<td>15.22</td>
<td>8.34</td>
<td>11.08</td>
</tr>
</tbody>
</table>
Figure 5.8  (A) Wavelength shifts measured as a function of concentration of antibodies. (B) Spectral position of peaks plotted against ellipsometric film thicknesses.
response (wavelength shift) is directly related to the amount of antigen-antibody complex formed at the surface, the adsorption strength of the antibodies to the BSA film was inferred by measuring the equilibrium wavelength shifts. The value of \( K \) was calculated as \( 3.02 \times 10^7 \) M\(^{-1} \) and \( 2.32 \times 10^7 \) M\(^{-1} \) for the data collected at angles of incidence of 15° and 50°, respectively. The values of the affinity constant \( K \) have been previously reported for BSA–anti-BSA system as \( 3.5 \times 10^7 \) M\(^{-1} \), \( 3.7 \times 10^8 \) M\(^{-1} \) and \( 0.25 \times 10^8 \) M\(^{-1} \) – \( 1.2 \times 10^8 \) M\(^{-1} \).[66-68]

Figure 5.8B shows a plot of numerous wavelength shifts and film thickness measurements that were performed after the incubation steps. The thickness of covalently attached BSA film was measured to be 3.54 nm which is similar to those reported in literature[37, 69, 70] and agrees well with the 14×4×4 nm ellipsoidal shape of BSA.[71] The thickness of the fully-formed film of antigen-antibody complexes is 9.74 nm which agrees well with the dimensions of antigen antibody-complexes measured by AFM[72] and the thickness of film made up of antigen-antibody complexes.[73]

The sensitivity of this SPR sensing platform can be measured in terms of refractive index units using Equation 5.2 for a direct comparison with other SPR based methods. The sensitivity of the reported SPR sensing corresponds to \(~600\) nm RIU\(^{-1} \) which is comparable to those calculated for grating-based (300 – 630 nm RIU\(^{-1} \))[74] and nanostructure-based (150 – 400 nm RIU\(^{-1} \))[75, 76] SPR sensors.

The gold gratings presented in this report can also be used in numerous other optical applications. For example, we tested the feasibility of using these gratings as narrow band-pass filters since they exhibit transmission peaks for a narrow selection of wavelengths. The position of grating and polarizer were interchanged in the experimental setup such that unpolarized white light illuminated the grating and the transmitted light went through a linear
polarizer (analyzer) into the detector. Figure 5.9A shows the transmission characteristics as a function of the angle ($\phi$) between the polarization axis of the analyzer and the direction of grooves in the grating. The transmission was recorded at an angle of incidence of $48^\circ$ which revealed a transmission peak at 666 nm. A plot of transmission as function of $\phi$ at 666 nm is shown in Figure 5.9B. The light intensity that is transmitted through the grating and the analyzer followed a squared sinusoidal dependence on $\phi$ which means that the light becomes strongly polarized after passing through the grating.[77] The ratio of the intensities of $p$-polarized light to $s$-polarized light at 666 nm is ~22 which means these gratings can be used as polarizing band-pass filters and have applications in the development of advanced optical devices such as tunable laser filters.[78] The polarization selectivity demonstrated by the periodically modulated thin gold films in this study is similar to the ability of elliptical subwavelength hole arrays in polarizing the unpolarized light.[79]

5.5 Conclusions

We have described a simple, inexpensive and robust sensor platform based upon surface plasmon resonance enhanced transmission of light at grating surfaces. We were able to detect ultrathin alkanethiol self-assembled monolayers as well as formation of antibody-antigen complexes at gold surfaces using this sensitive detection scheme. Several advantages of this detection platform is that the minimal alignment of optics is required due to collinear geometry and the signal that is measured is a peak and not a dip. In addition, the ability to tune the wavelength of surface plasmon resonance enhanced transmission on the same surface has numerous applications. For example, coupling of surface plasmon modes to
Figure 5.9  (A) A surface plot that shows the polarizing nature of the gold gratings. (B) A transmission intensity profile as a function of $\phi$ extracted from the surface plot at $\lambda = 666$ nm.
semiconductor quantum dots[80] and electronic resonances of molecules[81-83] as well as surface plasmon enhanced fluorescence[84] requires wavelength tuning.[85]

The plastic grating sensor chips used in this study can be mass produced using commercial injection molding techniques that are used in the production of optical discs. Commercially available DVD-Rs provide a limited selection of grating periods, but one could readily fabricate gratings with other sizes and shapes using standard photolithography and etching techniques[86] as well as soft lithography methods.[87]

This SPR sensing method can also be easily extended to other systems for biomolecular detection and numerous other applications.

5.6 References


CHAPTER 6. GRATING-COUPLER ASSISTED INFRARED REFLECTION ABSORPTION SPECTROSCOPY FOR THE CHARACTERIZATION OF ORGANIC THIN FILMS

Adapted from Analytical Chemistry, 78(20), 7335-7340 (2006).

Bipin K. Singh and Andrew C. Hillier

6.1 Summary

In this chapter we demonstrate how grating-coupler assisted infrared reflection absorption spectroscopy can be used to simultaneously determine the chemical identity and relative thickness of organic thin films. With a grating substrate, a threshold anomaly associated with passing off of the $-1$ diffracted order occurs at grazing angles of incidence, resulting in a sharp absorbance in the infrared. The position of this peak is sensitive to the grating geometry as well as the dielectric environment near its surface. Thus, shifts in the peak position can be used to determine the relative thickness of adsorbed films or quantify molecular adsorption events. To illustrate the characteristics and sensitivity of this phenomenon, several samples were prepared and tested, including self-assembled alkanethiolate monolayers with 11-mercaptoundecanoic acid, 11-mercaptop-1-undecanol, decanethiol, and a covalently linked layer of bovine serum albumin on a commercial, gold-coated grating. For these samples, the position of the threshold absorbance peak shifted to lower wavenumbers as film thickness increased, which is consistent with calculated shifts based upon an increasing refractive index at the interface. The sensitivity of this shift was
measured to be 3.7 cm$^{-1}$ nm$^{-1}$. These results illustrate how a grating substrate can be exploited in a standard infrared reflectance measurement to provide additional information about the relative thickness of adsorbed surface films.

**6.2 Introduction**

Infrared reflection absorption spectroscopy (IRRAS) is a popular surface-sensitive technique that can be used to provide both chemical and structural information about thin organic films and adsorbates on surfaces.[1] Typically, metal substrates are used for these measurements due to their large infrared extinction coefficients.[2] High extinction coefficients are necessary to detect the intrinsically low absorbances of thin films. The ability of IRRAS to interrogate molecular structure and composition has been routinely used in the characterization of various films,[3] including self-assembled alkanethiolate monolayers,[4] polymers,[5] and glasses.[6] IRRAS has also been applied to biological systems, such as protein adsorption,[7, 8] avidin-biotin binding,[9, 10] and the assembly of peptide nucleic acids.[11] Most of these studies have exploited the fact that IRRAS provides information about film properties, such as chemical composition, bond orientation, conformation, and details of the local interfacial environment. However, quantitative determination of film thicknesses in these systems requires an additional measurement, such as ellipsometry,[4] surface plasmon resonance,[12] or atomic force microscopy. Although there are examples combining infrared spectroscopy with ellipsometry,[13] the ability to quantitatively measure film thickness directly with the infrared signal has not been reported.

Diffraction methods are widely used to interrogate structure and ordering in materials at various length scales.[14] Grating-based diffraction sensor strategies have generated renewed
interest due to their surface-sensitive optical properties. Examples include two-photon fluorescence enhancement[15] and light reflection spectroscopy,[16] which have been used for the characterization of colloids,[17] and nanoparticles[18] as well as the determination of bulk refractive indices.[16] The detection of volatile organic compounds by chemoselective polymeric gratings has recently been demonstrated[19] as well as “tunable” diffraction gratings constructed from electroactive materials.[20-22] The ability of metal gratings to excite surface plasmons has also been used in surface-enhanced Raman spectroscopy,[23] in traditional grating-based surface plasmon resonance sensors,[24, 25] and for developing new geometries for biosensing such as surface plasmon-enhanced diffraction.[20, 26]

In this work, we demonstrate how coupling thin-film samples to a diffraction grating substrate can be used to yield information about film thickness simultaneously during IRRAS measurements. Although grating-coupler assisted infrared spectroscopy has been reported for investigation of the excitation spectrum of inorganic multilayers,[27-29] it has not previously been combined with IRRAS for film thickness measurements. In this work, film thickness information is obtained through an anomalous absorbance in the infrared spectrum that appears at grazing angles of incidence. This absorbance occurs as the $-1$ diffracted order becomes parallel to the grating surface and disappears, as shown in Figure 6.1. This disappearance, which is referred to as passing off of the diffracted order, is a threshold anomaly that significantly impacts the diffraction efficiency of the other propagating orders.[30] The threshold conditions are sensitive to the refractive index at the grating interface, which is modified by the adsorption of ultrathin molecular films. We provide both experimental and theoretical evidence of the properties of this threshold absorbance, including tests with several thin molecular films. This work demonstrates a new capability
Figure 6.1  Schematic depicting propagating orders near grazing angles of incidence.
for IRRAS measurements, which can be simply employed in a standard instrument to provide additional characterization for thin films.

6.3 Experimental Section

6.3.1 Materials and Reagents

Absolute ethanol, decanethiol (DT), 11-mercaptoundecanoic acid (MUA), 11-mercapto-1-undecanol (MUL), HEPES, \(N\)-hydroxysuccinimide (NHS), \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarbodiimide hydrochloride (EDC), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Nitric acid was from J. T. Baker (Phillipsburg, NJ). All chemicals and reagents were used as received. Recordable compact disks (MAM-A Gold CD-R) were purchased from Inkjet Art Solutions (Salt Lake City, UT). Gold (99.999\%) was purchased from Ernest Fullam (Latham, NY). All experiments used 18 M\(\Omega\), deionized water (NANOPure, Barnstead, Dubuque, IA). The 10 mM solutions of DT, MUA, and MUL were made in ethanol. HEPES-buffered saline (HBS, 10 mM HEPES, 150 mM sodium chloride) was prepared with the pH adjusted to 7.4 using 10 mM NaOH and stored at 4 °C.

6.3.2 Grating Preparation

Gold gratings were prepared from commercially available, gold-coated recordable compact disks (CD-R) as described in a previous publication.[25] Following removal of the protective polymer and lacquer layers by wet chemical treatment, the underlying gold grating was cleaned in an oxygen plasma for 1 min to remove any residual organic impurities (plasma cleaner PDC-32G, Harrick Scientific, Ossining, NY). It was subsequently washed several
times with water and ethanol followed by drying with a nitrogen stream. The grating was then placed in a vacuum chamber for deposition of additional gold (Denton Vacuum Turbo III, Morristown, NJ). Approximately 50 nm of gold was deposited onto the existing ~50 nm at a rate of 1-2 Å/s and a pressure of \(7 \times 10^{-5}\) Torr under nitrogen atmosphere.

### 6.3.3 Atomic Force Microscope (AFM) Imaging

AFM images of the sample surfaces were acquired with a Dimension 3100 scanning probe microscope and Nanoscope IV controller (Veeco Metrology, LLC, Santa Barbara, CA). Imaging was performed in tapping mode using silicon TESP7 AFM tips (Veeco Metrology, LLC, Santa Barbara, CA) with a spring constant of ~70 N m\(^{-1}\) and a resonance frequency of ~280 kHz.

### 6.3.4 Formation of Thin Organic Films

Gold gratings were kept under a UV lamp (Spectroline model SB-100P, Spectronics Corp., Westbury, NY) for 30 min and then cleaned in an oxygen plasma for 1 min before soaking in 10 mM ethanolic solutions of DT, MUL, or MUA for 4 h. After incubation, the gratings were rinsed vigorously with ethanol and dried in a nitrogen stream immediately prior to characterization. Activation of carboxylic acid terminated surfaces of MUA was achieved by exposing the sample to an aqueous solution containing 150 mM EDC and 30 mM NHS for 30 min. This reaction resulted in the formation of reactive succinimide esters on the surface. Subsequent attachment of BSA to the array surface was achieved by exposing the surface for 90 min to a 0.5 mg mL\(^{-1}\) solution of BSA in HBS. The sample was then rinsed with HBS and dried under nitrogen.
6.3.5  Infrared Reflection Absorption Spectroscopy

A Nicolet MAGNA 750 Fourier transform infrared spectrometer using a liquid nitrogen-cooled MCT detector was used to obtain infrared spectra. Reflectance spectra were collected with $p$-polarized light incident at 80° with respect to the surface normal. These spectra are presented as $-\log(R/R_0)$, where $R$ is the sample reflectance and $R_0$ is that from a reference sample. Each spectrum represents an average of 512 individual sample and reference scans acquired at 2-cm$^{-1}$ resolution. An octadecanethiolate-$d_{37}$ monolayer on a gold-coated glass slide served as the reference sample. IRRAS spectra were collected for each sample with a grating orientation both parallel and perpendicular to the incident light.

6.3.6  Ellipsometry

Film thickness measurements were performed using ellipsometry. A motorized, variable-angle null ellipsometer (Multiskop, Optrel GbR), operating in the PCSA configuration at a single wavelength (632.8 nm) at 70° angle of incidence, was used to measure ellipsometric parameters $\Delta$ and $\Psi$. The values of $\Delta$ and $\Psi$ were translated into equivalent optical thicknesses using a three-medium model. The optical constants of the gold substrate (refractive index $n$ and absorption coefficient $k$) were first determined using a two-phase model (air/substrate). The thickness of the adsorbed film was then found using a three-phase model (air/film/substrate) with a value of 1.45 for the refractive index of the film.[31]

6.3.7  Simulation of the Grating Response

We used PCGrate-S(X) v. 6.1 (International Intellectual Group, Inc., Penfield, NY) to model the reflectivity response (which is related to the diffraction efficiencies) of gold gratings.
Gratings covered with thin and thick layers of arbitrary shape can be modeled with the software. The modeling involves setting up of the grating geometry and defining the independent variables to be scanned. The solver is started once the system to be solved is completely defined (Table 6.1). The software prompts to save the results once the calculations are finished.

### 6.4 Results and Discussion

The gratings used in this study were prepared from gold CD-Rs as previously reported.[25] Briefly, square pieces were cut from a CD-R such that one side was parallel to the grating direction. The size of the pieces was kept small (50×50 mm) to minimize the influence of grating curvature. Cut pieces were exposed to concentrated nitric acid solution for 5 min to remove the protective layers. The gold gratings prepared in this manner were visibly translucent with a ~50-nm layer of as-received gold. To minimize artifacts due to underlying dye and polycarbonate layers, 50 nm of additional gold was deposited on top of these gratings to render them opaque. The topography of the grating samples was visualized by atomic force microscopy operating in tapping mode. Figure 6.2A shows the topography of a typical grating surface. Roughness analysis revealed a root-mean-squared roughness of 1.2 nm μm⁻². A cross-sectional profile perpendicular to the grating direction (Figure 6.2B) depicts a pitch of ~1500 nm and an amplitude of ~80 nm. Although it is possible that AFM tip convolution could impact the measured cross section, the shallowness of the grating and the sharpness of the AFM tips used for measurement suggest that these data provide an accurate representation of the sample profile. The shape of this surface profile approximates that of a trapezium with rounded corners.
### Table 6.1 Modeling and Simulation of the Grating Response in PCGrate.

<table>
<thead>
<tr>
<th>General</th>
<th>Accuracy optimization</th>
<th>Calculation mode: <em>Resonance</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type of low border conductivity: <em>Finite</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Check all boxes corresponding to Green functions and their normal derivatives</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of terms = 50% of points N</td>
</tr>
<tr>
<td>Grating shape</td>
<td></td>
<td>Type of grating shape: <em>Plane</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grating shape parameters: Grooved width = 100 mm, height = 50mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of plane section: Meridional = 1, Sagittal = 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Period = 1500 nm</td>
</tr>
<tr>
<td>Beam and</td>
<td></td>
<td>Wavelength = 800 nm</td>
</tr>
<tr>
<td>mounting</td>
<td>Wave front type = <em>Plane</em></td>
<td></td>
</tr>
<tr>
<td>geometry</td>
<td>Type of diffraction = <em>Classical (in-plane)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type of polarization = <em>NP ((P+S)/2)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optical mount configuration = <em>General</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polar Angle = 80 deg</td>
<td></td>
</tr>
<tr>
<td>Scanning options</td>
<td>Wavelength range = 2500 nm to 14285 nm</td>
<td></td>
</tr>
<tr>
<td>Layers</td>
<td>Layer 0</td>
<td>Refractive index = 1.000</td>
</tr>
<tr>
<td></td>
<td>Border 1</td>
<td>Load the border profile from the saved profile as recorded by the AFM.</td>
</tr>
<tr>
<td></td>
<td>Layer 1</td>
<td>Load <em>Au_Palik.RI</em> from the in-built library of RIs.</td>
</tr>
</tbody>
</table>
Figure 6.2  (A) AFM image of gold grating. (B) Line profile showing cross-section of the grating.
The gold gratings were subsequently used as substrates to prepare alkanethiolate self-assembled monolayers. A variety of surface functionalities can be readily fabricated on gold surfaces by tailoring the headgroup, X, of an \( \omega \)-functionalized \( n \)-alkanethiol, HS-(CH\(_2\))\(_n\)-X, and allowing it to self-assemble on gold.[32, 33] Spontaneous chemisorption of alkanethiol molecules occurs from ethanol solutions.[25, 26] The 10 mM solutions of DT, MUL, and MUA were prepared in ethanol, and the gold gratings were immersed for 4 h. The samples were rinsed with ethanol to remove unbound and weakly attached molecules from the surface. The films thus formed showed a wetting behavior that was consistent with the literature.[34] DT film showed strong hydrophobic behavior due to exposed methyl and methylene groups as indicated by the beading of water droplets. MUL and MUA films were hydrophilic and completely wetted by water. These preliminary results indicated well-formed surface films.

The formation of well-defined alkanethiolate monolayers was confirmed by IRRAS measurements. Figure 6.3 shows absorbance spectra for the various alkanethiolate films with the grating oriented such that the groove direction was parallel to the incident light beam (vide infra). Spectra for the C-H stretching region (2800–3000 cm\(^{-1}\)) are shown in Figure 6.3A while the positions of the absorbance peaks are provided in Table 6.2. The various peaks are consistent with previous reports.[4, 35] The peak at 2965 cm\(^{-1}\) in DT spectra corresponds to asymmetric in-plane C-H stretching (\( \nu_a \)) of the terminal methyl groups. The absence of this peak in spectra of MUL and MUA films clearly distinguishes them from DT. The two peaks at 2935 and 2879 cm\(^{-1}\) in DT are due to Fermi splitting of symmetric methyl C–H stretching mode (\( \nu_s \)). The peaks at 2921 and 2851 cm\(^{-1}\), which are present for all three films, are due to symmetric (\( \nu_s \)) and asymmetric (\( \nu_a \)) C–H stretching modes in the substituent
Figure 6.3  (A) Infrared reflection absorption spectra for decanethiol (DT), 11-mercaptopo-1-undecanol (MUL), and 11-mercaptopoundecanoic acid (MUA) films on gold grating in C-H stretching region. (B) Spectra for MUL monolayer in the O-H stretching region. (C) Infrared reflection absorption spectra for MUA film and Bovine Serum Albumin (BSA) covalently attached on MUA surface between 1600–1800 cm⁻¹.
Table 6.2  C-H Stretching Mode Peak Positions (cm$^{-1}$) for Functionalized Alkanethiolate Monolayers Chemisorbed on Gold Gratings.

<table>
<thead>
<tr>
<th></th>
<th>DT</th>
<th>MUL</th>
<th>MUA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$-v$_s$</td>
<td>2851</td>
<td>2850</td>
<td>2854</td>
</tr>
<tr>
<td>CH$_2$-v$_a$</td>
<td>2921</td>
<td>2918</td>
<td>2923</td>
</tr>
<tr>
<td>CH$_3$-v$_s$</td>
<td>2879, 2935*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_3$-v$_a$</td>
<td>2965</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
methylenic groups. In MUL, the hydroxylterminated surface is reflected by a broad absorbance in the OH stretching region (Figure 6.3B).[36] MUA shows peaks at 1740 and 1721 cm$^{-1}$ due to non-hydrogen-bonded and hydrogen-bonded C=O stretching modes associated with the terminal carboxylic acid group (Figure 6.3C). MUA surfaces can be readily activated with NHS/EDC to create reactive succinimide esters that can combine with primary amine groups on BSA.[25, 37] A sample was made using this method in which BSA protein was covalently attached to the chemisorbed MUA on gold. Infrared absorption spectra of this sample (Figure 6.3C) show a strong absorption centered at 1662 cm$^{-1}$, which can be attributed to hydrogen-bonded C=O mode in bundled internal R-helices.[36, 38] Loss of the majority of the signal from the C=O peaks at 1740 and 1721 cm$^{-1}$ indicates formation of a dense layer of protein on MUA. These results illustrate that gold gratings provide suitable substrates for construction of molecular alkanethiolate films that are consistent with films formed on typical gold-coated glass.[34]

In addition to the expected vibrational spectra for these adsorbed films, a strong absorbance was also observed at ~3400 cm$^{-1}$ when the grating direction was oriented perpendicular to the plane of incidence of the light beam (Figure 6.4A). No such absorbance was observed when the sample was rotated with the grooves parallel to the plane of incidence. This suggests that this “anomalous” absorbance was related to diffraction from the grating. To investigate the origin and characteristics of this absorbance, the optical response of the grating was simulated using a commercial solver (PCGrate, International Intellectual Group, Inc., Penfield, NY) that employs an integral method to numerically solve the electromagnetism equations. The profile of the gold grating was modeled as that obtained from the AFM scan of the surface (Figure 6.2B). The grating solver used a frequency-
Figure 6.4: (A) Measured absorbance spectra for gold grating oriented parallel and perpendicular to incident light. (B) Calculated efficiency of 0 order reflection of $p$- and $s$-polarized light from the gold grating. (C) Calculated efficiency of $-1$ order reflection of $p$- and $s$-polarized light from the gold grating.
dependent refractive index for gold.[39] This calculation revealed that, under these experimental conditions, only the 0 and −1 orders appear for the wavenumbers between 3213 and 4000 cm\(^{-1}\) while no propagating diffracted orders appear at 700–3213 cm\(^{-1}\). As the wavelength is increased (wavenumber is decreased), the propagating −1 diffraction order becomes parallel to the grating surface. Once parallel, this order passes off and becomes evanescent (Figure 6.1). This threshold phenomenon[30] can occur on metallic as well as dielectric gratings. Indeed, one can estimate the conditions at which this threshold occurs with the grating equation

\[
\sin(\theta_i) + m\lambda / d = \sin(\theta_r)
\]  

(6.1)

where \(\theta_i\) and \(\theta_r\) are the angles of incident and reflected light, \(m\) is the diffracted order, \(\lambda\) is the light wavelength, and \(d\) is the grating pitch. Using an incident angle of \(\sim 80^\circ\) and a pitch of \(\sim 1500 \text{ nm}\), Equation 6.1 predicts that the −1 order passes off at \(\sim 3350 \text{ cm}^{-1}\), which is similar to the position of the anomalous absorbance observed in Figure 3A. This loss of the −1 order results in a redistribution of energy into the 0th order and, consequently, a strong absorbance at the associated wavelength (or wavenumber).

Although Equation 6.1 can be used to estimate the conditions of this threshold, it does not take into account several important details. For instance, threshold phenomena are especially sensitive to the refractive index of the medium next to the grating surface.[16] Any changes in the dielectric environment at the grating surface are capable of changing the properties of evanescent waves generated and, thus, the threshold characteristics. Indeed, sensing methods based on threshold phenomena have been reported for determination of bulk refractive indices[16] and size characterization of colloidal suspensions.[17] It has been established that strong changes in reflectivity of highly conducting gratings can occur in the vicinity of
threshold phenomena when \( p \)-polarized light is used, which explains the sharp absorption noted on the grating samples.[30]

The diffraction behavior of gratings is typically reported in terms of diffraction efficiencies. The efficiency of a diffracted order is the fraction of incident energy that is distributed in that order. The diffraction efficiencies are known to be reliable and sensitive parameters and are frequently employed to measure the response of grating-based sensors.[19, 40] Thus, we chose diffraction efficiencies to compare between experimental data and theoretical simulations. Figure 6.4B depicts the calculated reflection efficiencies of the order reflection for incident \( s \)- and \( p \)-polarized infrared light. A near-perfect reflection efficiency appears for \( s \)-polarized light over these energies, which reflects the fact that gold is an excellent mirror for infrared light. For \( p \)-polarized light, however, a significant loss in reflection efficiency is observed in the calculations with a peak near \( \sim 3300 \) cm\(^{-1} \). This loss in efficiency of the 0th-order reflection is equivalent to an absorbance at these energies. Although the exact peak position for the calculated response is shifted slightly from that observed experimentally, the shape and character of these responses is very similar. Notably, both the pitch and the angle of incidence play a large role in the exact value of the calculated absorbance, so small differences can result in large shifts in the position of the absorbance.

Figure 6.4C depicts the calculated reflection efficiency of the \( -1 \)st diffracted order. Note that both \( p \)- and \( s \)-polarized infrared light disappear for wavenumbers less than 3213 cm\(^{-1} \), which marks the threshold transition of this diffraction order from a propagating to an evanescent wave. This passing off of the \( -1 \)st order is the source of the redistribution of energy into the other orders and the large change in efficiency of the 0th reflected order, which is the origin of the absorbance seen in the measured infrared spectrum.
Since a threshold phenomenon such as this is sensitive to changes in the refractive index near the grating surface,[16] we chose to test the sensitivity of the measured peak position to the thickness of the alkanethiolate molecular films. The position of the threshold absorbance peak was measured for the DT, MUL, MUA, and BSA on MUA films. For all the samples, the spectra obtained with the grating oriented parallel to the incident light were featureless except for the molecular absorption regions as shown in Figure 6.3. However, a strong absorbance was observed near ~3300 cm\(^{-1}\) for all films with the grating oriented perpendicular to the incident light. Figure 6.5A depicts the absorbance peaks for each of the samples under perpendicular illumination. A spectrum from a bare gold grating is also shown for comparison. The peak appears at the highest wavenumber for bare gold and shifts to lower wavenumbers in the presence of the absorbed films. The shift in wavenumber was compared to the absolute film thickness as determined by ellipsometry (Figure 4B and Table 6.3). The DT, MUL, and MUA films showed film thicknesses that are consistent with those reported in the literature at 1.62, 1.58, and 1.55 nm.[4] The thickness of the film containing BSA covalently attached to MUA was 5.17 nm, which is in agreement with earlier reports.[25, 41] A comparison of the film thickness and infrared absorbance provides a near-linear relationship (Figure 6.5B). With increasing film thickness, the absorbance peak shifts to lower wavenumbers. A linear fit of this trend gives a sensitivity of 3.7 cm\(^{-1}\) nm\(^{-1}\).

The response of the grating was simulated to theoretically evaluate the sensitivity of peak position toward changes in the index of refraction at the grating surface. Figure 6.6 shows that the position of the absorbance peak shifts to lower wavenumbers in a linear fashion as the refractive index is increased, which is in agreement with the trend observed in the experimental results. The slope of the line gives a sensitivity of 3100 cm\(^{-1}\) RIU\(^{-1}\). In terms of
Figure 6.5  (A) Absorbance spectra in threshold region for decanethiol (DT), 11-mercapto-1-undecanol (MUL), 11-mercaptoundecanoic acid (MUA) and Bovine Serum Albumin (BSA) on MUA films. (B) Ellipsometrically measured film thickness versus shift in threshold
Table 6.3  Position of Threshold Absorbance Peak of Various Samples.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Peak Position (cm⁻¹)</th>
<th>Ellipsometric Film Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>3430</td>
<td>0</td>
</tr>
<tr>
<td>Au/HS(CH₂)₉CH₃</td>
<td>3419</td>
<td>1.62</td>
</tr>
<tr>
<td>Au/HS(CH₂)₁₀OH</td>
<td>3421</td>
<td>1.58</td>
</tr>
<tr>
<td>Au/HS(CH₂)₆COOH</td>
<td>3421</td>
<td>1.55</td>
</tr>
<tr>
<td>Au/HS(CH₂)₉COOH + NHS + BSA</td>
<td>3411</td>
<td>5.17</td>
</tr>
</tbody>
</table>
Figure 6.6 Calculated shift in threshold absorbance versus refractive index of surrounding environment.
wavelength, this translates to ~3000 nm RIU⁻¹, which is comparable to the sensitivity of surface plasmon resonance-based sensors (3100-8000 nm RIU⁻¹).[42] We also investigated the impact of changing the angle of incidence and grating pitch on the position of this peak. It was found that an increase of 1° in angle of incidence shifted the peak position by 6 cm⁻¹ while a 10-nm increase in pitch shifted the peak position by 20 cm⁻¹. Although this sensitivity of peak position to system geometry limits the ability to quantitatively predict the exact position of this absorbance, relative changes due to absorbed films or refractive index changes were not very sensitive to these differences. Indeed, calculations using three different grating geometries with sinusoidal, triangular, and trapezoidal profiles exhibited the exact same shift in wavenumber with increasing refractive index. Thus, with proper calibration, relative film thicknesses can be quantitatively determined by shifts in this threshold absorbance peak. In addition, the ability to shift the peak position by tuning the pitch of the grating or the angle of incidence can be used to provide flexibility to the platform. For example, pitch modulation could be used to shift the absorption peak outside the spectral region where molecular absorbance peaks appear.

6.5 Conclusions

We have described a grating-coupler platform as a simple and sensitive method for determination of film thicknesses using a conventional IRRAS instrument. With a grating substrate, a sharp absorbance is observed at grazing angles of incidence associated with passing off of the −1 diffracted order. Both experimental measurements and theoretical calculations demonstrated that the position of this peak was a strong function of the dielectric properties of the grating interface. Although the exact position of this peak was found to be
highly dependent upon the details of the grating geometry, the shift in wavenumber due to a change in the refractive index near the grating interface could be used to quantitatively determine relative film thicknesses. This behavior provides a simple sensing platform for thin films or molecular adsorption events. The sensitivity of this absorbance shift was found to be $3.7 \text{ cm}^{-1} \text{ nm}^{-1}$, which is sufficient to detect the formation of monomolecular films. These results illustrate how a grating substrate can be exploited in a standard infrared reflectance measurement to independently determine the chemical identify and relative thickness of adsorbed surface films.

6.6 References


CHAPTER 7. GENERAL CONCLUSIONS

The work presented in this dissertation is a contribution to the field of label-free biosensing. Proof-of-concept experiments demonstrate that optical phenomena such as diffraction and surface plasmon resonance can be used as signal-transduction mechanisms for reporting the biomolecular recognition events occurring at surfaces. The techniques presented in this work are shown to be compatible with microarray-based detection technologies and represent a fast and robust method of performing high-throughput biosensing.

The development of a grating-based SPR imaging method is described for microarray samples in Chapter 3. Several innovative approaches have been presented in this chapter to address the issues regarding the construction of SPR-active substrate and patterning of surfaces to create microarray samples. Inexpensive plastic gratings were shown to support surface plasmons very efficiently. Gold surfaces were patterned with functionalized alkanethiol molecules to create microarrays in a single print and wash step that took advantage of high viscosity and low vapor pressure of glycerol. The covalent attachment of proteins by an amine coupling chemistry was clearly observed by simply photographing the microarray surface using a digital camera.

The details of a multicolor SPR imaging method are presented in Chapter 4. A simple color-readout mechanism is used to deduce the response of the SPR sensor in a microarray format. A chemical inkjet printing method was used to deliver precise amounts of proteins in predetermined spatial locations on the sensor chip. The method can also be extended to ascertain both the refractive index and thickness of adsorbed films in a way similar to the well-known two-color SPR method. The need for narrow band-pass filters is obviated which
also facilitates the use of natural light as illumination source. One can envision a portable microsensor based on the described method which utilizes sunlight as the source of light and detects the presence of chemical and biological agents in environment, food and water samples.

Chapter 5 describes a new type of sensor which is based on enhanced transmission of light through modulated gold films under the conditions of SPR. The wavelengths that couple to the surface plasmons are selectively tunneled from one surface of gold film to the other. This results in a peak in the transmission spectra. As with other SPR sensors, this peak is sensitive to the formation of molecular films at the sensor surface. A model immunoreaction between BSA and anti-BSA was monitored using this sensor platform and the binding affinity for this system was measured quantitatively. The successful demonstration of this new sensor platform as an immunosensor is just one of the many potential applications for which it can be used.

A grating-coupler assisted infrared reflection absorption spectroscopy (IRRAS) for the characterization of organic thin films is described in Chapter 6. It is shown that by using gold-coated gratings are used instead of conventional gold-coated glass slides, the information about the thickness of the molecular films can be ascertained in addition to the usual information about the molecular vibrations that the technique provides. This is made possible due to existence of a “threshold anomaly” near the conditions of the disappearance of propagating diffracted orders into evanescent modes. An additional absorption peak is observed at the threshold conditions in the reflection spectra whose position is sensitive to the thickness of the adsorbed film. Thus, a single measurement can accurately describe the chemical identity as well as thickness of molecular films.
In the original piece of research that is described in this dissertation, we have built several new approaches for the development of biodetection platforms and sensor chips using protein samples. These approaches are readily adaptable to the emerging areas of functional proteomics using protein microarrays in addition to the fields of microarray-based label-free detection. The progress in these areas will directly impact the advances that would enable a better understanding of the relationship between biological molecules which can subsequently propel the scientific community towards the discovery of better drugs and mitigation of diseases.

Future directions of this work include applying the developed techniques towards specific applications. For example, the described sensors can be utilized to develop portable diagnostic devices for homeland security that can enable easy detection of multiple chemical and biological agents.