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Evaluating biofilm performance using surface plasmon resonance

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EVALUATING BIOFILM PERFORMANCE USING SURFACE PLASMON RESONANCE

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Master of Science

by Kimberly Ann Kroiss

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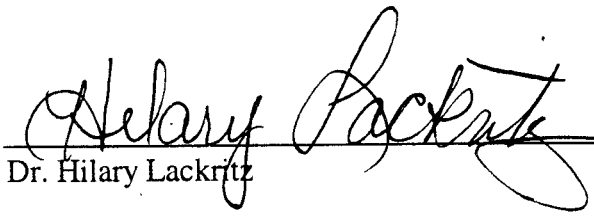

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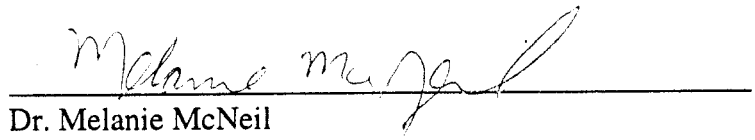

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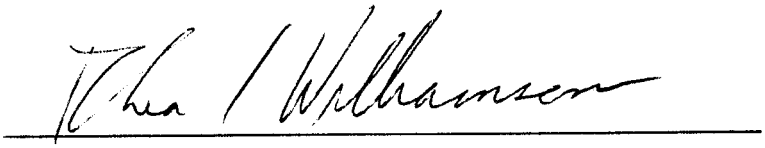

ABSTRACT

EVALUATION OF BIOFILOMS USING SURFACE PLASMON RESONANCE

by Kimberly A. Kroiss

Surface plasmon resonance (SPR) has been used as a diagnostic tool for over thirty years. Current technology is capable of the label-free characterization of both chemical and biological systems, however commercially available SPR systems have limited sensitivity and are difficult to use. A low cost biosensor with increased sensitivity is desired. A Metricon Prism Coupler was modified to function as an SPR spectrophotometer and a flow cell was designed for characterizing the binding of biotinylated β-galactosidase to streptavidin. Instrumentation capability was determined using 633nm and 1550nm wavelength lasers to detect streptavidin-biotin chemistry on a biosensor. Streptavidin was bound to a self assembled monolayer of thiol groups and exposed to a dilute β-galactosidase solution to assess the limit of detection. The flow cell design and injection system used to observe protein binding were validated and the instrumentation demonstrated an increased sensitivity at longer wavelengths.

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ADS	Aldehyde Dextran Sulfonate
AFM	Atomic Force Microscopy
BG	B-Galactosidase
CD	Carboxylated Dextran
CMD	Carboxymethylated Dextran
CV	Coefficient of Variance
DI	Deionized Water
DOE	Design of Experiment
GBP	Gold Binding Peptide
GPCR	G-Protein Coupled Receptor
IPA	Isopropyl Alcohol, 2-Propanol
mM	Millimolar
RIU	Refractive Index Unit
SPR	Surface Plasmon Resonance
SPW	Surface Plasmon Wave
ssDNA	Single Stranded Oligonucleotides

1.0 INTRODUCTION

1.1 Background

Biosensors utilize electronic, photonic, biologic, chemical, or mechanical means to produce signals that can be used for the identification or monitoring of biological phenomena. Applications include health, environment, and the identification of chemical or biological agents. .Surface plasmon resonance (SPR) is a technique that can be used for biosensing applications. It is a label free method that does not require modification for detection.

This technique has been used for over thirty years. SPR originated in the 1960's when experiments demonstrated that packets of electrons were optically excited by attenuated total reflection [3]. Since that time, the technique has undergone extensive research and development. Currently, SPR is used for the label free characterization of chemical and biological reactions. Table I contains a list of some common SPR biosensor applications.

Table I. SPR Biosensor Applications [23].

Qualitative	**Quantitative**
Monitor Purification	Active Concentration
Specificity	Kinetics (k_a, k_d)
Epitope Mapping	Equilibrium Constants (K_D)
Molecular Assembly	Thermodynamics ($\Delta H^{Vant\ Hoff}$)
Ligand Fishing	Stoichiometry
Small Molecule Screening	Mechanism

Commercially available SPR biosensors used for characterization and quantification of biomolecular interactions in real-time are expensive to purchase and difficult to use [4]. Development of a more sensitive biosensor at reduced cost would have potential applications ranging from the electronic nose to pharmaceutical development.

The objective of this work is to refine the technique to increase the efficacy and sensitivity of SPR by using a non-standard laser light source, and to test this improved method on different sensor chemistries. The capabilities of a prism coupler using SPR to detect protein binding were assessed.

1.2 Surface Plasmon Resonance

SPR is a charge-density oscillation occurring at the interface of dielectrically different materials. Plasmons, packets of excited electrons, on a metal will absorb the

energy of incident photons at a specific resonance wavelength [24]. The surface plasmons on a metal film (usually silver or gold) are excited by an exposure to light. The polarized laser light source is directed through a prism onto a sensor chip. The light reflected by the metal surface is directed back thru the prism and measured by a reflective spectrophotometer [4]. The molecules of interest are located on the sensor surface [6]. Material bound to or in contact with the metal surface will affect the resonance frequency and resulting absorbance. Data is most commonly acquired by varying the angle of incidence while using light source of fixed wavelength. The process is detailed in Figure 1.

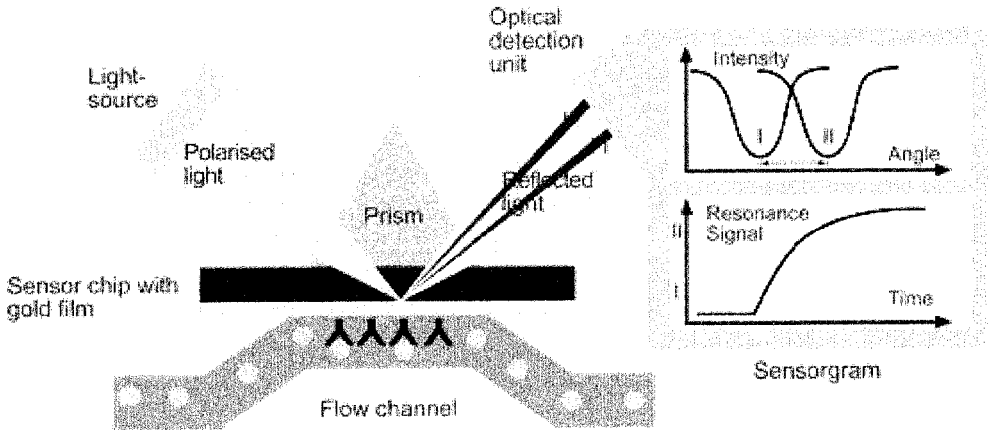


Figure 1. SPR based biosensor [6].

Traditionally, lasers having wavelengths between 600 and 800nm have been used. Utilization of longer wavelengths would potentially give increased sensitivity, because small changes in refractive index would have a more pronounced effect [4]. Sensor capability would be greatly improved because smaller changes on the surface would be

detected. This is critical because improved sensitivity would allow lower concentrations to be detected.

1.3 Theory

A fully reflected beam of p-polarized light does not have a net loss of energy after contacting the sensor surface [27]. The impact of light on the surface exposes the low refractive medium to an electric field, or evanescent field wave. The amplitude of the evanescent wave decreases exponentially to a depth of roughly one light wavelength, or about 37% of the maximum intensity. A metal interface is penetrated by the p-polarized component of the evanescent wave, which excites the electromagnetic surface plasmon waves (SPW) and results in an enhanced evanescent wave. The SPR phenomenon is sensitive to the refractive index at the gold surface. The wave vector bound to the surface of the conductor and the vector component of the incident light are parallel to the conductor surface and are equivalent to the wave vector of the surface plasmons. The magnitude of the surface parallel wave vector is a function of the wavelength and angle of incidence per Equation 1 [27].

$$k_x = \frac{(2\pi)}{\lambda} n_1 \sin\theta \qquad \text{Equation 1}$$

Where k_x = Surface parallel wave vector
n_1 = Prism index of refraction
θ = Angle of incident light from a vector normal to the surface
λ = Wavelength of light

The wavelength of the surface plasmon wave is dependent on the refractive indices of the conductor and the sample medium per Equation 2 [27].

$$k_{SP} = \left(\frac{2\pi}{\lambda}\right)\left(\frac{n_{gold}^2 n_2^2}{n_{gold}^2 + n_2}\right)^{0.5} \qquad \text{Equation 2}$$

Where k_{SP} = Surface plasmon wave vector
n_{gold} = Gold index of refraction
n_2 = Sample medium index of refraction
λ = Wavelength of light in a vacuum

An increase in sample index of refraction and penetration of a plasmon enhanced evanescent wave will increase the surface plasmon wave vector. The increase in sample index of refraction will occur when something binds to the surface. The light wave vector can be adjusted to match the plasmon wave vector by varying either the polarized light wavelength or the angle of incidence. The dielectric equations for this phenomenon are detailed by Knoll *et al.* When the wave vector and light energy are equivalent, a resonant absorption of the light occurs and a characteristic drop in the reflected light intensity is observed. The biomolecular interactions on the sensor surface change the refractive index within the evanescent wave penetration depth. The angle of incidence required for the SPR phenomenon is changed. This change is the measured response signal. Proteins have similar refractive indices, allowing SPR to function as a label free mass detector independent of the analyte and ligand. Also, the evanescent wave is penetrating the sample, so opaque sensor chips can be used [27].

1.4 Biofilms

A biofilm consists of microorganisms attached to a solid surface. Microorganisms capable of attachment include bacteria, fungi, yeasts, protozoa, and others. Biofilms can be used to grow biochemicals that are then purified and utilized for medicines, food additives, or chemical additives for cleaning products [3].

Streptavidin is a tetrameric protein (4×10^{13}kDa) used for various biochemical applications. The protein has a high affinity for biotin ($K_a \sim 10^{13}\ M^{-1}$) and is capable of one hundred percent binding [1]. The structure of biotin is shown in Figure 2.

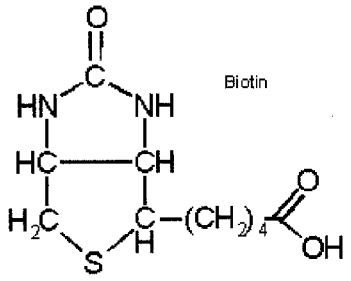


Figure 2. Biotin structure [30].

A label free detection process would be advantageous compared to the fluorescent labeling process, because the affect labeling has on the phenomena of interest in unknown. The label free technique ensures the method of detection does not affect the behavior observed. In this way, the characterization of biofilms with a sensitive label free sensor would further membrane development.

1.5 Optical Waveguiding Techniques

A Metricon prism coupler has been modified to use SPR. Both the instrumentation and the samples are different than those used in a traditional SPR instrument. A prism coupler uses optical waveguiding techniques to measure the thickness and refractive index of dielectric and polymer films [2]. Measurement of a film is conducted by bringing a sample into contact with the base of a prism by means of a pneumatically-operated coupling head. A laser beam is directed at the base of the prism and is reflected onto a photodetector. The intensity and angle of deflection are analyzed to determine the thickness and index of the sample. The process is detailed in Figure 3.

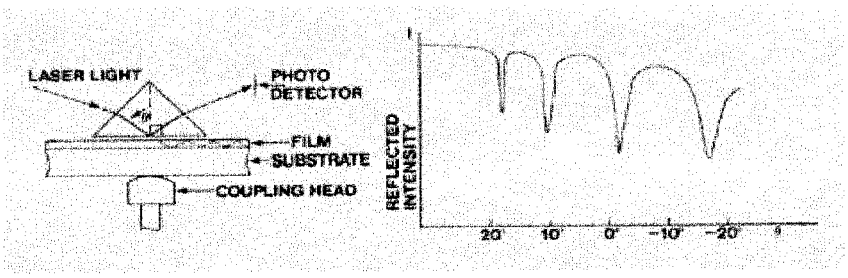


Figure 3. Schematic of a prism coupler and reflected light versus angle of incidence [2].

The modified instrument is intended as a baseline configuration for the characterization of biofilms. Binding of an analyte to the biofilm will affect the index of refraction. This is a result of the change in mass and is not specific to the biofilm, providing kinetic information. The sensor chips are single use, and are the only disposable component of the system. Sensor capability could be more effectively

evaluated using longer wavelength SPR. Future work includes designing an improved instrument prototype.

2.0 LITERATURE REVIEW

2.1 Topic of Research

Surface plasmon resonance is an excellent tool for characterizing biological systems, and therefore has direct applications for the pharmaceutical industry. Increasing sensor capability would be a benefit to the research and development of drug compounds. Currently available commercial instrumentation is complex, having integrated software systems for data analysis, report generation and automated sampling.

The wavelength of light is typically fixed while the angle of incidence is varied to detect changes in the sensor surface. The fixed wavelength results in a small change in resonance angle, and detection of these changes limits sensor capability. Using longer wavelengths of light should result in a sharper response and larger shifts in resonance angle. A review of the literature has shown that low cost units can be manufactured and used to generate reproducible experimental data. A Metricon Prism coupler has been modified to function as an SPR. Once the instrumental capabilities of the unit are assessed, it can be used to evaluate novel chemistries.

2.2 Current Commercial Methods

A large fraction of research publications using SPR to study biomolecular interactions utilize Biacore Instrumentation [7]. Biacore Instruments developed the first

commercial SPR biosensor for analyzing biomolecular interactions in 1990. These instruments can be used to determine kinetic rate constants and affinity. The capabilities include fully automated sampling, integrated software for generating data reports and analyzing data, and flow cells operated in series or in parallel maintaining a reference on the sensor chip and a baseline for each test. Microrecovery units recover material bound to the sensor surface for mass spectrometry analysis. The most common sensor surface has a thin film of carboxymethyl dextran matrix [6]. The S51 is Biacore's most advanced sensor. It has increased sensitivity, throughput, automation, and ease of use. Referencing is improved by having one reference and two ligand surfaces within a single flow cell [6]. The Biacore units are expensive, averaging $400,000.00.

Aviv Instruments has a biosensor based on plasmon waveguide resonance. Their system employs two He/Ne lasers for probing the sensor surface and a polarizer that produces plane-polarized light having two orientation options for examining individual biomolecular interactions or large changes on the surface [7]. Anisotropic examination of the surface yields information about ligand response to environmental changes. This sensor was used to detect conformational changes in agonists and antagonist on a sensor surface. This capability could benefit drug research.

Leica Microsystems biosensor uses modular components and fluidics similar to the traditional HPLC configuration, while incorporating their core technology of reflected light critical angle refractometers. The fluidics enable the recirculation of ligand or analyte during binding characterization. A large sensor surface allows interfacing with a mass spectrometer. The sensor has self-assembled monolayers (SAM's) of ligand. This

is an affinity based biosensor that can be configured for recirculation of ligand during immobilization or analyte during binding energy characterization [7].

There are significant current commercial applications for this technique. Improvements in the capability would be commercially viable.

2.3 Drug Research and Development Utilizing SPR

The elimination of labeling and rapid screening of SPR make it a tool of choice for characterizing drug compounds. Ahmad, *et al.* used SPR in an attempt to determine the binding energy of enantiomeric drug compounds to immobilized albumins. Subtle differences in the binding energy of enantiomers to human tissue were obtained, however the differences could have been attributed to the dilution process used to obtain low analyte concentrations, or non-specific binding to pipettes or vessels. Additional data runs would have allowed a more thorough statistical analysis and perhaps lowered the coefficient of variance to reveal statistically different binding energies. Three runs were obtained for each concentration and enantiomer and coefficient of variance values less than 25% were considered acceptable. This suggests more than the three-to five runs are required to differentiate between similar chemistries. Additionally, a more conservative coefficient of variance of less than 10% would result in greater data confidence.

Thurmond *et al.* used SPR to measure and quantify the binding of inhibitors to protein p38 kinase, a potential drug for autoimmune diseases. The characterization of inhibitor binding to this protein has been done in efforts to develop treatments for

inflammatory diseases. The physical binding of an inhibitor is an indication of its potency and efficacy. The dissociation constants obtained from the experiment were consistent for both kinetic and equilibrium analyses and comparable to those found in the literature. The extraction of binding constants allowed direct comparison of the inhibitors and provided insight into the role these proteins play in controlling inflammatory response. This type of experiment demonstrates the contribution of SPR to drug discovery.

SPR has also been researched as a potential glucose detection device by Hsieh *et al.* A modified glucose/galactose protein having a binding affinity matched to the glucose concentrations in the human body (1-30mM) was developed. The results suggest that miniaturization of such biosensors could be developed as continuous glucose monitoring devices.

The characterization of drug compounds, detection of protein binding, and potential use as a diagnostic tool all emphasize that this is a promising area of research. Improving the sensitivity of the technique would have a positive impact on drug research and development.

2.4 Biosensor Surfaces

Sensor surfaces should be chemically inactive and remain mechanically intact during the fabrication process [17]. The types of surfaces commonly used were investigated prior to selection of the surface for this experiment.

Gold is the most common metal used for plasmon resonance because of its free electrons. The angular shift resolution of a gold plated sensor chip is approximately 0.05 millidegrees, and a gold plated chip has a refractive index resolution less than $1x10^{-6}$ [16]. The angular shift resolution of a coated gold surface is sufficient for the characterization of most biomolecular interactions [16].

The metal surface required for plasmon resonance is incompatible with biological systems and has to be passivated for ligand compatibility [11]. The surface is commonly modified using a dextran layer. The desired ligand is then bound to the dextran layer [17]. Some researchers feel the contribution of the dextran surface is inseparable from the response of the biosensor, but a significant amount of data validates their use and experienced researchers offer suggestions for obtaining the best possible data [10].

Several methods of surface modification have been employed to functionalize SPR metal surfaces. Thin, dense, and uniform oxide coatings capable of functionalization have been used on silver SPR sensors [19]. Silver films have a higher sensitivity than gold films, because the SPR peak is narrower in the visible light region. However, poor Ag chemical properties have made it impractical as a stable sensor surface.

Metal oxide coatings were tested to stabilize the Ag surface, and were evaluated for robot printing DNA and SPR. Small variations in film thickness (less than one nanometer) can alter the index of refraction. Also, porosity could make the film vulnerable to chemicals used in binding experiments. Three metal films, chromium, aluminum, and niobium, were successfully deposited onto gold by evaporation and then oxidized. They were then hydroxylated by heating in air or immersion in acid, and

functionalized. The films were silanized by attachment of amine-modified silane and used in robot printing of DNA or protein arrays, demonstrating Ag films can be stabilized and made compatible with biological systems.

Biacore sensors have a 100nm thick hydrophilic matrix of 2-3% carboxymethylated dextran [17]. The carboxymethylated dextran (CMD) coating reduces non-specific analyte binding, but also contributes steric effects and limits diffusion of analyte [20]. The significance of these effects is dependent on the biological system being characterized, because steric hindrance and mass transport are both determined by molecular size. The surface capacity and sensitivity of a self assembled monolayer and a CMD hydrogel matrix were compared on Au films. Diagrams of the sensor chips are located in Figure 4. Affinity results indicated the self-assembled monolayer had less steric effects than the CMD hydrogel matrix.

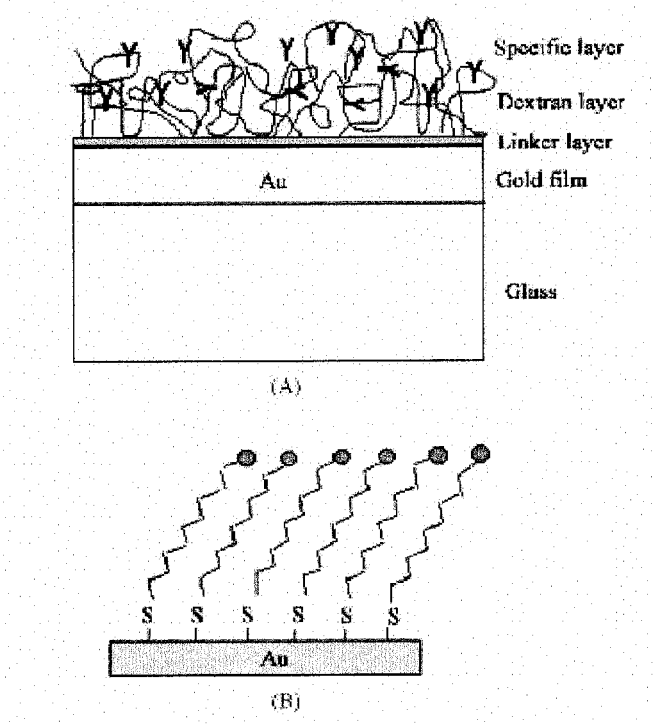


Figure 4. Sensor chip diagram. (A) Three-dimensional structure of CMD. (B) Two-dimensional structure of self-assembled monolayer [20].

Chegal *et al.* developed an aldehyde dextran sulfonate matrix structure (ADS) for ligand attachment to gold. The aldehyde functional group is responsible for covalent bonding to the ligand, and the sulfonate functional group is electrically attracted to the ligand. The affect of varying the aldehyde and sulfonate ratio on the binding mode was determined and results indicated ADS was superior to simple dextran, aldehyde dextran, and aldehyde ethylcellulose for protein immobilization.

Thiol based self-assembled monolayers (SAM's) are also used for binding of biotin ligands [18]. The immobilization and reconstitution of G-protein coupled receptors (GPCR) on a carboxylated dextran (CD) surface has been done using flow cells [9]. The

substantial fraction of the glucose moieties in the CD matrix were substituted with long alkyl groups, giving the film amphiphillic characteristics. Liposomes were efficiently captured when injected over the surface. This experiment was the first to demonstrate the immobilization and reconstitution of rhodopsin on a biosensor via a flow cell.

Based on this investigation, similar chemistry was used in this experiment to prove the methodology. Streptavidin was bonded to a thiol modified SAM, creating a biosensor surface for use with biotinylated compounds.

2.5 Experimental Technique

Published biosensor data frequently does not fit the simple bimolecular interaction model A+B=AB [10]. This has caused some investigators to consider biosensor data as invalid, however there are several experimental factors that complicate biosensor analysis. According to Myzska *et al.*, performing experiments under non-ideal conditions is more likely to be the cause for erroneous results, rather than flaws in the technique. Experimental techniques for avoiding surface-imposed heterogeneity, aggregation, avidity, nonspecific binding, mass transport limitation, and crowding will ensure the best possible data is obtained [10].

Several factors can lead to improvements in the experimental design. It is essential to use high quality reagents. Both the analyte and the ligand should be free of contaminants and the exact concentration established. The selected reagents should be monomeric in solution and have a 1:1 stoichiometry when mixed. Analytical

centrifugation is recommended to accurately determine if there are assemblages in the reagents.

Proper cleaning of the instrument will reduce the chance of contamination by foreign compounds or build ups in the flow system. Weekly washing of fluidics and syringe pumps is recommended to keep the system in good working order. Multiple flushing with the following four reagents is recommended: 0.5% SDS, 6M urea, 1% acetic acid, and 0.2M $NaHCO_3$. The system should be primed with water for extended down time. Three flushes are recommended whenever experimental reagents are changed.

Baseline data should be established before each experiment by sampling buffer solution without analyte. A flat response is expected, however a minor positive bulk refractive index change of 5-10RU is sometimes observed on the Biocore 2000 and 3000 instruments. If this is an inconsistent result, the fluid cartridge may need to be replaced. Bias can be reduced by ensuring test order is random and replicate runs are performed. Three to five repetitions are recommended for statistical analysis in addition to the baseline.

The amount of nonspecific absorption must be established. This can be done by injecting the analyte solution through the system with a ligand free biosensor surface. The highest concentration to be used for the experiment should also be used to check for nonspecific binding to increase the likely hood of this event to occur. Any low level nonspecific binding can be used as a reference point. Avidity is the agglomeration of molecules on the sensor. The assay must be designed to reduce the probability of bivalent

molecules cross linking. If the analyte is bivalent as in Figure 5a the molecules could potentially crosslink on the surface of the sensor, resulting in an apparent higher affinity. It is recommended that the bivalent molecule be bonded to the surface as in Figure 5b, ensuring a simple reaction model.

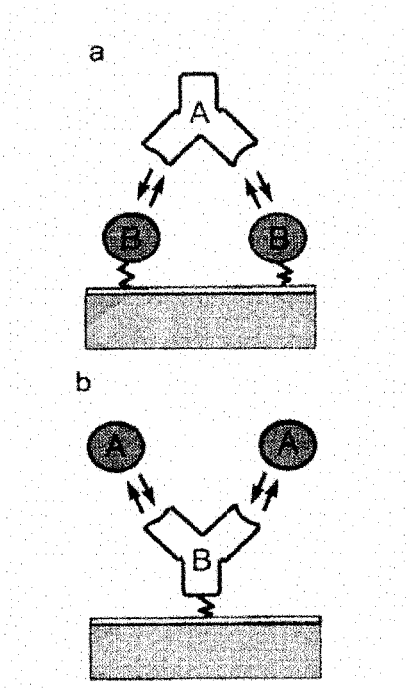


Figure 5. Assay orientation. (a) Binding a bivalent analyte (A) to a monovalent ligand (B) immobilized on the biosensor chip. The analyte could potentially cross-link on the surface. (b) Binding monovalent analyte (A) to a bivalent ligand (B) immobilized on the sensor chip ensures a simple reaction model [10].

These complications of the chemistry can make interpretation of the results problematic. Direct immobilization of the analyte onto the ligand is optimal and will create a more stable surface. Amines, aldehydes, or free thiols are commonly used on the surface of the ligand. Capturing the ligand with a molecule on the surface and then

capturing the analyte will complicate analysis. These immobilization methods are depicted in Figure 6a and b.

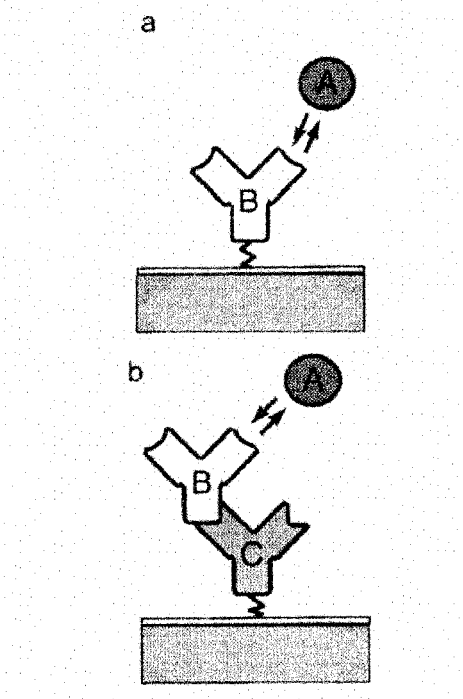


Figure 6. Immobilization methods. (a) Direct coupling of ligand (B) to sensor chip. (b) Capture of ligand (B) by molecule (C) bonded to chip surface [10].

Crowding, steric hindrance, and aggregation on the sensor surface can be minimized by having a low capacity on the surface. Biacore instruments are able to detect a response of less than 50 RU. The lower detection limit of the Metricon SPR will have to be determined.

Mass transfer limitations can be determined by running samples at varying flow rates. If changes in binding rate are detected, the system is mass transfer limited. In such

cases, using the fastest possible flow rate will minimize such limitations and increase the possibility of obtaining kinetic data. If the binding response reaches equilibrium or the observed rate decreases, the injection volume should be lowered, to avoid dispersion of the sample.

A biosensor was characterized by numerical analysis of SPR spectra [22]. The researchers found that resonance angle shift is proportional to surface density of analyte and the difference between protein layer and buffer solution refractive indices, Figure 7.

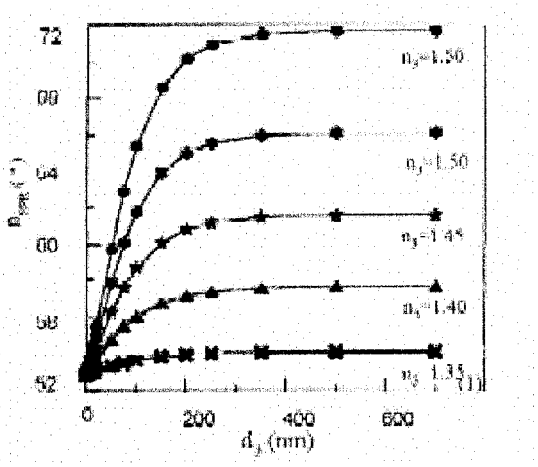


Figure 7. The effects of the bound analyte thickness (d_3) and refractive index (n_3) on resonance angle (θ_3) as determined by numerical analysis [22].

The gold film thickness also affected the resonance angle, but did not affect the correlation of resonance angle shift with surface density of analyte, Figure 8. Variation in the thickness of the glass chip did not significantly affect the resonance angle.

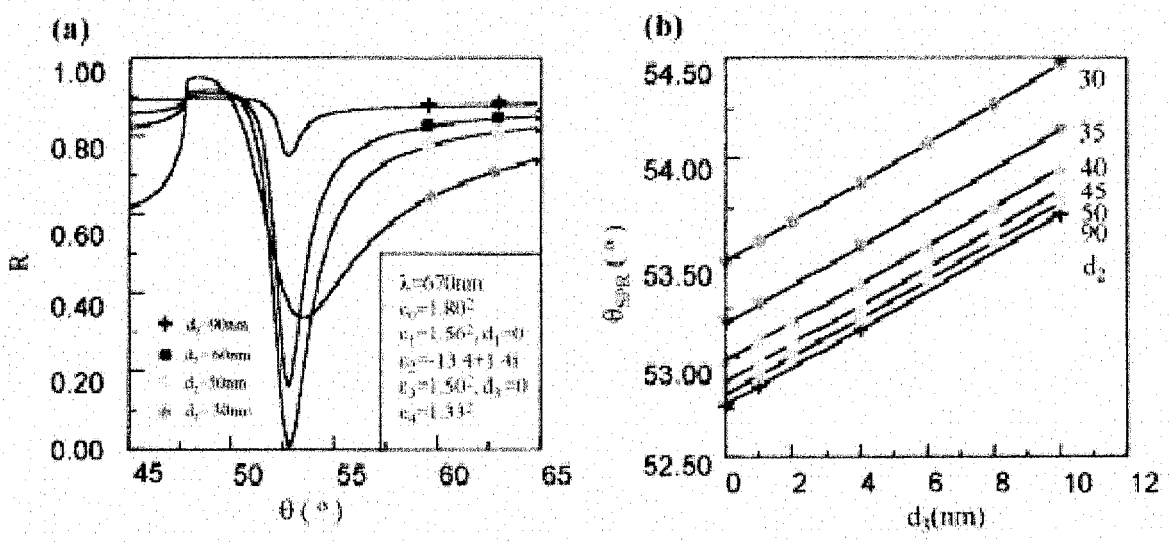


Figure 8. The effect of gold film thickness (d_2) on resonance angle (θ_{SPR}) according to numerical analysis. (a) SPR spectra under different gold thickness (d_2). (b) Angle of incidence (θ_{SPR}) via analyte thickness (d_3) under different gold thickness (d_2) [22].

2.6 Low Cost Instrumentation

Low cost, functional SPR instrumentation can be manufactured in the laboratory. An SPR spectrometer was developed for detection of DNA binding [13]. Biotinylated single-stranded oligonucleotides (ssDNA) were bound to the surface of a gold plated sensor chip via an avidin-biotin linkage. A shift in resonance wavelength was detected as analyte ssDNA hybridized with the ligand ssDNA bound onto the chip surface. A schematic of this simple SPR is shown in Figure 9.

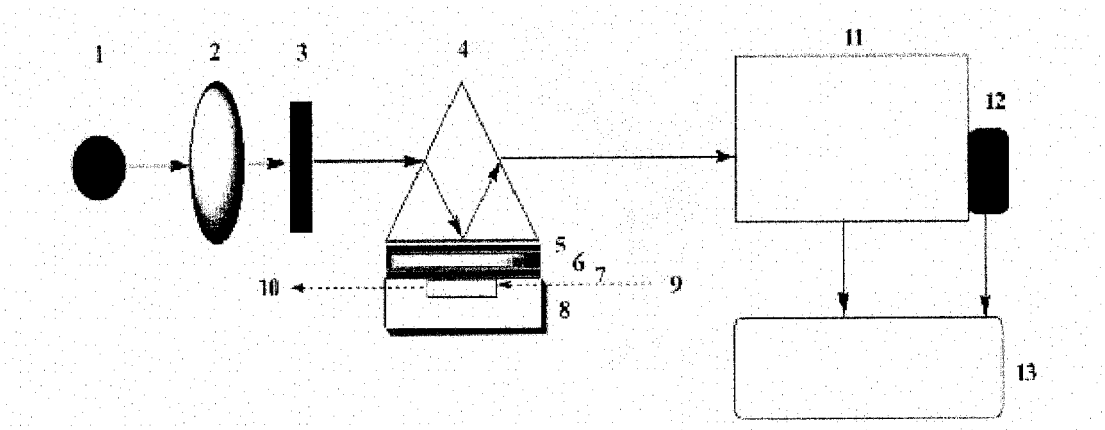


Figure 9. Schematic of a SPR instrument. (1) Tungsten halogen lamp. (2) Convergent lens. (3) Polarizer. (4) Prism, η=1.516. (5) Cedar Oil, η=1.515. (6) Glass slide. (7) Gold-coated film, η=1.516. (8) Flow-cell. (9) Sample entrance. (10) Sample exit. (11) Monochromator. (12) CCD. (13) Computer [13].

A thin (50±2nm) gold film was vacuum deposited onto a glass slide. The gold surface was coated with a layer of 6-Mercaptohexanoic acid to make it compatible with the ssDNA. After soaking the gold plated chip in acid for 2 hours, it was rinsed first with ethanol, then with deionized water, and finally dried. The refractive indices of the prism, cedar oil, and gold film were matched to allow coupling of the polarized light. The incident angle of light was optimized and then fixed. Analyte solution was then passed through the flow cell and changes in the resonance wavelength were noted upon exposure to various concentrations of ssDNA. While the apparatus is simpler than that proposed here, some of the substrates can be prepared similarly and the refractive indices of will be matched.

Due to the nature of sensor materials and the refractive index of aqueous sample solutions, SPR is sensitive to changes in temperature [22]. An inexpensive, temperature

regulated, and portable SPR was developed [15]. It was intended for both laboratory use as well as for the identification of food pollutants and environmental toxins in the field. The sensor is enclosed in a small temperature controlled enclosure. The refractive index of aqueous solutions is dependent on temperature, and a change of 0.1°C will change the refractive index by a factor of $1x10^{-5}$. This is a significant difference because the smallest detectable change in an SPR system is of similar magnitude. Temperature often also affects the ability of the ligand to bind to analyte. If an optimum binding temperature is known for a sensor, temperature control in a portable device will ensure optimum performance regardless of changes in ambient conditions.

Temperature effects on protein (Staphylococcus aureus enterotoxin B) binding rate and refractive index were studied using a Texas Instruments SPR module (Spreeta). Sensor chip surfaces were pre-functionalized with a gold binding peptide (GBP). This passivates the sensor surface for covalent ligand attachment and reduces nonspecific binding. These sensor chips are stable for up to one month at 4°C if stored in buffer solution.

The change in refractive index of temperature can be seen in Figure 10. Temperature was increased in steps of 6°C. Solutions were equilibrated after each increase and six repetitions were performed for each temperature.

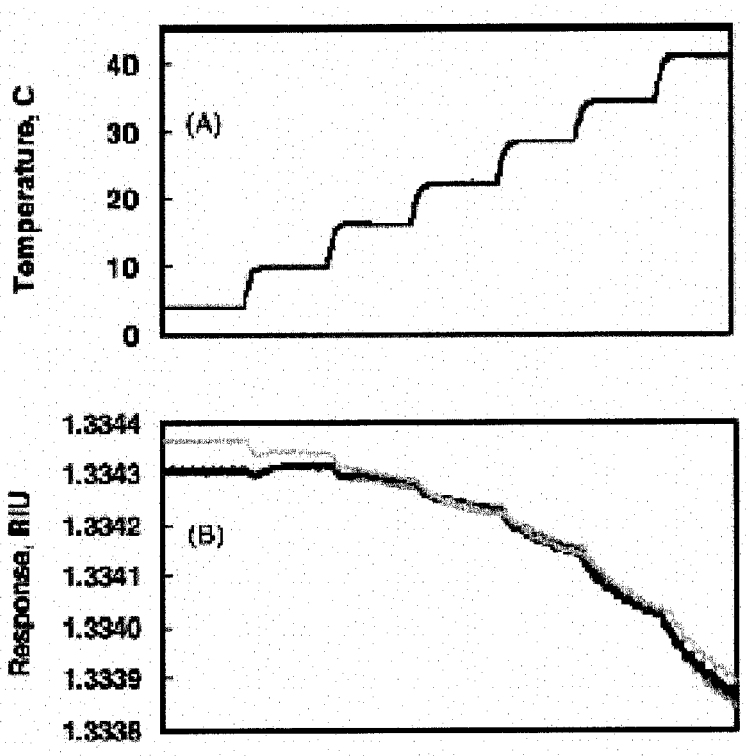


Figure 10. Response of a three channeled sensor during temperature variation from 4-40°C. (A) Sensor housing temperature. (B) Sensor response, in refractive index unit (RIU) [15].

The affect on protein binding rate can be seen in Figure 11. The temperature was varied between 4 and 56°C. Binding rates increased up to 46°C.

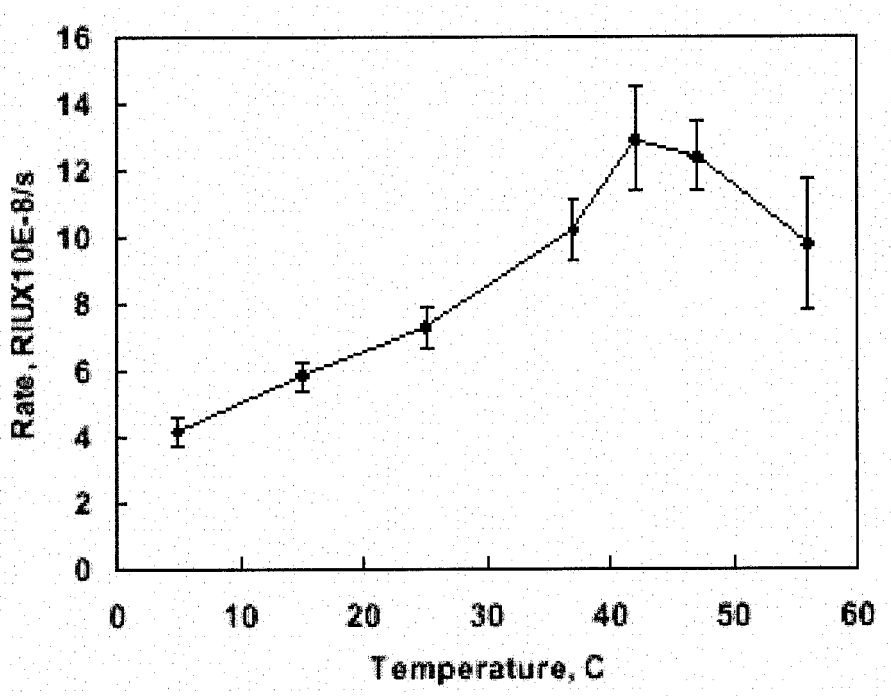


Figure 11. Effect of temperature on binding rate. The average of 3 values of the linear initial slopes of the binding curves was calculated for each test temperature [15].

This data suggests the effect of temperature will increase the rate. It is unclear whether the reaction rate reaches a maximum and then decreases, or whether the rate levels off with increasing temperature, due to the significant error bars at higher temperatures. It is clear that ambient temperature should be taken into consideration when characterizing biosensors.

3.0 RESEARCH OBJECTIVE

Assess the capabilities of a prism coupler utilizing surface plasmon resonance to detect surface changes by varying the angle of incidence at a fixed wavelength. Design a flow cell that would allow exposure of the biosensor surface to injected solutions, while in contact with the prism. Attempt to detect protein binding to the surface of the biosensor using the prism coupler and flow cell at ambient conditions. Write an experimental procedure utilizing previous research and follow it to ensure data integrity. The following hypothesis was tested after the instrumental capabilities were assessed:

- Using a longer wavelength laser will increase sensor resolution by conservation of momentum. This will result in larger changes in the angle of refraction and allow changes in mass on the sensor surface to be detected.

4.0 RESEARCH APPROACH

4.1 Testing

The experimental work included optimizing the substrate, assessing instrument capability, designing a flow cell, and evaluating the binding of β-galactosidase to streptavidin. Preliminary testing included optimizing substrate surface preparation and determining feasibility. Baseline testing was done to determine the instrument capability, substrate selection, a laboratory procedure for creating a streptavidin SAM was written, and the flow cell was designed. Reliability and data analysis are also discussed.

Feasibility can be assessed after the instrumentation capabilities are known, the substrate preparation is optimized, and the flow cell is designed. The project is detailed in Figure 12.

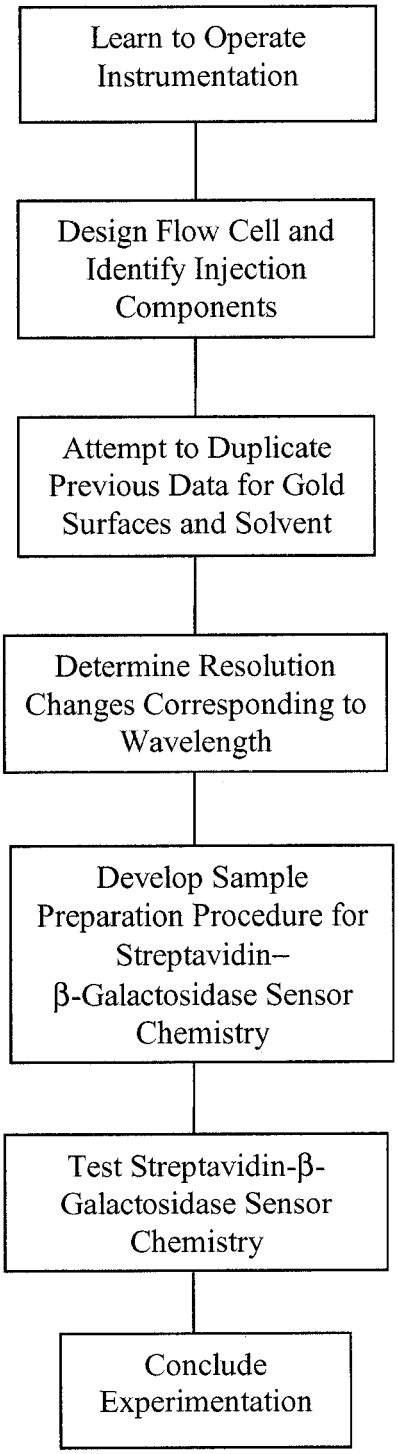


Figure 12. Flow chart.

4.1.1 Preliminary Testing

Samples were prepared to identify the optimum glass cleaning method, to improve adhesion of gold to glass and give a smoother gold surface for bonding of thiol groups. Three different cleaning methods were used to clean the glass slides prior to the sputter deposition of a gold surface. Solvent wiping with either ethanol or acetone was compared to the previous method using Dawn soap, because solvents are better for cleaning of the substrate. Gold was then sputtered onto the glass surface per the procedure located in Appendix A and the SPR peak resolution was compared.

Piranha cleaning of the glass was compared to solvent cleaning, to determine if acid etching the surface would improve surface roughness. Piranha is a 3 to 1 mixture of sulfuric acid and hydrogen peroxide. Atomic Force Microscopy (AFM) images were generated and surface roughness was calculated. These results are also compared to the previous method using Dawn soap.

Preliminary testing was done to determine if the instrumentation was able to use surface plasmon resonance technology to measure total internal reflectance of a gold surface and if the effects of exposure to solvent could be detected. A Denton Vacuum Sputterer was used to deposit a layer of gold onto glass slides cleaned with ethanol per the procedure in Appendix A. The slides were then mounted on top of flow cells and exposed to varying concentrations of isopropyl alcohol (0, 0.05, and 0.10% IPA in deionized water, DI). These measurements were compared to those of the dry slide taken prior to exposure.

4.1.2 Baseline of Instrumentation

The instrumentation capabilities were determined using streptavidin-β-galactosidase biotin sensor chemistry. The reaction behavior is well established, has a 1:1 stoichiometry, and a strong covalent affinity. Wavelength and ligand concentration were the two parameters that could be varied to determine if the instrumentation could detect the resulting changes on the index of refraction. The wavelength was changed to determine if longer wavelength was able to detect small amounts of binding on the surface obtained by using increasingly dilute solutions. A two level two factor design of experiment was planned for varying wavelength (633 and 1550nm) and concentration (0.1, 1.0 and 10.0μg/ml) per the test matrix in Table II.

Table II. Baseline Test Matrix.

Concentration β-Galactosidase (μg/ml)	**Wavelength (nm)**
-1	1
1	-1
-1	-1
1	1

A series of multiple runs was conducted to determine reproducibility. Similar test configurations were run at the beginning and end of the experiment to make sure there weren't any instrumentation or sample changes that took place during the length of the

entire project. Peak intensity, width, and angle of refraction were generated by the instrumentation for each run. Peak width was the key indicator for increased resolution. The average, standard deviation, and coefficient of variance are reported for identical data sets run at the beginning and at the end of the DOE to determine error for each measurement and to ensure the level of error remained consistent throughout the experiment. An optimum concentration of 10μg/ml was expected based on previous research. Improved peak resolution was expected at longer wavelength.

4.2 Substrate

For preliminary testing the gold was sputter deposited onto glass microscope slides using a Denton Vacuum Sputterer. The ideal sputter time of 35 seconds was determined in previous experiments. The resultant 45nm film thickness gave optimum peak intensity. The gold film was uniform, but was flaking was problematic because the gold has poor adhesion to glass. The gold surface is shown in Figure 13. The circular mark on the center of the slide was left by the edge of the interaction well after being mounted onto the flow cell. One corner of the glass slide is not coated with gold, because that area was taped during sputtering to secure the slide.

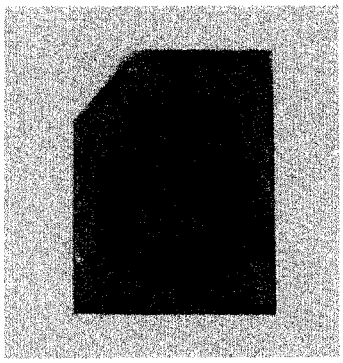


Figure 13. Gold sputter deposited onto glass microscope slide.

The adhesion of the gold sputtered onto glass was problematic during preliminary testing so substrate was purchased for the Streptavidin/β-Galactosidase testing. Slides having a titanium layer deposited between the glass and gold were purchased from Platypus Technologies. Titanium is commonly used between gold and glass to improve adhesion without risk of migration to the surface [26]. The slides were 15mm O.D. and had 100 angstroms of gold deposited onto glass. A titanium layer was deposited prior to the gold, to improve adhesion. The Platypus substrate is shown in Figure 14.

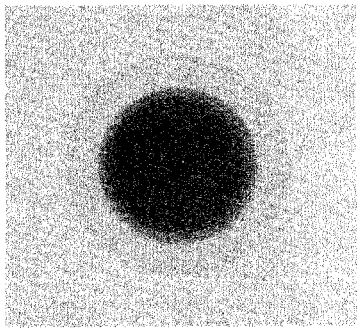


Figure 14. Gold coated substrate from Platypus Technologies.

4.3 Streptavidin-Biotin Sensor Chip Preparation

The proteins and chemicals required to create a SAM on the surface of the gold slide and the ligand solution are listed in Table III. A detailed table listing reagents, lot numbers, and concentrations is located in Appendix D.

Table III. Chemicals.

Chemical/Protein	**Function**
Mercaptoundecanoic Acid (MUA)	Thiol Acid
Streptavidin Streptomyces Avidinii	Ligand (Receptor)
β-Galactosidase, Biotin Labeled from Escherichia Coli (BG)	Analyte
Sodium Hydroxide (NaOH)	Base for Adjusting pH
Bovine Serum Albumin (BSA)	Protein for Non-Specific Binding
Phosphate Buffered Saline (PBS)	Buffer Solution
N-Hydroxysuccinimide	Biotinylating Reagent Creates NHS Ester, replaced by receptor
2-mercaptoethanesulfonic acid (MES-Na)	Sulfonated Buffer Solution
1-ethyl-3-(3-dimethylaminopropyl carbodiimide (EDCl)	Condensing Agent

The following steps were completed to create a SAM on gold substrates. The substrate was exposed to 10mM MUA in ethanol (a thiol acid which is hydrophobic and

bonds to gold) for approximately 24 hours. The substrate was then washed with (1) ethanol, (2) 50% ethanol/water, (3) 25% ethanol/water, and (4) water to remove unbound reagent without causing it to precipitate in water. Alkane chains with NHS ester end groups form a self assembled monolayer on the thiol modified gold surface. These Alkane chains were converting a carboxylic acid to an ester by adding NHS (a transthioesterification agent) to MES buffer. Subsequent addition of EDCI causes a dehydration reaction where the carboxylic acid group is converted to a NHS ester and a water byproduct. This weak ester group was then replaced by the analyte, streptavidin, upon exposure for 30 minutes. The slides were then washed with PBS/BSA so the albumin would adhere to portions of the substrate that weren't covered with Streptavidin. The substrate was then ready for testing. They were stored in PBS/BSA at <40°F and used within 90 days to prevent bacterial growth.

The ligand used was biotin labeled β-Galactosidase. This protein is larger than biotin, and therefore it's binding to the analyte would be more easily detected, than a small molecule such as biotin. The ligand solutions were made by first dissolving the protein in 1ml of deionized water, and then adding to 0.1M phosphate buffered saline. Three different concentrations of ligand were made for determining the limit of detection, 0.1, 1.0, and 10μg/ml. They were stored in PBS/BSA at <40°F and used within 90 days to prevent bacterial growth.

A procedure was written for bonding the analyte to the gold surface and making the ligand solution. This procedure was followed for the preparation of all substrates, which were prepared at the same time using the same solutions to reduce potential differences in the samples. The complete procedure is located in Appendix C.

4.4 Flow Cell

A flow cell was designed that would allow the ligand immobilized on the gold to be exposed to dilute analyte solutions. The flow cell was machined out of white Delrin. The material provided a smooth surface that was relatively nonporous and is easy to machine. The slide is exposed to an interaction well holding 0.74 mL of analyte solution. The solution is injected through an injection port located on one side of the flow cell, and exits through a port located at the top of the flow cell. The substrate is mounted vertically on the front of the flow cell. This design ensures there won't be air entrapped in the well and the entire ligand surface will be exposed to solution. A CAD drawing of the flow cell design is located in Figure 15.

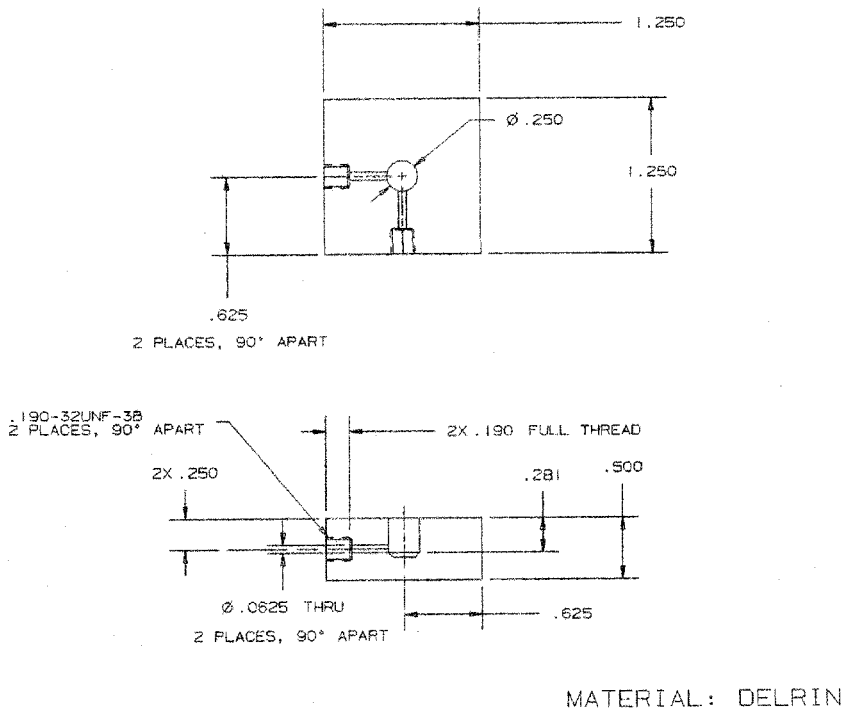


Figure 15. Flow cell design.

Adapters were required for connecting an injection system to the flow cell. Polypropylene threaded tube fittings were used because metal components in contact with liquid may have interfered with the resonating plasmons. The tube fittings had a shrouded barb for supporting 1/16 inch I.D. PVC tubing. This allowed the parts to be re-used and tubing to be changed if necessary. The fittings had a 10-32 thread, a 1/4 inch hex for tightening, and were 0.172 inches long. The length was slightly shorter than the 0.190 inch depth of the holes machined into the sides of the flow cell. Photos of the machined parts with adapters are located in Figure 16.

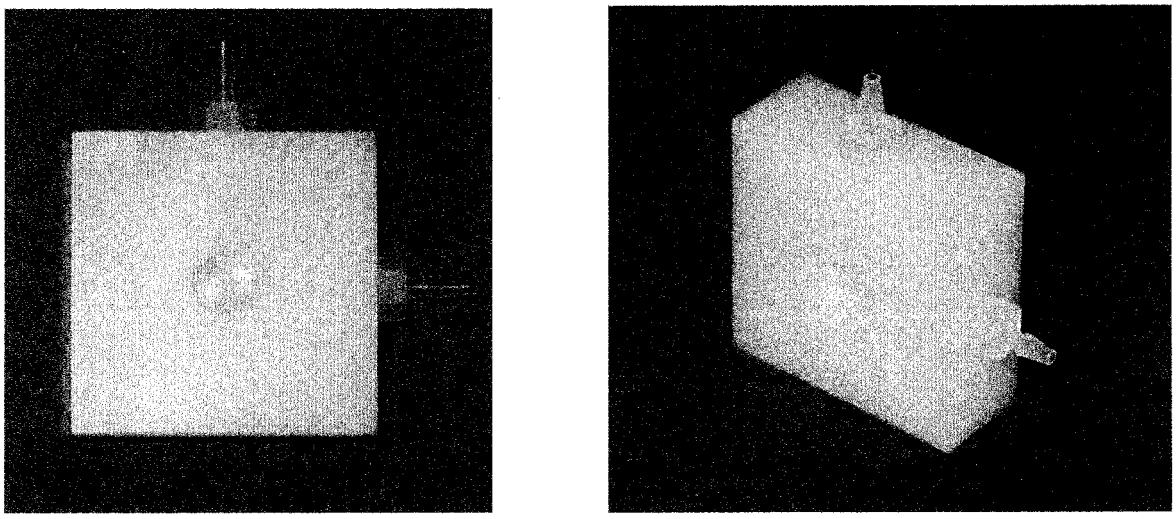


Figure 16. Flow cell with tube fittings. (a) Front view. (b) Angle view.

The injection system included a syringe and PVC tubing. The analyte solution was loaded into a 6cc polypropylene syringe having a polyisoprene male luer lock tip. A nylon female luer lock fitting with a shrouded barb for supporting 1/16 inch I.D. tubing was used to connect the syringe to the tubing. Similar tubing was attached at the outlet port so solutions exiting the flow cell could be contained. The entire injection flow cell system was designed to contain the solutions during testing and prevent any liquids from leaking onto the electronics within the prism coupler. The substrate was attached to the front of the flow cell with tape. Sufficient area of the substrate was left uncovered so the prism face would be in contact with the back side of the sensor. Care was taken align the substrate with the injection well and the prism. The injection system and attachment of the substrate to the flow cell are depicted in Figure 17.

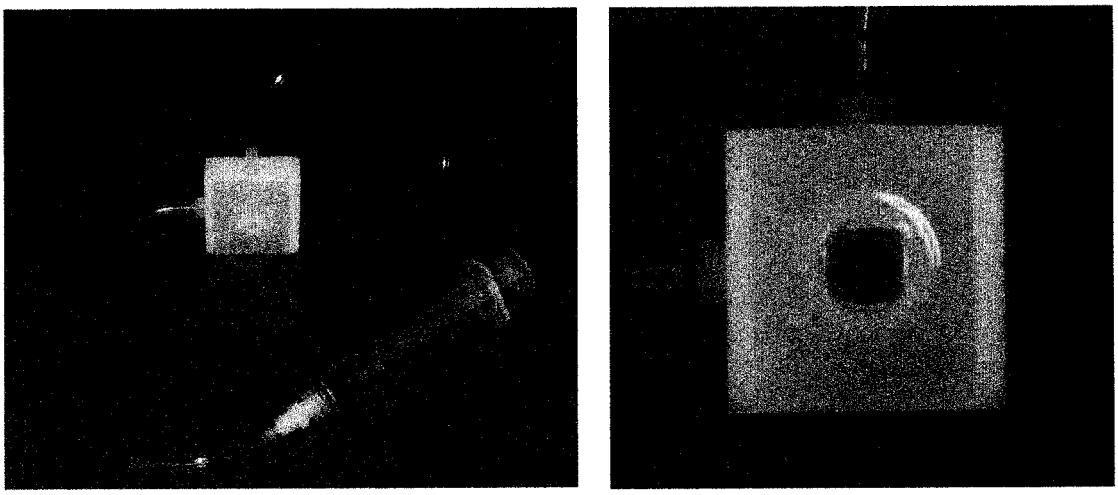


Figure 17. Injection system and flow cell. (a) Complete system. (b) Attachment of substrate.

Prior to testing and attachment of the substrate, the flow cells were flushed with a 10mg/ml bovine serum albumin in 0.1M phosphate buffer solution. This was done so all non-specific surfaces susceptible to protein attachment would be coated with albumin, and the analyte solution injected during testing would bind only to the substrate. This would ensure the test results would reflect the concentration injected as closely as possible.

4.5 Equipment

Specifications for the equipment that will be used are listed in Table IV. Product specifications are located in Appendix B.

Table IV. Equipment.

Instrument	**Function**	**Tolerance**
Denton Vacuum Desk II	Sputter Deposition	±10 milliamps
Metricon 2010 Prism Coupler	SPR Instrument	Index Accuracy ± 0.001 Index Resolution ± 0.0005
Glass Prisms	BK7	Unknown

The Denton Vacuum used for sputter deposition was used as specified, with the exception of an Argon gas purge prior to deposition. It was assumed that the vacuum chamber was sufficiently emptied by bringing it to 50 millitorr prior to deposition.

The Metricon prism coupler was operated per the manufacturers recommendations. The only exception was that the film thickness being measured was located on the backside of a glass slide in contact with the prism, rather than the film being in contact with the prism as intended. The instrument motor and circuit board both had to be repaired during the course of the experiment.

4.6 Reliability and Data Analysis

Potential sources of experimental error include aggregates in solution, non-specific binding, mass transfer limitations, contamination, poor solution preparation, lack of temperature control, and binding of buffer solution. The error was determined by performing multiple runs under similar conditions. Average, standard deviation, and

coefficient of variance (C.V.) are reported. A C.V. of 10% or less indicates reproducible results.

Ideally, this analysis would have been performed at the beginning and end of the DOE to ensure there weren't changes in reliability during the course of the experiment. Analysis of the design of experiment matrix would have provided a multiple regression equation, with coefficients showing the significance of each factor. This would have been used to describe the affect of the test variables on the results. However, protein binding was not detected at high concentrations, so the tests at lower concentrations in the original DOE were not performed. Analysis of the solvent study and high protein concentrations are reported.

5.0 RESULTS AND DISCUSSION

5.1 Preliminary Testing

Samples were prepared for the identification of the optimum glass cleaning method. Three different cleaning methods were used to clean the glass slides prior to the sputter deposition of gold onto the glass surface. Solvent wiping with either ethanol or acetone was compared to the previous method using Dawn soap, because solvent cleaning is more effective. Gold was then sputtered onto the glass surface per the procedure located in Appendix A and the SPR peak resolution was compared. Results indicate cleaning with ethanol resulted in greater peak intensity than cleaning with acetone, so ethanol is recommended. The peaks are shown in Figure 18.

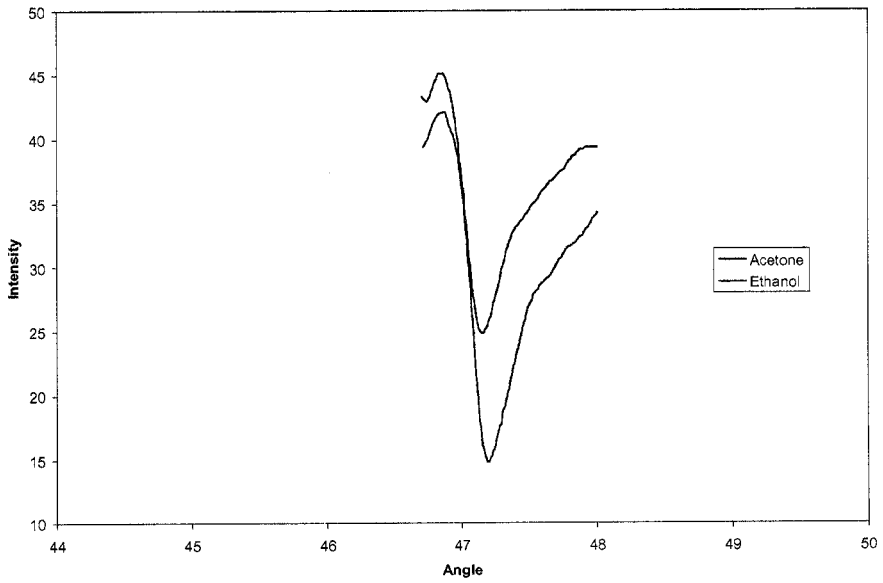


Figure 18. SPR peaks for substrate cleaned with acetone and ethanol.

To optimize the gold film thickness, 12 glass slides were cleaned and gold was sputtered on the surface of the slides. The sputter time was varied between 20 and 75 seconds in intervals of five seconds. The Metricon was set at a constant angle and all settings were the same for each run. The maximum intensity is observed for a film thickness resulting from a 45 second sputter time. The optimal gold film thickness is expected to be below 0.3 of the incident wavelength of light [25]. The results are shown in Figure 19.

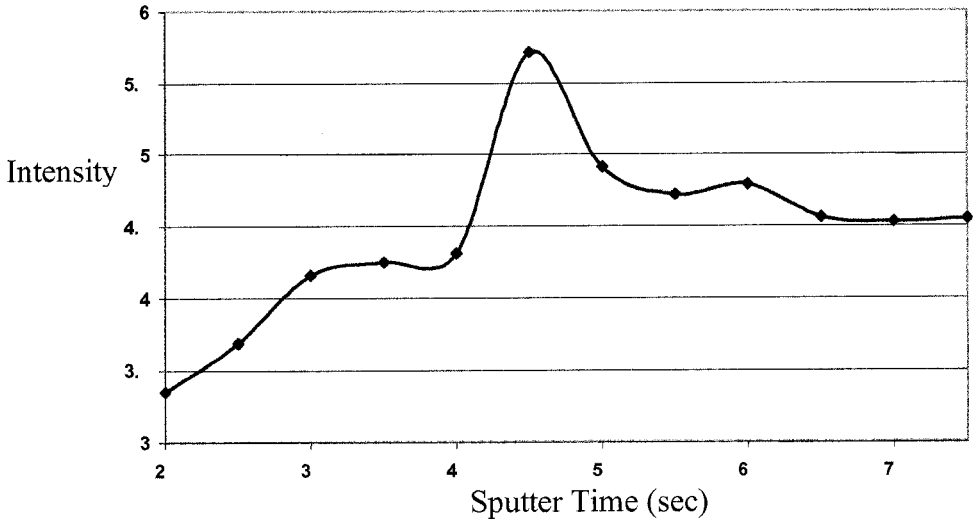


Figure 19. Gold film thickness optimization [25].

The prism coupler takes measurements while rotating the sample on a turntable. The direction of sample rotation was thought to have an effect on the SPR angle. To test this theory, measurements were taken rotating the sample in different directions. A directional effect is observed and the resulting SPR angles are summarized in Table V.

To avoid this affect, all samples must be measured by rotating the sample from left to right.

Table V. Effect of Table Rotation on SPR Angle.

Solvent	**SPR Peak Table Rotating Left**	**SPR Peak Table Rotating Right**
Air	163	158
DIH2O	163	159
1% IPA	146	142
	147	142
Pure IPA	146	140
	146	140

Testing was then done to determine if the instrumentation was able to use surface plasmon resonance technology to measure total internal reflectance of a gold surface and if the effects of exposure to solvent could be detected. A Denton Vacuum Sputterer was used to deposit a layer of gold onto glass slides cleaned with ethanol per the procedure in Appendix C. The thickness of the gold film was not measured for any of the samples. The slides were mounted on top of flow cells and exposed to air, DI, and 0.1wt% IPA. The SPR peaks show a relative shift in resonance angle, indicating that when a 1550nm laser was used, the instrumentation was sensitive enough to detect changes in solvent

within the flow cell. The peaks are shown in Figure 20, SPR angle at the minimum intensity are noted in parenthesis after the data label.

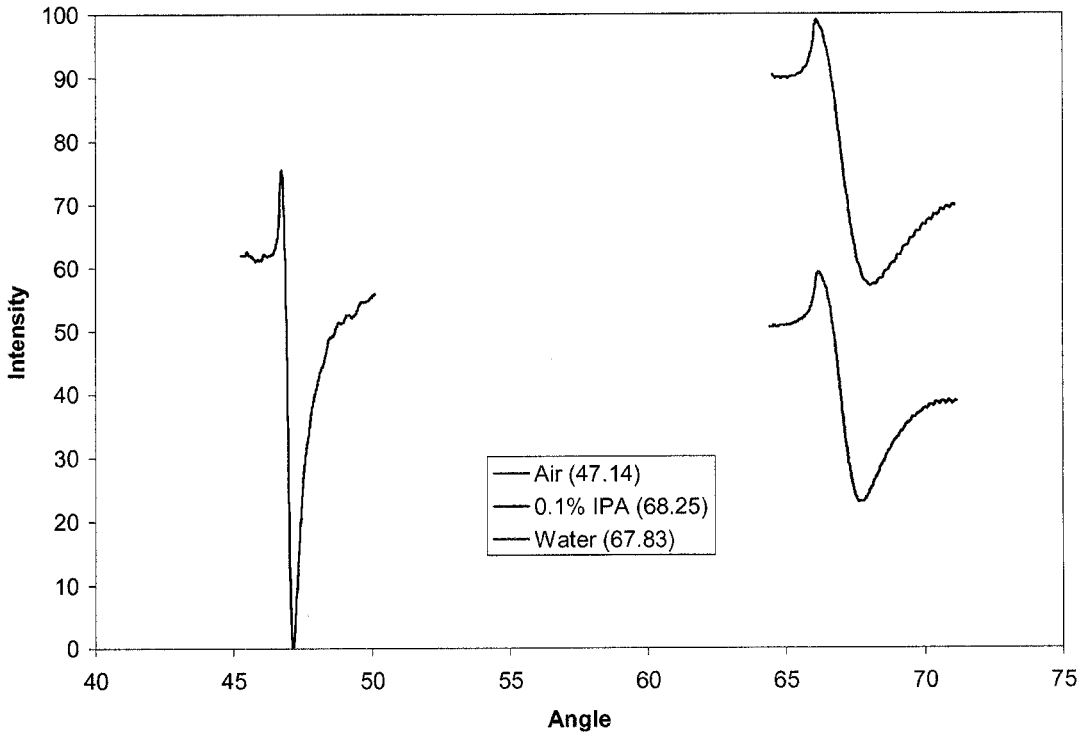


Figure 20. Relative change in resonance angle as a result of solvent at 1550nm.

The sample was first tested dry, then after injection with water, and finally after injection with 0.1% IPA. Testing in the order of increasing concentrations reduced contamination effects, because there wouldn't be residual materials on the biosensor surface. It was not practical to repeat the initial dry run at the end of the experiment because the surface was contaminated with water and isopropanol. The quality of the data is established by comparing results to those from a statistical study. To determine

the standard deviation of the angle occurring at minimum intensity and to establish an effective procedure for injecting solutions into the flow cell, the standard deviation of resonance angles was determined for tests using the same flow cell for multiple runs. The injection procedure studied required flushing the flow cell with the following in between each test of 0.1% IPA: a) 1mL air, b) 0.5 mL DI, c) 1 mL air. The same substrate was used for five tests. Changes in resonance angle and peak width are the only differences of interest. The differences in amplitude are due to instrumental adjustments. The results are shown in Figure 21, SPR angle at the minimum intensity are noted in parenthesis after the data label.

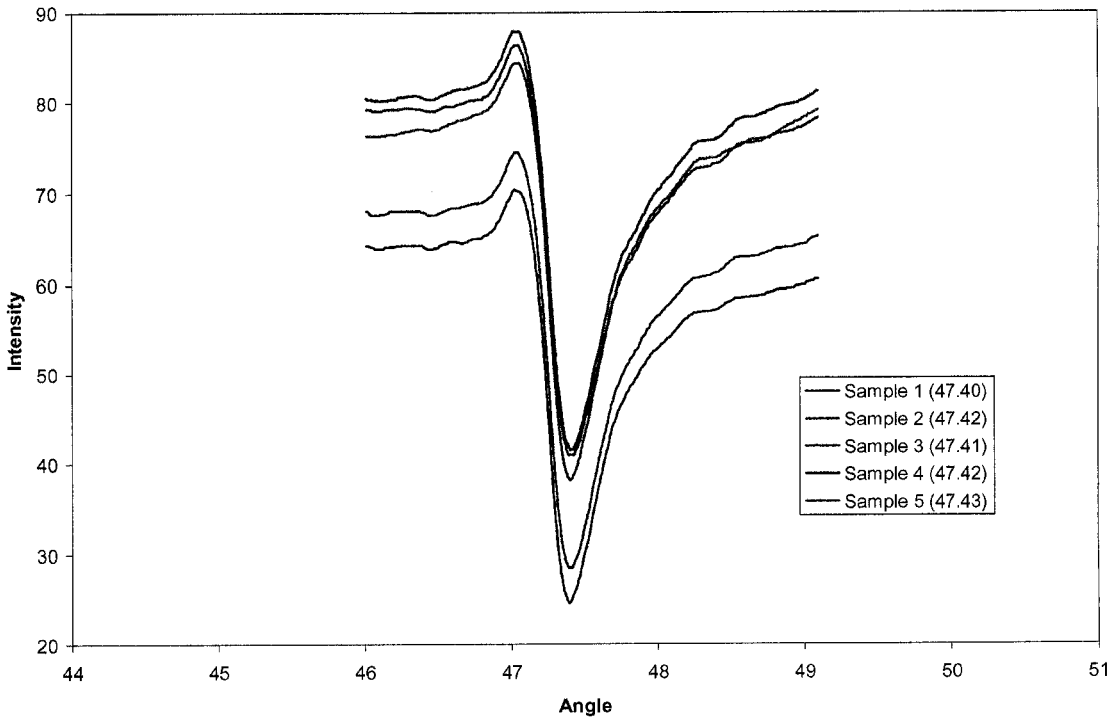


Figure 21. Repeatability of flow cell injection procedure.

The angles at intensity minima have a standard deviation of 0.01 and the coefficient of variance for five runs is 0.02%. The results are considered repeatable and any results from subsequent tests are regarded as statistically valid if the change in SPR angle resulting from binding on the surface is greater than 0.01 (the standard deviation of this experiment).

Varying concentrations of isopropyl alcohol (IPA) in DI were then used to determine the limit of IPA detection. The concentrations of IPA, subsequent SPR peaks, and the SPR angle at minimum intensity are shown in Figure 22, SPR angle at the minimum intensity are noted in parenthesis after the data label.

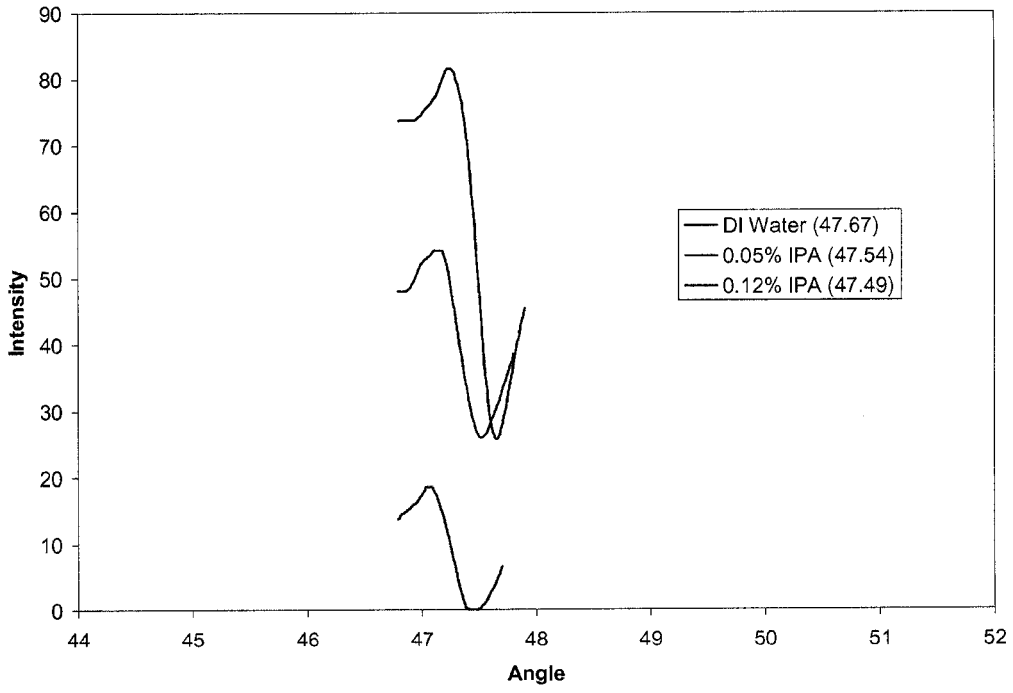
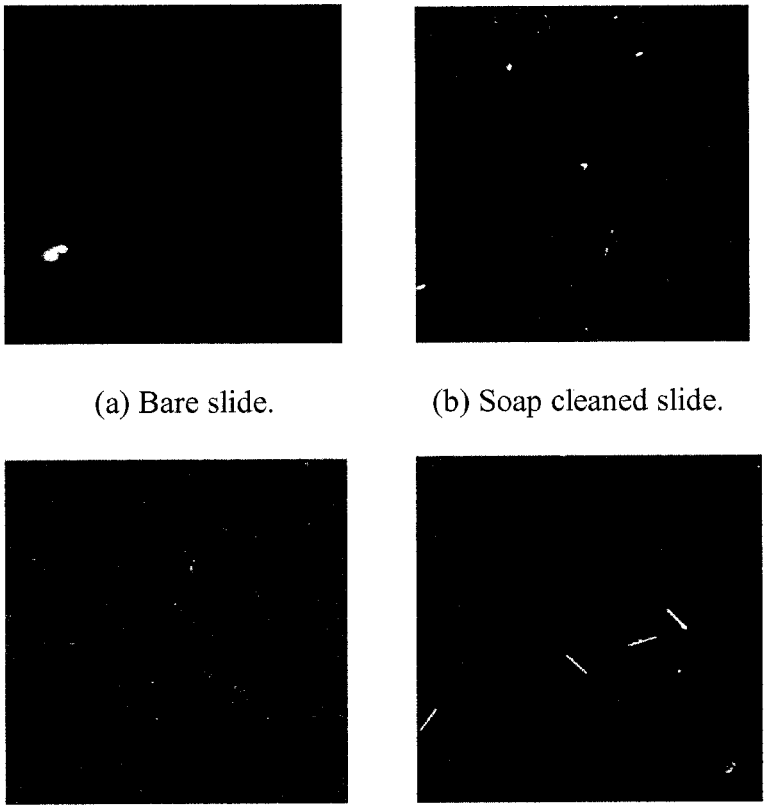


Figure 22. Prism coupler limit of detection.

The difference between peaks for DI, 0.05% IPA, and 0.12% IPA all differ by an amount greater than the standard deviation for the repeatability study, suggesting that the shifts in SPR angle are due to the change in solvent concentration. The limit of detection would therefore be 0.05% IPA.

The poor adhesion of the gold film on the glass was becoming problematic. At this stage of the project gold was still being sputtered onto glass using the Denton Vacuum Sputterer. The gold film was lifting off of the surface of the glass during some of the experiments, resulting in lost work time and data. Methods of improving the adhesion of gold to glass by modifying the surface prior to gold deposition were investigated. Solvent cleaning of the glass with ethanol was compared to Piranha cleaning. Piranha is a 3 to 1 mixture of sulfuric acid and hydrogen peroxide. Atomic Force Microscopy (AFM) images were generated and surface roughness was calculated. The results of three cleaning methods are compared, including the previous method using Dawn soap. The AFM images are shown in Figure 23.



(a) Bare slide. (b) Soap cleaned slide.

(c) Ethanol cleaned slide. (d) Piranha cleaned slide.

Figure 23. AFM images depicting surface roughness [25].

Values for the roughness of the surface were also calculated. The surface roughness data is shown in Figure 24.

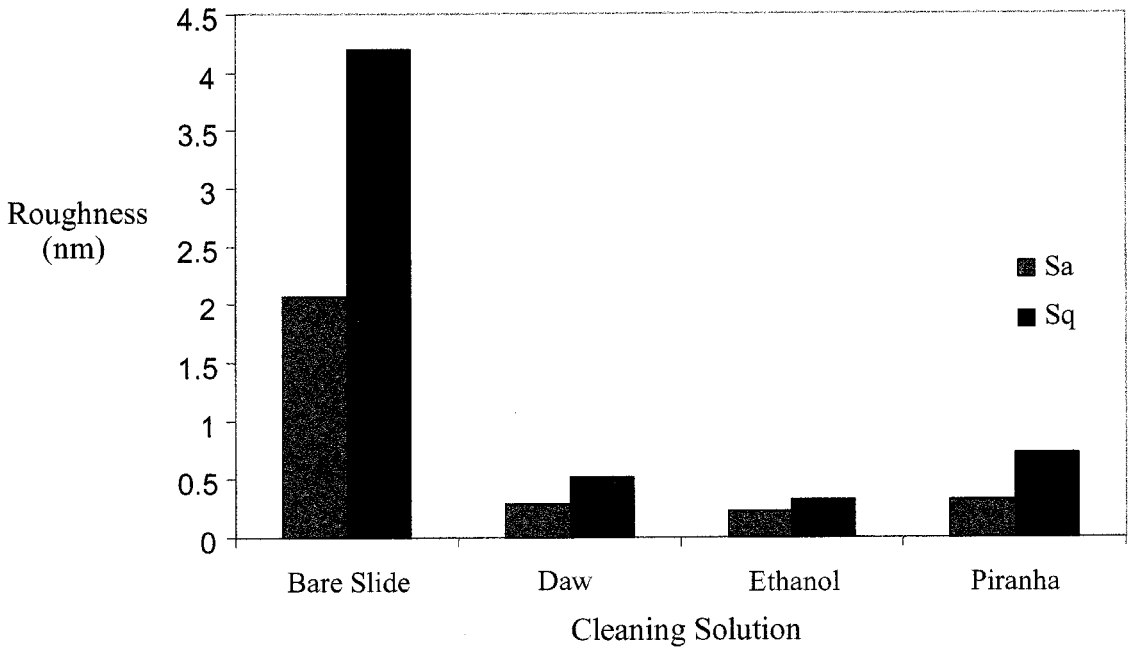


Figure 24. Surface roughness as a result of cleaning method. Sa corresponds to the average surface roughness and Sq corresponds to the root mean square roughness [25].

The data suggest that cleaning improves the roughness of the surface by an order of magnitude. The ethanol cleaning improves the roughness of the surface more than the previous cleaning method using soap, while the Piranha clean increases the root mean square roughness, thus not making an improvement on the cleanliness of the surface. From this study, it is determined that the optimum cleaning method was being used, and adhesion issues must be resolved by other means.

Commercially available slides used for SPR were purchased from Platypus Technologies. These samples were 15mm O.D. and had 100 angstroms of gold deposited onto glass. A titanium layer was deposited prior to the gold, to improve adhesion. This was previously discussed in Section 4.2.

5.2 Baseline of Instrumentation

The instrumentation capabilities were determined using streptavidin-BG biotin sensor chemistry. An instrumental failure caused these tests to be done on a different instrument. The first test was for pure PBS, the protein carrier solution. This was done to determine if there were any changes to the SPR peak resulting from contact with the PBS solution. The second test was with an injection of 10μg/ml BG in PBS. The results of five tests are summarized in Figures 25-29, SPR angle at the minimum intensity are noted in parenthesis after the data label.

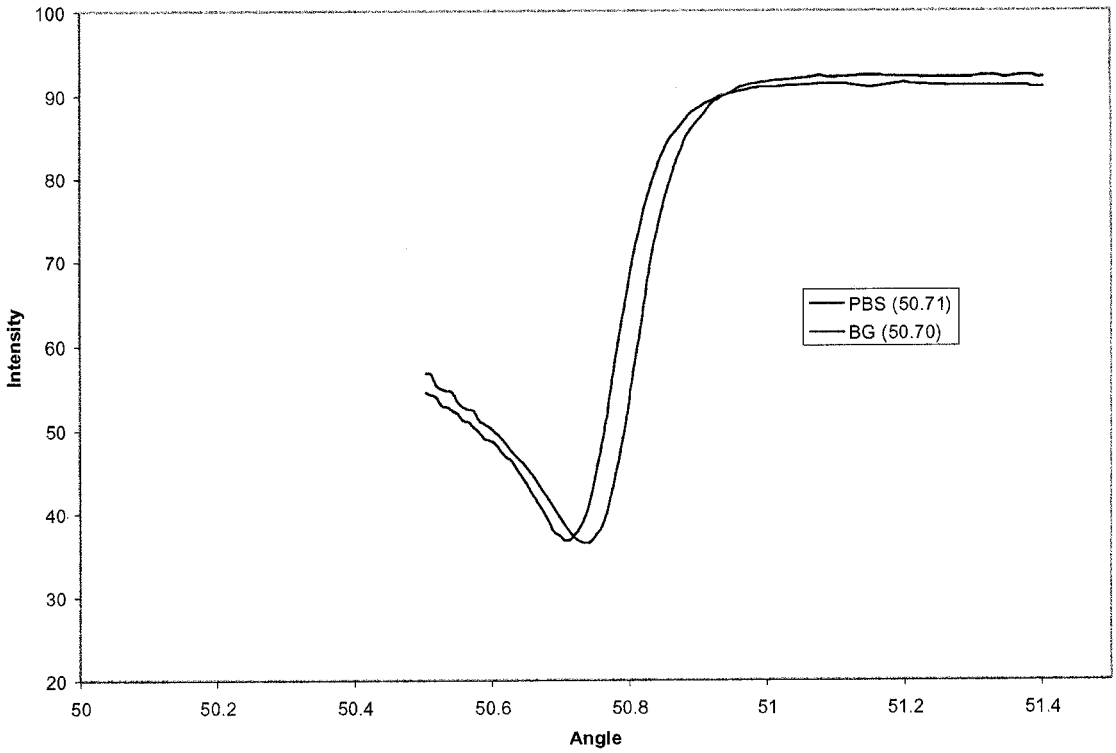


Figure 25. Streptavidin-BG Test A.

The previous chart shows the test results from a streptavidin substrate before and after five minutes exposure to BG. The resulting shift in SPR angle was only 0.01, which is equivalent to the standard deviation calculated from the repeatability study. This suggests the binding of BG to streptavidin was not detected. Additional runs were performed to determine if binding could be detected.

Figure 26 shows the results from another test exposing streptavidin to BG. A larger, more statistically significant shift of 0.08 is observed, suggesting binding of BG to streptavidin was detected.

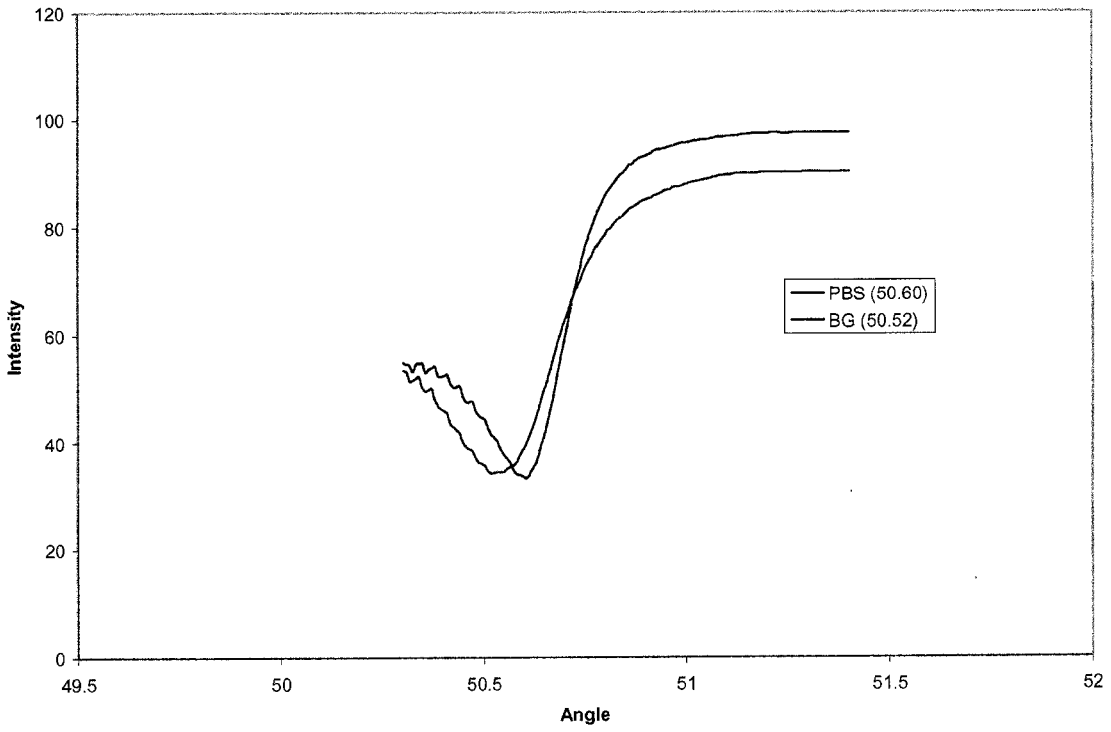


Figure 26. Streptavidin-BG Test B.

This chart shows the results of another test of streptavidin before and after exposure to BG. The shift also seems statistically significant, because it is larger than the standard deviation from the repeatability study. However, this shift is in the negative direction compared to a positive angle shift shown in Figure 27. This data suggest that the standard deviation of tests run using streptavidin-BG is larger than when running tests using solvent on a gold film. It should also be noted that the solution the streptavidin substrates were stored in had become cloudy, indicating that the protein had bacterial growth.

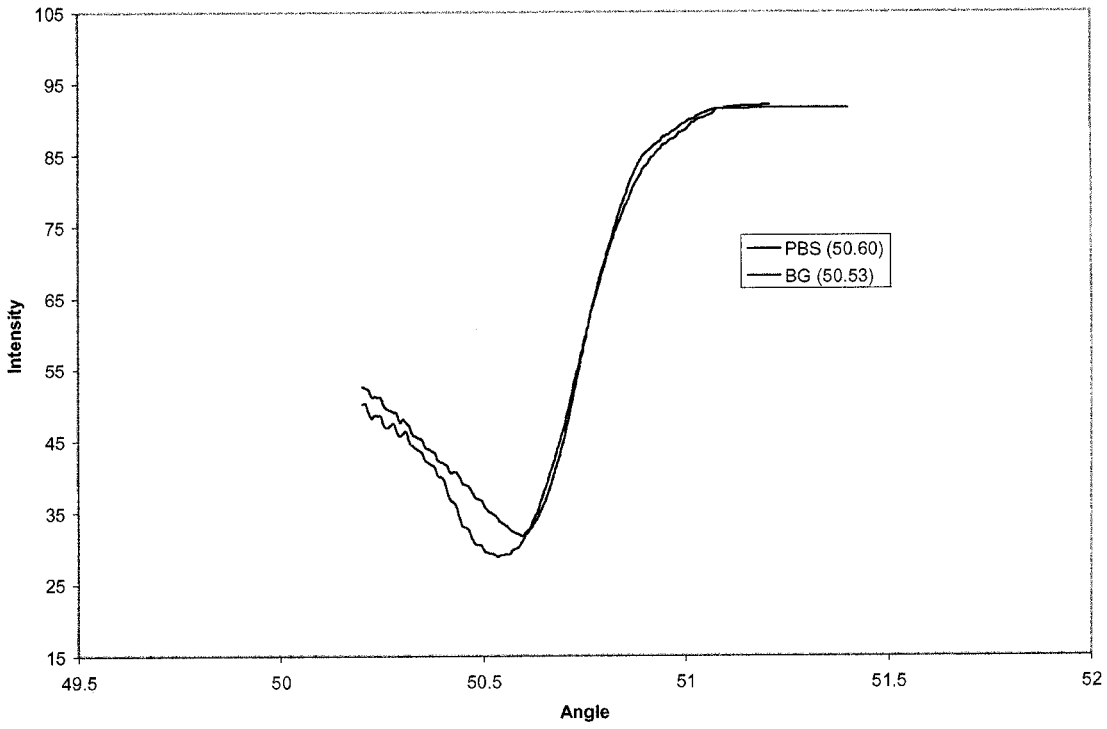


Figure 27. Streptavidin-BG Test C.

Preliminary tests were then conducted on the instrument to assess whether the poor results were due to bacterial contamination of the substrate or the instrumentation differences. The streptavidin-BG tests were being conducted on a different instrument than the preliminary tests.

Tests involving the exposure of a gold film to air, DI, and 1% IPA were repeated. A stronger concentration of IPA used, the previous concentration was 0.1% IPA. The performance of the commercial slides purchased from Platypus Technologies was also compared to that of gold on glass substrate substrate deposited with the Denton. The results are shown in Figure 28.

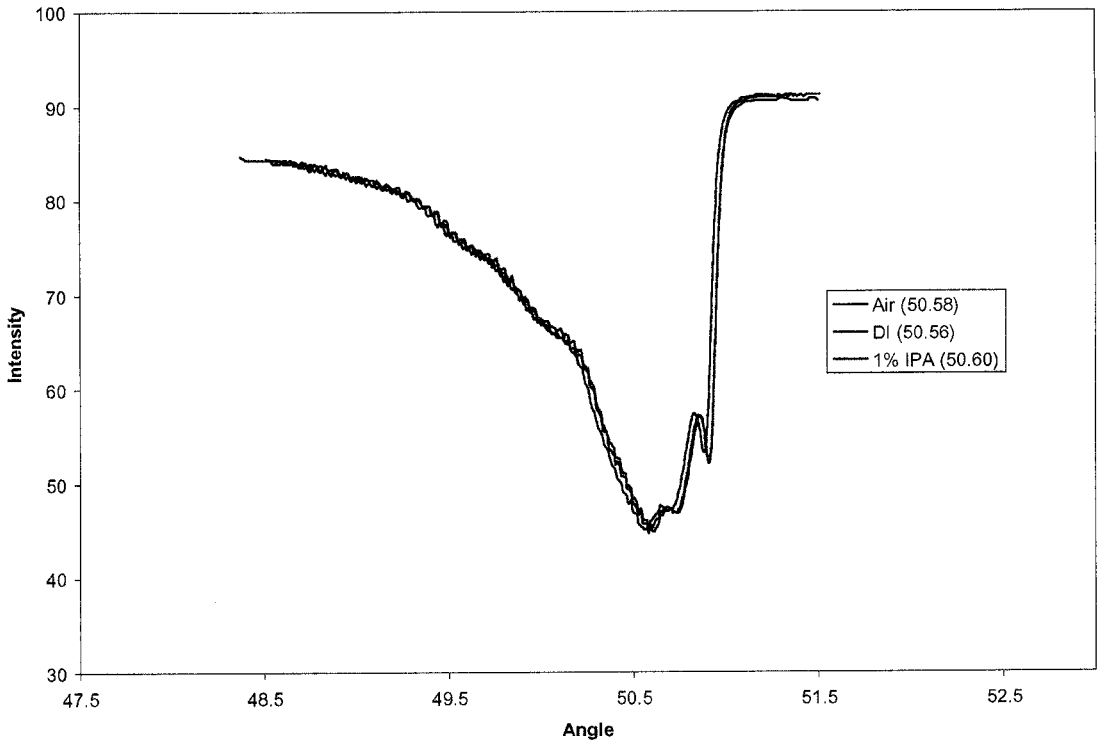


Figure 28. Sputtered slide I tested 110204.

This slide shows the results of exposing a gold slide to air, DI, and IPA. The resulting shift in SPR angle is not statistically valid when compared to the previous results, because it is equivalent to the standard deviation. This test was repeated approximately two weeks later and the results are shown in Figure 29.

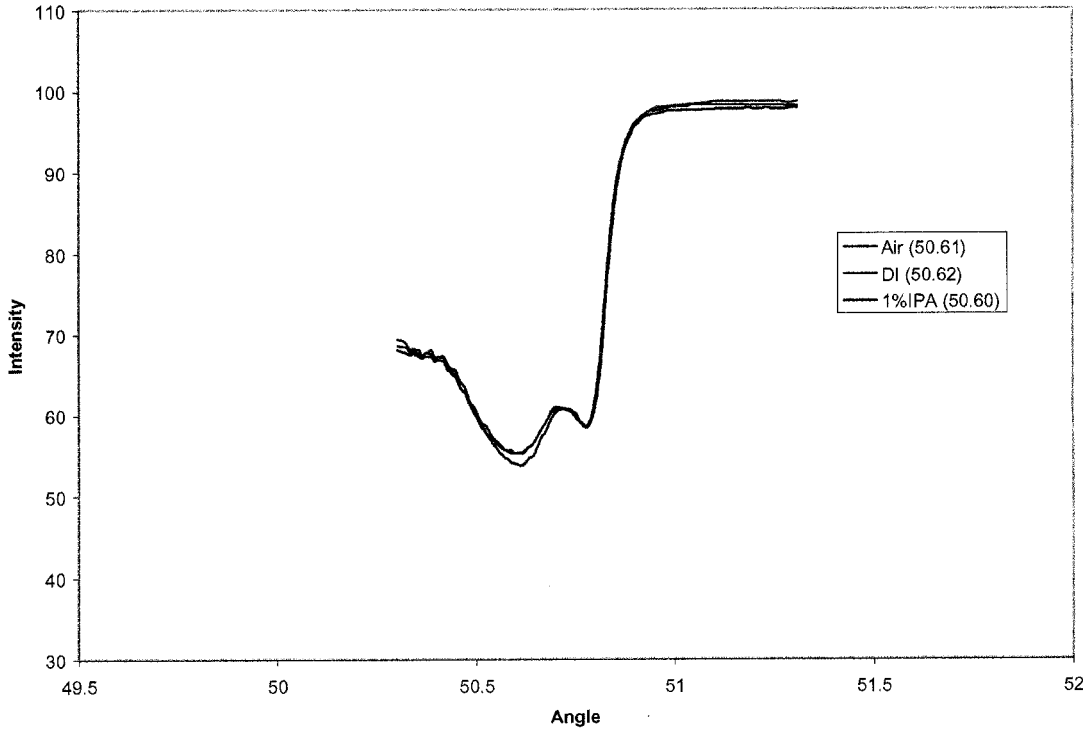


Figure 29. Sputtered slide II tested 112304.

The data also suggest that exposure to air, DI, and IPA were undetected. The resulting shift in SPR angle is equivalent to the standard deviation previously determined.

Tests were then conducted to determine if the change in substrate was the cause for poor results. Clean Platypus slides were exposed to air, DI, and 1% IPA to determine

if the change in solvent could be detected. Unfortunately, the resulting changes in SPR angle were also inconclusive. The peaks are shown in Figure 30.

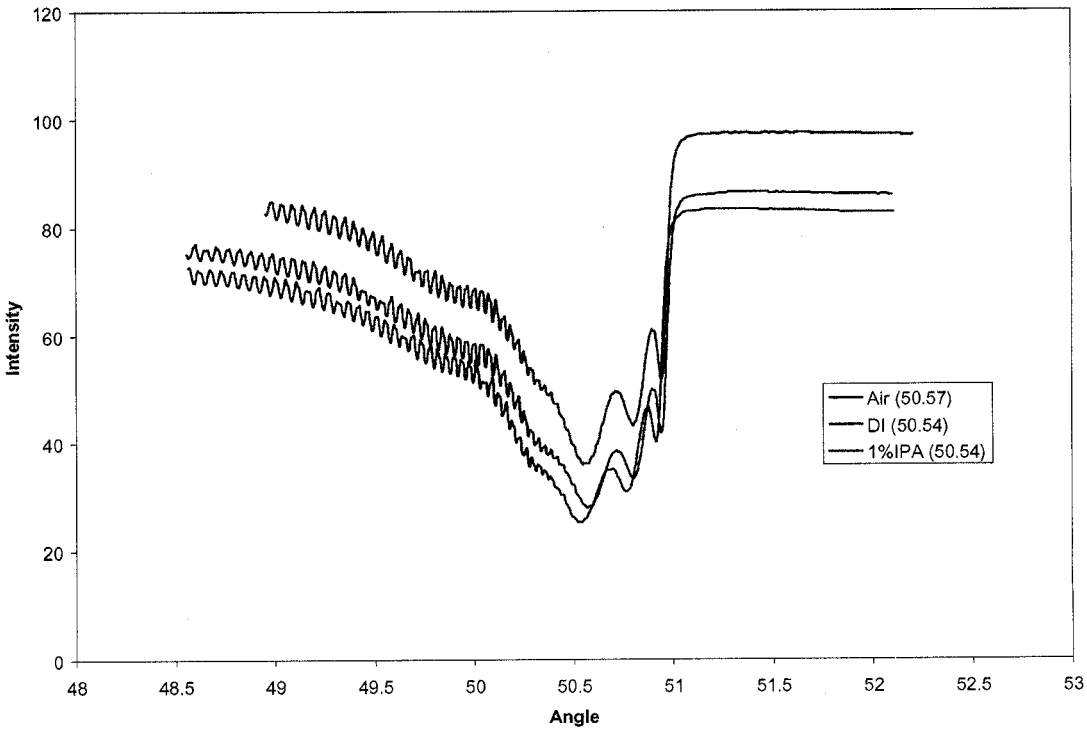


Figure 30. Platypus slide I tested 110204.

There is considerably more noise in this data than previous. However, the SPR peak is still well defined and the observable shift is not statistically significant. The oscillations are due to ringing. This phenomenon is caused by poor contact between the metal film and the substrate, resulting in changes in the intensity of the light being reflected. This test was repeated after approximately two weeks, to assess repeatability, and is shown in Figure 31.

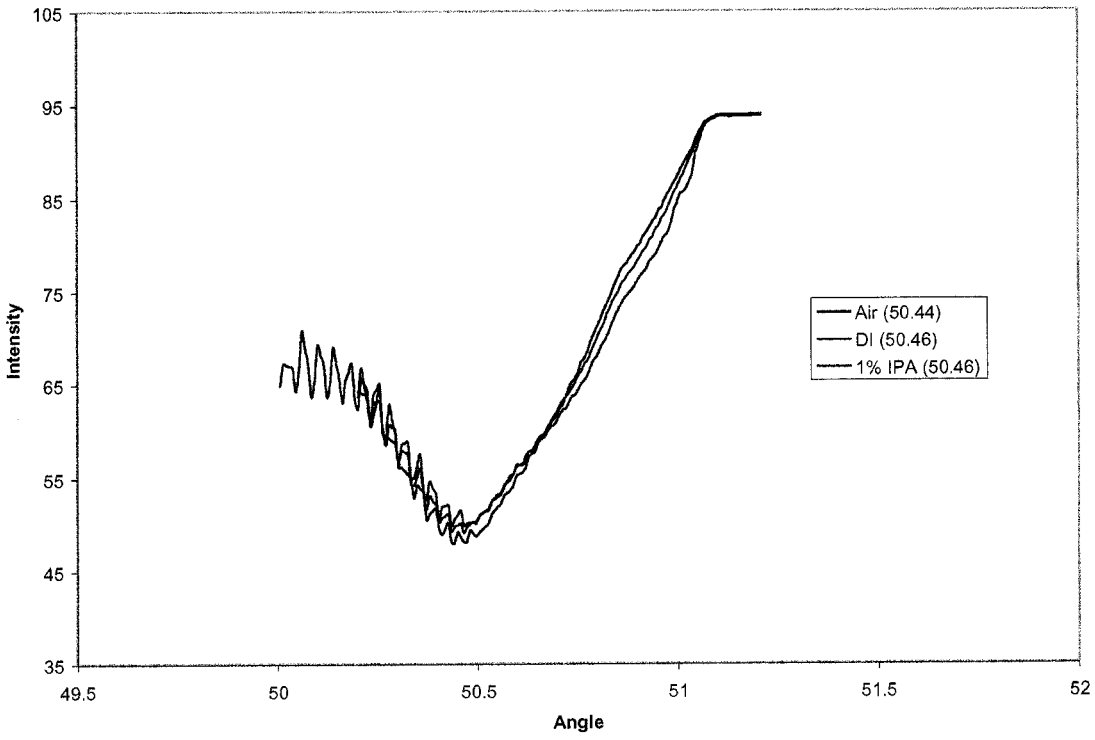


Figure 31. Platypus slide II tested 112304.

There is still more noise in this data and Figure 31 than previous. However, the SPR peak is still well defined and the observable shift is not statistically significant. The ringing indicates this sample also had poor contact, perhaps between the sample and the prism.

The test results indicate that neither the substrate sputtered at SJSU nor the Platypus slide showed any shift in SPR angle resulting from exposure to DI or IPA. This does not correlate with data previously obtained using the first instrument, indicating there was an instrumentation problem. However, the cloudiness of the streptavidin

substrate is indicative of an additional problem. Lack of reproducible shifts in SPR angle could be a result of either the instrumentation or bacterial contamination.

The remaining tests specified in the DOE were not performed because they were less robust than those performed initially. Poor results were contributed to problems with the instrumentation and possibly bacterial contamination of the protein solution.

6.0 CONCLUSIONS AND RECOMMENDATIONS

Preliminary testing on the Metricon at SJSU showed that a longer wavelength of light, 1550 nm, could be used for surface plasmon resonance on a modified prism coupler. The wavelength could be further optimized by testing a range of wavelengths around 1550nm. The ideal wavelength would increase the limit of detection by providing greater resolution.

Data showed a reproducible shift in SPR angle as a result of exposure to both deionized water and solvent. The instrumentation limit of detection was determined to be 0.05%. This could be used as a basis for determining analyte and ligand concentrations in future work. It could also be a starting point for determining the optimum wavelength. Lower concentrations could be evaluated over a range of wavelengths, allowing the limit of detection to be determined for an optimum wavelength.

The cleaning method was optimized to ensure the most uniform gold film of an ideal thickness was deposited and the maximum adhesion to glass was obtained. SPR peak resolution and atomic force microscopy identified ethanol as the best solvent for cleaning glass prior to sputter deposition. The use of Dawn soap for cleaning the substrate is not recommended. Using a sputter time of 45 seconds will provide the highest intensity, but the thickness of that film was not measured. Measuring the thickness of the film would aid in selecting a commercial substrate that would give optimum performance on this instrument. The gold thickness of 100 angstroms on the commercial substrate was not correlated to the optimum thickness obtained when using

the Denton and a 45 second sputter time. Identifying the film thickness and obtaining substrate having both a gold film of that thickness and a titanium adhesion layer would further increase instrumentation sensitivity.

Flow cell design was improved and effectively demonstrated. The design allowed injection of solutions and solution contact with the biosensor surface. Coating the surfaces with bovine serum albumin seemed an effective way to prevent loss of ligand due to non-specific binding within the flow cell, tubing, and syringe. This should be verified with an optimized substrate and functional chemistry.

The instrumentation failures were problematic, causing a loss of work time and forcing the transfer of work to a different location during the experiment. This adversely affected the results of the streptavidin-BG testing. Results were inconclusive and supplemental testing left the failure with two possible causes, instrumentation and bacterial contamination. The chemistry procedure should be further studied eliminate the cause of cloudiness in the protein solutions. Autoclaving solutions and ensuring proper raw material storage are recommended.

This experiment demonstrates that longer wavelength light can be used to detect slight changes in film thickness. The detection of protein binding would be improved by eliminating the potential sources of bacterial contamination in the samples and ensuring good contact between the prism and substrate. Once these issues are remedied and the improved limit of detection is further demonstrated, this useful approach can be used with novel chemistries.

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APPENDICES

APPENDIX A. EQUIPMENT SPECIFICATIONS

Metricon
CORPORATION

MODEL 2010 PRISM COUPLER FEATURES AND SPECIFICATIONS

Measurement accuracy and resolution:

index accuracy: ±.001	index resolution: ±.0005
thickness accuracy: ±(0.5% + 50 Å)	thickness resolution: ±0.3%

Precise accuracy and resolution values depend on film type, thickness range, and rotary table resolution. Figures above are worst case and typical of films in the 0.5-1.0 micron thickness range with low resolution rotary table. In many cases index resolution can be improved to ±.00005 by use of a high resolution rotary table, a no-cost option (see below). Index absolute accuracy is limited primarily by uncertainties in determining the angle and refractive index of the measuring prism. If the user is willing to perform a simple calibration procedure with each prism, absolute index accuracy of ±.0001 can be achieved. Direct NIST and NIST-traceable standards are available, respectively, for index and thickness.

Refractive index measuring range: With standard prisms, films and bulk materials with refractive index 2.65 and below are measurable. In addition, films with index up to 2.80 are sometimes measurable, depending on film thickness.

Film types/thickness ranges measurable: The Model 2010 can measure virtually any film type which is not metallic or very highly absorbing at the operating wavelength. Thickness and index of one or both films of dual film layers are measurable, provided the top film has higher refractive index. Thickness must exceed a minimum threshhold which depends on film and substrate (or underlying film) index. Examples of thickness ranges measurable* for common single or upper film types at the standard (633 nm) operating wavelength (for other film types, interpolate between example films with closest index):

Typical measurement time: 10-25 seconds with standard table, 15-75 seconds with high resolution table.

Index-only measurement of bulk materials/thick films: Material

**High-index films over substrates or underlying films of lower index other than Silicon dioxide are sometimes measurable at thicknesses up to half as thin as the above limits. Optional shorter wavelengths are also available to extend the measuring range to thinner films. Please consult Metricon for details.*

Maximum thickness for which simultaneous thickness/index measurement is possible is approximately 15μ for all film types. However, using bulk index measurement (see below), accurate index-only measurements of films thicker than 5-7μ may be made, and, with the VAMFO option, thickness-only measurements may be made on films as thick as 100μ .

Film type/index	Thickness & one index measurement	Thickness or index (assume other parameters)
low-kdielectrics (n=1.35) on Si	0.65-15 μ	0.25-65 μ
Silicon dioxide (n=1.46) over Si	0.48-15 μ	0.20-0.48 μ
Photoresist (n=1.63) over Si	0.42-15 μ	0.18-0.42 μ
Photoresist (n=1.63) over SiO_2/quartz	0.70-15 μ	0.30-0.70 μ
Polyimide (n=1.72) over Si	0.38-15 μ	0.15-0.38 μ
Polyimide (n=1.72) over SiO_2/quartz	0.50-15 μ	0.16-0.50 μ
Si oxynitride (n=1.80) over Si	0.50-15 μ	0.14-0.35 μ
Si oxynitride (n=1.80) over SiO_2/quartz	0.45-15 μ	0.13-0.45 μ
Si nitride (n=2.0) over Si	0.32-15 μ	0.12-0.32 μ
Si nitride (n=2.0) over SiO_2/quartz	0.30-15 μ	0.15-0.30 μ

s must be transparent/semi-transparent. Maximum index measurable with standard prisms is 2.65. Accuracy, resolution, and measurement time are same as for thin film measurement

(see above).

Operating wavelength: Low power (0.5 mw nominal) He-Ne laser (632.8 nm), CDRH/BRH Class II. Optional shorter wavelengths for measurement of thinner films, and near-IR (780/830, 1064, 1300, 1550 nm) wavelengths for fiber/integrated optics applications, are available. A wideband (75 W arc lamp) source is also available to permit measurement at any wavelength from 400-1000 nm. Optional sources change CDRH safety class to IIIa or IIIb.

Substrate materials/sizes: Film measurements may be made on virtually any polished substrate material including silicon, GaAs, glass, quartz, sapphire, GGG, and lithium niobate. Standard unit accepts substrates up to 8" (200 mm) square.

Measurement area: While the film and measuring prism are in contact over an area roughly 8 mm square, film area actually measured is only 1 mm diameter.

Prism types: Four general purpose prisms (see below) are available, each covering a broad index range. Eleven additional prism types are stocked for specialized purposes such as very high index measurements or measurements at very short or long wavelengths (consult Metricon for details). Prisms are easily interchangable in less than one minute to permit use of more than one prism type with a single system:

Prism type	Index range	Comments
200-P-1	<1.80	low wear, optimum for low index (<1.80) films
200-P-2	1.70-2.45	optimum for high index (>2.0) films. Optional 200-P-2-60 prism increases index measuring range from 2.1-2.65.
200-P-3	<2.10	covers wide index range, but less durable and cannot be used for TM measurement
200-P-4	<2.02	low wear, useful over a wide index range (1.4-2.0)

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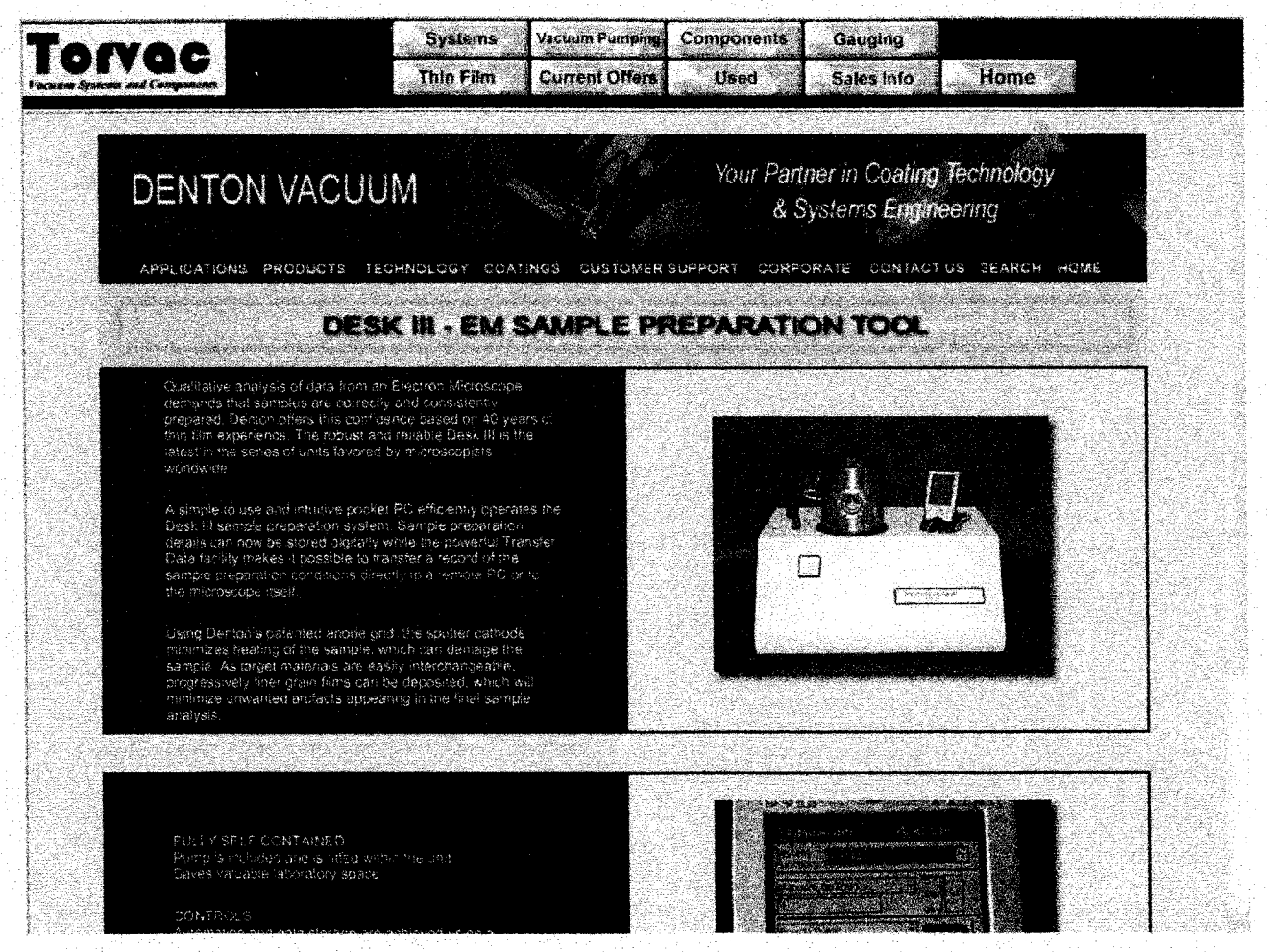
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Qualitative analysis of data from an Electron Microscope demands that samples are correctly and consistently prepared. Denton offers this confidence based on 40 years of thin film experience. The robust and reliable Desk III is the latest in the series of units favored by microscopists worldwide.
A simple to use and intuitive pocket PC efficiently operates the Desk III sample preparation system. Sample preparation details can now be stored digitally while the powerful Transfer Data facility makes it possible to transfer a record of the sample preparation conditions directly to a remote PC or to the microscope itself.
Using Denton's patented anode grid, the sputter cathode minimizes heating of the sample, which can damage the sample. As target materials are easily interchangeable, progressively finer grain films can be deposited, which will minimize unwanted artifacts appearing in the final sample analysis.
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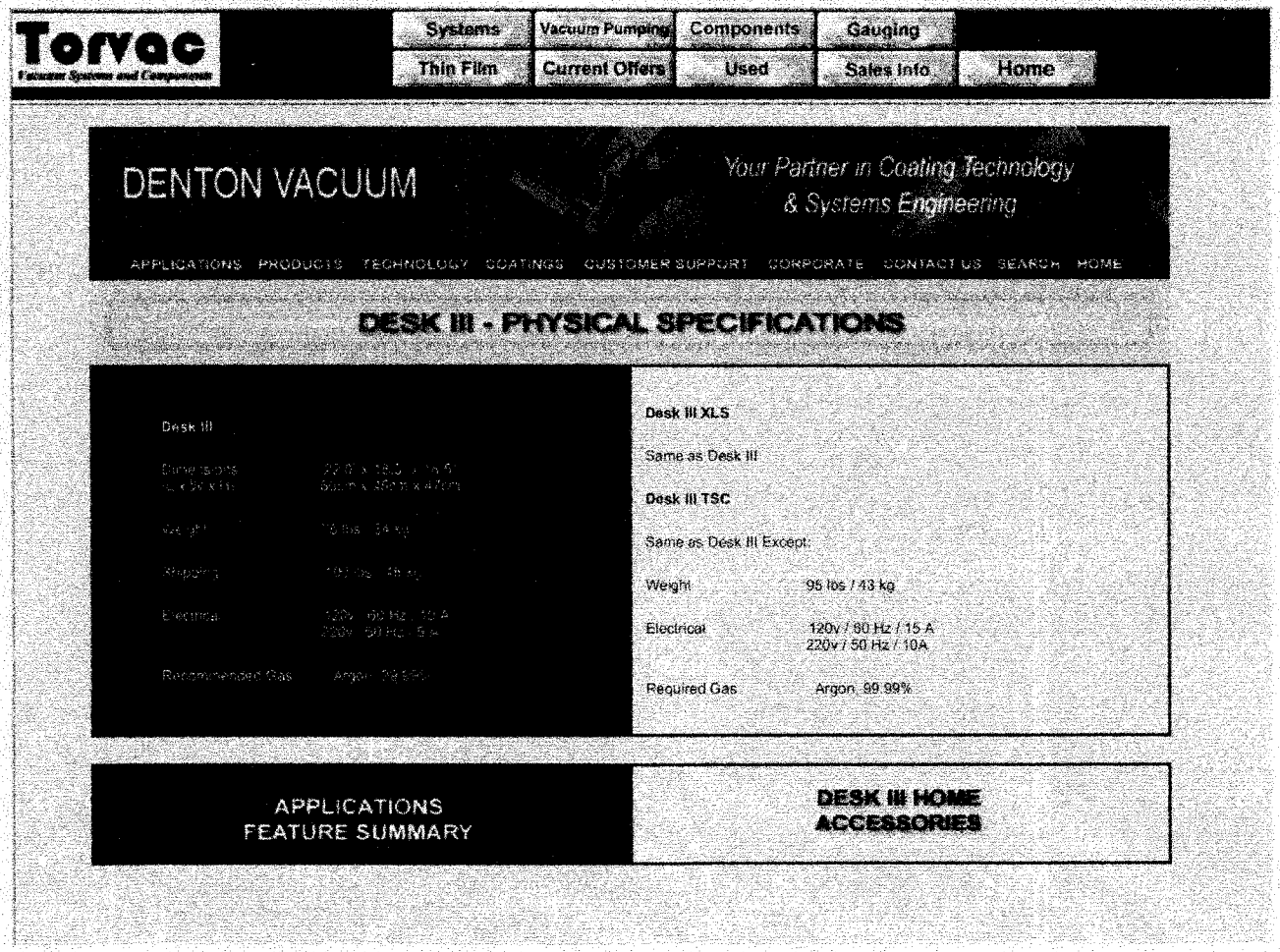
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APPENDIX B. SAMPLE PREPARATION PROCEDURE

1.0 Streptavidin Substrate Preparation

1.1 Prepare Glass Slide

A. Clean both sides of a glass slide with ethanol.

B. Sputter the clean glass slide with gold for 35 seconds at 35 milliamps and 50 military.

1.2 Prepare Stock Solutions

To keep errors to a minimum, all solutions used should be consistent throughout the entire experiment. Solutions should be frozen and subsequently thawed only once.

C. Prepare 10X 0.1M Phosphate Buffered Saline (PBS) solution by adding the following to 1L deionized water (H_2O):

Compound	**Quantity (g)**
Disodium hydrogen phosphate, dihydrate (Na_2HPO_4)	10.9
Monosodium dihydrogen phosphate, monohydrate (NaH_2PO_4)	3.2
Sodium Chloride (NaCl)	90

Verify solution pH is 7.2±0.2 after addition of both phosphates. Incorrect pH indicates an error has been made. Add NaCl and mix thoroughly. Addition of NaCl may

affect pH. If solution pH has changed, adjust pH to 7.2-7.4 using sodium hydroxide (NaOH). Solution is stable for days at ambient conditions or weeks if refrigerated, it can also separated into smaller volumes and then frozen for future use. Dilute 1:10 with H_2O prior to use and re-adjust pH as necessary. If the solution is refrigerated or frozen, thaw and dissolve again before use.

D. Prepare 100ml of 50mM MES buffer solution by adding 1.07g of MES to 100ml H_2O. Adjust pH to 5.0 ± 0.1 using sodium hydroxide (NaOH). The pH is vital and should be as close to 5.0 as possible. Solution is stable for a very long time if refrigerated.

E. Prepare 10mM 11-mercaptoundecanoic acid (MUA) solution by adding 21.84g of MUA to 10mL ethanol (ETOH). Solution is stable at room temperature. A small amount of precipitate is acceptable, but a solution containing a large amount of precipitate should be disposed.

F. Prepare 10mg/ml bovine serum albumin (BSA) solution by adding 1g BSA to 100mL PBS. Frothing is normal, however the solution should become clear after settling. Each cm of froth on the surface is indicative of 1mg/ml concentration. Refrigerate or freeze the solution and allow thawing before use. Dispose of solution if precipitate is observed.

G. Thaw 1mg of Streptavidin. Add a small amount of deionized water (1mL) to dissolve the Streptavidin. Prepare the 10µg Streptavidin/1 mL PBS solution by adding 1mg Streptavidin to 100mL PBS. Prepare ten 10ml aliquots of the diluted Streptavidin. Work with one aliquot at a time and freeze the rest. Thaw before use. Trace precipitate is acceptable. Dispose of solution if excess precipitate is observed. Not sure of ideal streptavidin concentration: could try 10, 1, or 0.1 µg/mL.

1.3. Prepare Substrate

H. If using pre-made, pre-sputtered slides, assume the slides are clean and skip this step because washing may add impurities. Cleanliness can be checked by observing wetting characteristics of water on the surface. Wash the gold surface of the slide using (1) water, (2) ethanol. This step will remove any impurities. The slide should look clean and uniform as it dries.

I. Expose the gold surface of the slide to 10mL of 10mM MUA overnight at RT. To protect the surface, immerse the slide in solution, and close the container to keep the ethanol from evaporating. Reaction time should be detectable by visible surface changes, overnight exposure may not be necessary.

J. Wash slide using (1) ethanol, (2) 50% ethanol/water by volume, (3) 25% ethanol/water by volume, and (4) water to remove unbound reagent (excess MUA,

thiol) without causing it to precipitate in water. Each wash should be approximately 5x the volume of the cell.

K. Dry slide in air or in oven. Slides will be stable for a few hours if stored in air or DI water or for a few days if stored under N_2, because oxygen may attack sulfur. Method: store in container filled with N_2. Argon is ideal because it is heavier than air and will settle on the substrate surface. Nitrogen is lighter and will rest on the surface of the substrate.

L. Thaw at least 78±8 mg (or 78±8μL) EDCI.

M. Take 5 mL of the MES buffer prepared earlier. Add 23±2 mg NHS and add 78±8 mg (or 78±8 μL) EDCI to the 5 mL of MES buffer. If EDCI does not dissolve, one might need to first dissolve the EDCI in a small quantity of water miscible organic solvent such as dimethylformamide (DMF), dimethyl sulfoxide (DMSO), or ETOH.

N. Note: EDCI is not stable once dissolved in water. Use within a few seconds or minutes.

O. Expose the slide to this NHS/EDCI/MES solution at RT for at least 30±10 minutes.

P. Wash the slide with PBS.

Q. Expose the slide to either 3, 10, or 30 µg/mL Streptavidin/PBS solutions for 30 minutes at room temperature. Determine the lowest detectable analyte concentration.

R. Wash the slide with PBS/BSA solution to remove unbound streptavidin and passivate the surface.

S. Store the slide in contact with fresh PBS/BSA at <40F until ready for use. Use slide within ninety days.

2.0 Ligand Preparation

2.1 Biotinylated B-Galactosidase (BG) Ligand Solution Preparation

A. Prepare the 10 µg/ 1mL BG in PBS by adding 0.5 mg BG to 50 mL DI.

B. Dissolve whole bottle (0.5 mg) with 1 mL distilled water into 10 aliquots. Add 0.1 mM Magnesium Chloride. Freeze 9 aliquots and work with only one aliquot at a time.

2.2 Biotin

C. Use PBS to clean flow cell.

D. Run plain buffer.

E. Run B-Galactosidase.

F. Run Biotin and BG.

G. Biotin will successfully bind before BG.

H. Will get small signal (able to detect Biotin) or no signal (unable to detect Biotin).

I. Lack of BG signal will indicate success of Biotin binding.

J. Check for presence with galacticide solution, will turn yellow.

APPENDIX C. MATERIAL QUANTITIES

Solution	Volume of Final Solution (mL)	Material	Acronym	Cat Lot	MW (g/mol)	Concentration in Final Solution (mM)	Quantity	RM Storage	RM Stability
Substrate Preparation									
MUA Solution	10	11-Mercaptoundecanoic Acid	MUA	45,056-1	218.4	10	21.84mg	RT	Yes
		Ethanol	ETOH			*	10 mL	RT	Yes
MES Buffer	100	MES-Hydrate	MES	M3671	213.3	50	1.07g	RT	Yes
		Deionized Water	DIH2O			*	100ml	RT	Yes
		Sodium Hydroxide	NaOH			to pH 5.0	Unk	RT	Yes
	5	MES Buffer	MES-Na			*	5ml	<40°F	Yes
		1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide	EDCI	39311	155.2	100	77.60mg	-30°F	Not in H2O
		N-Hydroxysuccinimide	NHS	130,672	115.1	40	23.02mg	RT	30 min in H2O
10X PBS	1000	Disodium hydrogen phospate, dihydrate	Na2HPO4	71643	178	100	10.9g	RT	Yes
		Monosodium dihydrogen phosphate, monohydrate	NaH2PO4	71506	138	100	3.2g	RT	Yes
		Dionized Water	DIH2O			*	1000ml	RT	Yes
		Sodium Chloride	NaCl		58.5	130	90g	RT	Yes
		Sodium Hydroxide	NaOH			topH 7.2	Unk	RT	Yes
		Dilute 1:10 with DIH2O prior to use							
Storage Solution	100	Bovine Serum Albumin	BSA		68000	10mg/ml	1000mg	RT	Yes
	100	Phosphate Buffered Saline	PBS			*	100ml	RT	Yes
Analyte	50	Streptavidin	MBP			10microgram/ml	0.5mg	-30°F	No
		Phosphate Buffered Saline	PBS			*	50ml		
Ligand Preparation									
Ligand	50	B-Galactosidase	BG			10microgram/ml	0.5mg	-30°F	No
		Phosphate Buffered Saline	PBS			*	50ml		