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Imobilization of antibodies on hydrazide activated glass surfaces via oxidized carbohydrate
residues

by

John Peter Gering

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
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Major: Analytical Chemistry
Major Professor: Edward S. Yeung

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This is to certify that the Master's thesis of

John Peter Gering

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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CHAPTER 1. INTRODUCTION

Thesis Organization

The purpose of the research discussed in this thesis was to covalently link antibodies to glass slides in a way that retains their ability to recognize and bind antigens. Its basic organizational structure consists of an introduction (Chapter 1), an article submitted to the journal Analytical Chemistry which contains an extensive literature review (Chapter 2), two appendixes to the paper, and concluding remarks presented in Chapter 3. The first appendix to the paper illustrates the attachment strategy utilized by Weiping et al. which is only described in the literature review of the journal article. The second appendix presents a calibration curve and discusses its development and how it was used to determine the percent of surface coverage with bound antibodies.

Introduction to Antibodies

Antibodies are proteins which are present in the blood and lymphatic systems. Their purpose is to attach to a foreign molecule (or antigen), thereby forming a complex which can be removed by macrophages in the course of phagocytosis. As is the case in the enzyme linked immunosorbent assays described below, an antibody obtained from another species can sometimes serve as the antigen. Antibodies recognize their respective antigens on the basis of a very specific configuration of amino acids and/or sugar residues on the antigen surface. This particular constellation of amino acids and carbohydrates is referred to as an epitope. Polyclonal antibodies are capable of recognizing more than one epitope on a particular antigen, whereas monoclonal antibodies will only respond to a single epitope.[1] Only polyclonal antibodies were used in the present study.

All antibodies used in this research were purified by immunoaffinity chromatography. This type of purification is accomplished by running the antisera though a column that contains an immobilized antigen that will retain the antibody of interest. After extraneous serum proteins (including antibodies that are not specific to the immobilized antigen) pass through the column, only the desired type of antibody is eluted.[2] In some cases, minimal cross reactivity to other species was insured by passing the purified antibody through a second column that contained immobilized antigens from other species. For example, minimal cross reactivity to human, bovine, and horse serum proteins in goat anti-

mouse immunoglobulin was assured by passing the latter through agarose gels that were activated with serum proteins from humans, cattle, and horses.[1]

Although several different classes of antibodies exist (e.g. IgG, IgM, IgA, IgE, IgD)[2] only the IgG class was utilized in the present study. A diagram of an IgG antibody molecule and its parts is presented in Figure 1.1 An immunoglobulin of this particular type consists of two heavy chains and two light chains with a combined mass of 150 to 160 kilodaltons.[2] The antigen binding sites correspond to the uppermost tips of the Y, and each antibody is therefore capable of binding two antigens. With the exception of the antigen binding sites, the amino acid sequence in the remainder of the molecule is relatively constant. Although they are not shown in Figure 1.1, each of the variable light and heavy regions typically contains at least one disulfide bond.[2]

Antibody Fragments

Although antibody fragments were not used in my research, some familiarity with such fragments is presupposed in the literature review presented in chapter 2. There appears to be some difference of opinion as to the uppermost boundary of the Fc region. Lu does not include the disulfide bonds of the hinge region as part of the Fc fragment[3], whereas Alarie[4] and Hermanson[2] do. Figure 1.1 follows the convention established by Lu.

Digestion with the proteolytic enzyme papain cleaves the antibody immediately above the two disulfide bonds in the hinge region, creating two Fab fragments (not shown in Figure 1.1) and an intact Fc fragment.[2] Enzymatic cleavage with pepsin leaves the disulfide bonds of the hinge region and everything above them intact, while the lower Fc portion is broken up.[2] This intact portion is variously referred to as an (Fab')$_2$ [Hermanson], or F(ab)'$_2$ [Alarie], or F(ab')$_2$ [Lu] fragment.[2-4] A reducing agent such as dithiothreitol[4] or 2-Mercaptoethylamine[2] will split the two disulfide bonds of the F(ab)$_2$ fragment yielding two Fab' fragments (shown in figure1.1). The Fab' fragment is similar to the Fab fragment except the former contains a sulfhydryl (-SH) group which can be utilized for various coupling strategies.
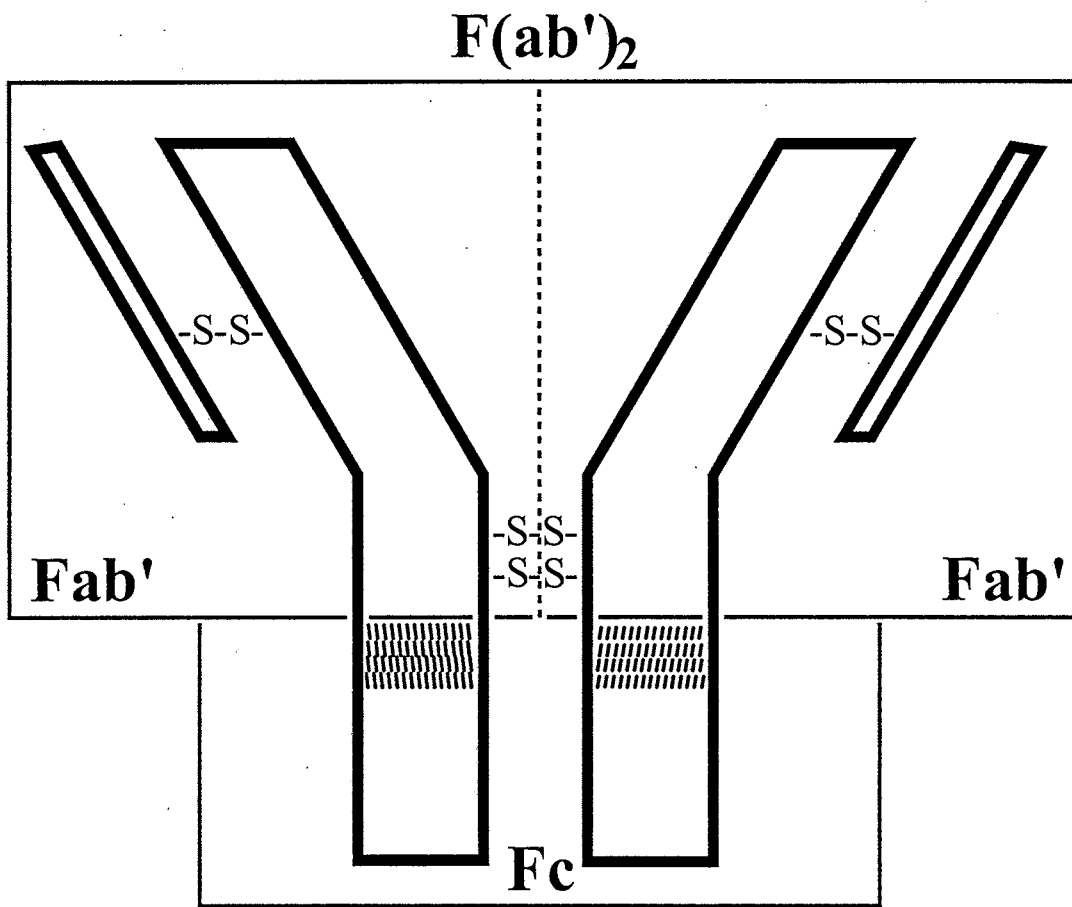
Figure 1.1 The C_H2 region with carbohydrate residues is indicated by crosshatching.

Introduction to Atomic Force Microscopy

Atomic force microscopy (AFM) was one of several methods employed for the detection of immobilized AB. With an enormous dynamic range and a spatial resolution ranging from several microns to less than 10 Angstroms[5], AFM has become a very desirable imaging tool. In the present case, an important advantage of AFM over scanning electron microscopy (SEM) is that, while the former works well on insulators and semi-conductors, the latter requires that the surface be either naturally conducting or rendered conducting by the deposition of a gold and palladium overcoating. The alloy overcoating used in SEM might obscure some surface features. AFM can be done in three different fundamental modes: contact, non-contact, and intermittent-contact (or tapping mode). In contact mode the probe tip remains in continuous contact with the surface. Hence forces resulting from friction and drag have the potential to alter the surface. A disadvantage of the non-contact mode is that if the surface being analyzed is covered by a thin layer of water (as is often the case under ambient conditions), the non-contact AFM image will fail to distinguish between the water layer and the underlying topography. Tapping mode AFM was used in the present study because it avoids both of these disadvantages. Furthermore, in the imaging of larger areas which show substantial topographical variation, its performance generally surpasses that of the non-contact mode.[6]

AFM images are generated as a piezoelectric scanner moves either a probe tip mounted on the end of a flexible cantilever or the sample itself to create a raster pattern. When van der Waals forces are plotted against the distance between the probe tip and the surface being imaged, it can be seen that, at a distance of several Angstroms, their magnitude is 0. At closer distances they increase rapidly and are repulsive. At distances greater than several Angstroms, the forces are attractive in nature. Their magnitude gradually decreases after reaching a maximum at a distance somewhat greater than the x intercept of several Angstroms. In contact mode the bending of the cantilever is commonly detected by alterations in the position of a laser beam that is reflected off of its mirrored surface. In non-contact mode the cantilever is vibrated at its resonant frequency (100 to 400 kHz). This frequency varies with the square root of the cantilever spring constant which in turn varies with the derivative of the force versus distance plot described above. The derivative of the

force versus distance curve is of course a function of the separation between the tip and sample. When operated in the tapping mode, changes in the amplitude of the cantilevers vibration are likewise a function of the tip to surface distance.[6]

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CHAPTER 2. IMMOBILIZATION OF ANTIBODIES ON GLASS SURFACES THROUGH SUGAR RESIDUES

A paper submitted to Analytical Chemistry

John P. Gering, Luca Quaroni, and George Chumanov

ABSTRACT

This work presents a step-by-step chemical procedure for attaching antibodies to glass surfaces via the oxidized sugar moiety. The method utilizes a condensation reaction between an aldehyde group on the antibody and a hydrazide group on the modified glass surface. ELISA tests were used to evaluate the activity and surface distribution of immobilized antibodies as well as nonspecific binding to surfaces after various modification steps. Light microscopy and AFM imaging in height and phase modes were performed revealing fairly uniform antibody coverage on micron and submicron scales. Exposure of the surfaces to a BSA solution minimized nonspecific binding to undetectable levels.

INTRODUCTION

Immunoassays have widespread applications not only in clinical and pharmaceutical chemistry but in environmental analysis as well.[1,2] The high specificity of the antibody/antigen interaction enables the relevant analyte to be isolated from complex mixtures with minimal sample preparation.[3] Immunoassays also offer the additional advantages of being relatively inexpensive and providing exceptionally low detection limits, which are made possible by enzymatic and radioactive labeling techniques.[3]

Although homogenous immunoassays are at least one order of magnitude faster than heterogeneous assays,[2] they are limited to the simultaneous analysis of at most 2-4 analytes. On the other hand, it is possible to develop a multi-analyte heterogeneous assay using various approaches to patterning antibodies (AB) at precise, spatially-addressable regions on

surfaces.[4-7] All heterogeneous assays require the immobilization of antibodies or antibody fragments on a solid support. The many immobilization protocols can be classified in terms of two basic types depending on whether the orientation of the immobilized antibodies is random or specific. Randomly immobilized AB may have one or both of the antigen binding sites positioned in such a way that the binding of the antigen is sterically hindered. Alternatively, when immobilization is done with a specific (or site selective) orientation, the binding regions of the AB can be made readily accessible to antigens by directing them upwards and away from the support.

Immobilization of AB with random orientation on surfaces can be achieved by spontaneous adsorption. Despite the simplicity, this approach can result in an over 90% loss of AB activity[8] and in desorption losses as high as 15% in a 16 hour time interval.[2]

Traditional covalent immobilization techniques utilize links to aminoacid side chains such as the amino groups of lysines, carboxyl groups from glutamic and aspartic acid, the aromatic ring of tyrosines, and sulfhydryl groups obtained by mild reduction of cystines, as reviewed by O'Shannessy and Quarles.[9] Since each of these groups is present on all parts of the antibody, random orientation is attained with these methods.[9] Even if the binding of the antigen is sterically unhindered, the coupling reactions can also alter the antigen recognition domain rendering that site inactive.[10] Furthermore, diminished flexibility resulting from multipoint attachment can interfere with the conformational changes required to adapt the immunoglobulin to a ligand.[11] A final consideration regarding randomly immobilized immunoglobulins is that improper orientation, denaturation, and attachment at multiple points, can accentuate the problem of nonspecific binding to the support.[12]

The disadvantages cited above have led to various approaches to immobilization in which attachment occurs only, or at least mainly, through the Fc portion of the antibody. In several schemes Fc receptors such as protein A and G are covalently linked to the support using techniques described above. A disadvantage of protein A is that it does not show an affinity to IgG from certain species.[13] Protein G will couple to antibodies from a wider range of species,[14] but exhibits weak attraction towards some of the immunoglobulins that are strongly bound by protein A.[15] Natural protein G also has albumin binding domains which increase non-specific attractions to the support.[16] The recombinant fusion of these two

proteins offers almost universal immunoglobulin binding capabilities while eliminating those regions of the G protein which contribute to non-specific binding.[16] However, random orientation of proteins A and G on the surface creates limitations similar to those encountered with immunoglobulins.

Proteolytic digestion of IgG results in Fab' and F(ab')$_2$ fragments in which sulfhydryl groups from the hinge region can be easily exposed for the vectorial immobilization on a surface. An additional advantage of using these fragments is that nonspecific binding to the Fc portion is eliminated.[16] Nevertheless, immunological activity of the fragments is not always preserved. Alarie et al. reduced F(ab')$_2$ fragments with dithiothreitol and immobilized the resulting Fab' fragments on silica beads activated with 2-fluoro-1-methylpyridinum toluene-4-sulfonate. They speculated that lower than expected activity (55-80%) was due to denaturation resulting from the unintended reduction of other disulfide bonds.[17]

Carbohydrate residues are largely located on the C_H2 domain of the Fc portion IgG.[16] In fact, only about 10% of IgG have carbohydrate residues on their Fab' fragments.[18] These observations lead to site selective immobilization strategies that rely on links between a support activated with either amine or hydrazide groups, and aldehyde groups produced from oxidizing oligosaccharide moieties. These strategies usually offer the highest retention of antigen binding ability.[16] Domen compared three coupling protocols: reduction of a Schiff base formed between an aldehyde activated support and an amine group on the AB, the reaction of a sulfhydryl group on the AB with an iodoacetic acid activated support, and attachment of AB to a dihydrazide support via oxidized carbohydrate residues. The respective mole ratios of bound antigen to immobilized AB were 1, 1.2, and 2; while the respective coupling efficiencies for all three methods were 79, 74, and 90%, respectively.[16] The specific activity of matrices obtained by reacting hydrazide activated agarose supports with aldehyde groups of antibodies was found to be four times greater than agarose supports which were coupled to IgG primary amines using either CNBr or esters.[9]

Methods which involve the attachment of biotinylated AB to avidin supports can result in either random or site selective orientation. Biotin conjugation to primary amine or sulfhydryl groups of the biomolecule will result in a haphazard orientation. Increased immunological activity resulting from selective orientation has been obtained by reacting

biotin-hydrazides with oxidized IgG carbohydrate residues.[19]

In the present study, we examine the covalent binding of antibodies to glass slides using a condensation reaction between an aldehyde group on the antibody and a hydrazide group on the modified surface. A similar procedure, used by David Hage to produce supports for immunoaffinity chromatography, served as the point of departure in this work.[12] This procedure was extensively modified to produce a high surface density of immobilized antibodies. The presence of covalently bound antibodies was assessed by ELISA using spectroscopic and tapping mode AFM measurements as well as light microscopy.

A similar site selective immobilization of antibodies was recently carried out by treating silicon wafers with aminopropyltriethoxysilane. The support was reacted with the oxidized IgG, and the resulting Schiff base was subsequently reduced by $NaBH_4$[20] (as shown in Appedix A). Although the protocol outlined by Weiping et al. involves fewer steps, the use of a hydrazide modified surface, utilized in the current study, offers several advantages for the attachment of oxidized biomolecules. First, sodium borohydride, as well as more specific reducing agents such as cyanoborohydride, are capable of reducing amino acids in the antigen binding site.[9] The hydrazone bond formed between aldehyde and hydrazide moieties is stable in a pH range of 3-10 and does not need to be further reduced. Second, since the pK's of the hydrazide and amino groups are ca. 2.6 and ca. 9.0, respectively, immobilization of Ab to a hydrazide support can be carried out at a pH sufficiently low to minimize cross linking between amino and oxidized carbohydrate residues on immunoglobulins. Third, hydrazide activated supports have also been shown to reduce non-specific binding to polystyrene microwell plates.[21] Finally, in our approach the adipic acid dihydrazide serves as a spacer that increases the distance between the bound IgG and the glass substrate. It was found that using spacer molecules of longer length increased the mole ratio from 0.53 to 0.7 of bound antigen to Ab immobilized on hydrazide modified agarose.[11]

EXPERIMENTAL SECTION

Materials. All antibodies, 3,3`- Diamino Benzdine tetrahydrochloride (DAB), and o-Phenylenediamine Dihydrochloride (OPD) were supplied by Pierce (Rockford, Ill.). 3-Glycidoxypropyltrimethoxy silane was obtained from Gelest (Tullytown, PA). Adipic acid

dihydrazide and sodium borohydride were purchased from Sigma (St. Louis, MO). Fisher (Fair Lawn, NJ) supplied both HPLC grade isopropanol and reagent grade periodic acid.

Surface modification. Slides (1 x 0.5 inch) made of quartz, soft glass, or glass covered with a layer of indium tin oxide, were used as supports. A cylindrical slide holder was made in house from Kel-F. The holder had an indentation on the bottom the size of a magnetic stir bar. This allowed the entire slide holder to spin as the various reactions were carried out. Slides were cleaned in concentrated sulfuric acid saturated with potassium persulfate. 2% HF can be used to remove previous depositions of silane from quartz.

Silanization was initially carried out by refluxing in 50 ml of 0.1M Na acetate buffer which contained 200 µl of 3-glycidoxypropyltrimethoxy silane for 5 hours. Since this approach did not provide highly uniform coverage we turned to a modified version of a silanization procedure used by Goss et al.[22] Slides were placed in a solution of 73.5 ml isopropanol and 1.5 ml water. This solution was brought to reflux temperature. 130 µl of 3-glycidoxypropyltrimethoxy silane was added. The slides were stirred and refluxed in this solution for 15 minutes. Next, the slides were gently agitated in pure isopropanol for about 15 to 30 seconds. The slides were air dried for 3 to 5 minutes (until they looked dry) and then cured in an oven for 15 minutes at 107 °C. In subsequent batches of slides the same procedure was followed but the amount of silane was reduced to 30 µl and the reflux time was also reduced to 5 minutes. Although one cycle of refluxing, drying, and curing provided satisfactory results, the most uniform depositions (as evidenced by the degree of hydrophobicity) were achieved with a total of three cycles.

After silanization, the epoxy group was oxidized to a diol by refluxing for 90 minutes with a dilute pH 3.0 sulfuric acid solution. The diol functionality was further oxidized to an aldehyde group by exposing the slides to a solution consisting of 6 ml water, 54 ml glacial acetic acid, and 1.0 g periodic acid for 2 hours at room temperature. Glacial acetic and periodic acids (as well as their vapors) can cause severe irritation of the skin and respiratory tract and irreversible eye damage. After two water rinses, the slides were sonicated in isopropanol for 10 to 15 minutes, and rinsed again four times with water. Since residual periodic acid may react with the sodium borohydride used in a subsequent step, care must be taken to ensure that it is removed from the intervening spaces between the slides and the

holder. Hydrazide activation was carried out in a solution of 0.04 g adipic acid dihydrazide in 60 ml of 0.1 M sodium phosphate buffer at pH 5.0 at room temperature. The probability that both ends of the dihydrazide molecule would attach to the aldehyde groups of the support was minimized by: (1) limiting the exposure time to one hour, (2) using a 5,000 fold mole excess of adipic acid dihydrazide relative to the estimated number of aldehyde groups on the support, and (3) reducing unreacted aldehyde groups to alcohol with $NaBH_4$ immediately after attaching the dihydrazide.

The reduction of unreacted aldehyde groups to alcohol was carried out by exposing the slides to a solution of 0.017M sodium borohydride and 0.1M, pH 7.5 sodium phosphate buffer for 90 minutes at room temperature. The prepared slides were stored in pH 7.0, 0.1M sodium phosphate buffer. It has been reported that dihydrazide supports retain 90% of their activity after 40 days if stored at 5 °C.[12]

Oxidation of antibodies. The extent of oxidation of the carbohydrate residues on the Fc portion of the antibodies depends on pH, periodate concentration, exposure time, and temperature.[23] The oxidation was carried out by agitating the IgG in 0.02 M sodium acetate buffer, pH 5.0, 0.15 M NaCl, and 0.010 M periodic acid. Exposure to light was minimized during the preparation of the periodic acid reagent as well as during the oxidation reaction. A typical concentration of AB used for oxidation was ca. 120 µg/ml. The reaction was stopped by the addition of ethylene glycol to produce a 20% final concentration and shaking for two minutes. Two oxidation protocols were used: 20 minutes at 4°C and 30 minutes at 25°C.

After oxidation unconsumed ethylene glycol and undesirable oxidation byproducts such as formaldehyde were removed by dialysis. Dialysis was carried out with four 2 l aliquots of buffer using Pierce "Slide-A-Lyzer" cassettes with a 10,000 MWCO and extra thick membrane for a total of 20 hours at 4 °C. The first dialysis was performed against 0.15 M NaCl, 0.02 M sodium acetate buffer pH 5.0. In the second and third dialysis cycles 0.1M sodium phosphate buffer at pH 7.0 with 0.1% (v/v) Triton X-100 was used. Finally, the oxidized antibodies were dialyzed against 0.1 M sodium phophate buffer at pH 7.0 without Triton X-100.

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Attaching oxidized antibodies to slides. Two approaches were employed for the covalent attachment of the oxidized antibodies to the hydrazide surface. In the first approach, 35 μl of a 2.7-4.4 mg/ml Ab solution was sandwiched in between two modified slides, and placed in an enclosed container with 100% humidity at 4 °C. The concentration of antibodies was estimated to be from 60 to 100 times larger than that required for monolayer coverage of both surfaces assuming 10^4 Å^2 footprint per antibody molecule. The slides were separated after 48 hours and gently washed with a 0.1M phosphate buffer at pH 7.0. In the second approach, a Teflon holder limiting the antibody exposure of the slides to only two ca. 6.6 mm spots was used. The holder was placed on ice and shaken for 48 hours. Both approaches yielded comparable results.

OPD test. An OPD based ELISA test using 500, 2,500 and 5,000 fold dilutions of a 3.2 μM solution of horse radish peroxidase (HRP) labeled Ab was carried out to detect the presence of covalently bound antibodies. HRP catalyzes the transformation of OPD to an intense golden brown soluble product, the concentration of which was determined spectroscopically. OPD is a possible carcinogen, and should be handled with gloves and eye protection. Under two-way recognition conditions, goat anti-rabbit immunoglobulin was detected by means of a HRP labeled rabbit anti-goat antibody. In the case of one way recognition, the immobilized Ab was rabbit anti-goat with goat anti-mouse (minimal cross reactivity to human, bovine, and horse serum proteins) serving as labeled Ab.

The tests were compared with a control slide that was activated with hydrazide groups but lacked immobilized Ab. Both slides were blocked for 1 hour with a 1% bovine serum albumin (BSA) solution in 0.1 M sodium phosphate buffer pH 7.0 (blocking buffer). Both slides were exposed to the HRP labeled Ab solution in blocking buffer for 1 hour. Three 15 minute washes were performed in 20 ml of blocking buffer with 0.05% (v/v) Tween-20 detergent. All steps (blocking, exposure, and washing) were carried out at room temperature under continuous shaking. The OPD solution was prepared according to manufacturer's directions (1mg OPD, 1 μl of 50% hydrogen peroxide per ml of 0.05 M citric acid, 0.05 M sodium phosphate buffer, pH 5.0).

After washing, slides were transferred to new containers and exposed to 10 ml of the OPD solution. The extent of the uncatalyzed reaction was monitored using pure OPD

solution. After 22 minutes the reaction was stopped with 7.5 ml of 2.5 M sulfuric acid. Absorbance of the colored product was measured at 490 nm. When necessary, dilutions were made with 0.03 M citric acid, 0.03 M sodium phosphate, 1 M sulfuric acid. A calibration curve was also developed by reacting 0 to 100 µl of the 3.2 nM labeled Ab with 10 ml of 1 mg/ml OPD.

OPD tests were carried out with plain glass, hydrazide activated glass, and glass covered with cellobiose in order to assess the susceptibility of these surfaces towards non-specific binding. Cellobiose was attached to a hydrazide activated surface in a way similar to the attachment of oxidized antibodies - specifically through the aldehyde group of the open chain form which exists in equilibrium with the cyclic hemiacetal.

DAB test procedure. The HRP enzyme was also used to catalyze the reaction of DAB to an insoluble brown colored precipitate that can be made darker by chelation with nickel and cobalt. The metal enhanced DAB concentrate is toxic and flammable. Tests using metal enhanced DAB were carried out to determine the uniformity of the coverage of the substrates with immobilized antibodies. The blocking, exposure to secondary antibody, and other treatment steps were identical to those used for the OPD tests. After the DAB was diluted to a concentration recommended by the manufacturer and heated to 37.5°C, the slides were immersed in the solution for a period of time ranging between 18 seconds and 10 minutes to obtain precipitates of different density.

AFM measurements. Atomic force microscopy (AFM) images were acquired with a Nanoscope III BioScope (Digital Instruments-Veeco, Santa Barbara, CA) equipped with a Dimension "G" scanner (100 micron x 100 micron x 6 micron; x, y, z scan maxima). All AFM images were collected in Tapping Mode under ambient room conditions, using a V-shaped probe, nominal spring constant kc = 3.2 nN/nm (ThermoMicroscopes, Sunnyvale, CA), at an oscillation frequency ca. 92 kHz, with free-air amplitudes ranging over 40-100 nm. The engage setpoint was adjusted such that 20% dampening of the free-air amplitude was considered as engagement with the surface. After minimizing the applied load (vertical force), the z-piezo position was centered, allowing maximal extension and retraction of the z-piezo. Height and phase mode images were collected at 1 Hz scan rate in the "scan down" direction, using one probe per specimen. When needed the images were corrected for z-

piezo drift with an offline 1st-order flattening program (Nanoscope Software, v 4.23r3, DI-Veeco). The "erase scan line" function was applied when necessary to remove scan artifacts. Surface plot images are presented in mixed mode at 45 degree rotation and 45 degree pitch.

RESULTS AND DISCUSSION

A diagram of the steps involved in the preparation of the support and Ab immobilization is presented in Figure 2.1. The first step is a condensation reaction between a silanol group of the support and 3-glycidoxypropyltrimethoxy silane. The next step is an acidic hydrolysis in which the epoxide group of the silane is converted to a diol. Treatment of the diol group with periodic acid yields formaldehyde and a support terminated with an aldehyde group. Subsequently, the terminal aldehyde group reacts with one of the two hydrazide groups of adipic acid dihydrazide, leaving the other hydrazide as the terminal functional group of the support. At this point the hydrocarbon chain could loop back towards the surface and condense with unreacted aldehyde groups. This reaction leads to inactivation of the support and was minimized by reducing any unreacted aldehyde groups to alcohol with sodium borohydride. Finally, the oxidized carbohydrate residue of the Ab is condensed with the hydrazide activated support to form a hydrazone.

Our experience indicates that the most uniform depositions of silane are obtained by refluxing with a non-aqueous solvent (isopropanol), followed by curing. This observation is in agreement with previosly reported results.[24] Silanized slides appeared to be thoroughly hydrophobic. Although one cycle of refluxing, drying, and curing provided satisfactory results, the most uniform deposition (as evidenced by the degree of hydrophobicity) was achieved with a total of three cycles.

An important question relates to the extent to which oligosaccharide moieties on the AB should be oxidized. On the one hand, it is desirable to increase the number of potential sites for attachment on each antibody. On the other hand, increasing the number of aldehyde sites likewise increases the possibility for interactions between antibodies through Schiff base linkages.[23] The oxidation of Ab was carried out at 4 °C for 20 minutes and at 25 °C for 30 minutes. These conditions are expected to produce an average of 1 and 2 aldehyde groups per antibody respectively.[23]

The uniformity of the surface coverage by Ab is affected to large extent by the presence of other molecules potentially competing with the oxidized Ab for active sites on the surface. Such competition may involve either non-specific binding, or the formation of a covalent bond as in the case of the oxidation byproduct formaldehyde. This argument was used to explain the failure of our initial attempts to immobilize fluorescein-labeled Ab. The preparation contained a large excess of bovine serum albumin as a stabilizing agent. The interference arising from the BSA might be attributed to both the nonspecific binding of the BSA to the hydrazide activated support, as well as to covalent bonding involving oxidized carbohydrate residues on the small percentage of BSA molecules which could be glycosilated.[25] The use of BSA as a stabilizing agent for the primary AB was consequently abandoned.

Triton X-100 in a concentration of 0.1% is commonly used to minimize aggregation of antibodies.[2] This is especially important for chemically modified and oxidized Ab that can form Schiff base linkages. However, the detergent strongly interferes with UV-Vis absorption measurements of antibody concentration. It is important to note that this amount of Triton is 7 times above the critical micelle concentration.[26] Attempts to remove the detergent by ultrafiltration through 10 and 30 kD MWCO membranes were unsuccessful because the average micelle size of about 90 kD (estimated from an aggregation number of 138 and a molecular weight of 650) exceeds the pore size.[26] Even though we do not have direct evidence that Triton X-100 interferes with Ab immobilization and subsequent chemistry, we attempted to keep the detergent concentration at a minimum by carrying out the last dialysis cycle of the oxidized Ab in a Triton free buffer. In spite of the fact that the two preceding dialysis cycles were carried out against a 0.1% Triton buffer, the Triton concentration of the Ab solution may have been less than 0.1% even before the start of the last cycle. (Since much of the Triton was bound up in micelles, the concentration of monomeric Triton actually capable of penetrating the membrane's 10kD MWCO pores, was less than 0.1%.)

Slides treated with BSA as well as immobilized and labeled antibodies were fully transparent and appear indistinguishable from clean glass or quartz. This fact is significant for the future development of immunoassays that rely on spectroscopic methods of detection.

The presence of immobilized antibodies was determined by ELISA tests using Ab with one and two way recognition, as described in the Experimental Section. No significant difference in the final product was noted between these two schemes indicating that, over a one-hour reaction time, the labeled antibodies saturated the binding sites of all Ab immobilized on the surface. Typical experimental results are presented in Table 2.1.

Methods for immobilization of antibodies on solid supports should be developed with a perspective towards minimizing nonspecific binding. We have explored several possibilities to reduce nonspecific adsorption of the labeled Ab. It is known that plain glass exhibits a high degree of protein adsorption.[27] Our measurements with HRP labeled Ab and plain glass yielded only ca. 3 times less OPD product as compared to glass with immobilized antibodies where special precautions were exercised to bring nonspecific binding to undetectable levels (see below). This result confirmed extensive nonspecific binding of Ab to the plain glass. A test for nonspecific binding was also performed for hydrazide modified glass surfaces. However, no significant differences were noted in the binding of the labeled Ab for the plain and the modified glass indicating a high degree of nonspecific protein adsorption to the hydrazide surface as well.

Cellulose coated surfaces are known to display a low adsorption of proteins.[28] An attempt was made to use cellobiose to mimic a cellulose surface and minimize adsorption of labeled Ab. An appealing feature of this approach is that the attachment of cellobiose to glass can be carried out using the same procedure as for the immobilization of Ab. Cellobiose modified surfaces consistently yielded less nonspecific adsorption of the labeled Ab than plain glass but the improvement (less than two times) is obviously insufficient for practical immunoassay applications.

The most efficient way for minimizing nonspecific binding of the labeled antibodies to glass substrates was blocking the surface with BSA after Ab immobilization. These surfaces exhibited undetectable levels of nonspecific binding as is evident from Table 2.1, where the absorbance measured from the OPD solutions containing control slides that were blocked with BSA was practically indistinguishable from that of OPD solutions where only the uncatalyzed reaction took place (compare column 3 with column 4).

An important question relates to the surface density of immobilized Ab on glass and

the uniformity of their distribution. The traditional way for imaging of immobilized antibodies by measuring fluorescence from a label is limited by optical resolution. Furthermore, the quantitative assessment of the antibodies present is ambiguous due to the possible quenching of the emission and uncertainty in the number of chromophores per Ab. In the current study, several methods were used to evaluate surface density and uniformity of Ab coverage. Measurements by scanning electron microscopy of 12 nm gold labeled antibodies that were bound to the Ab immobilized on the surface were inconclusive, presumably because of interference from the gold palladium overcoating needed to provide a conducting layer.

In the first method, a calibration curve was generated from data of the enzymatic reaction in solution containing known amounts of the HRP labeled Ab. An ELISA test was carried out with slides containing specifically bound HRP labeled Ab. The data were compared to the calibration curve (shown in Appendix B) and the amount of immobilized antibodies was thus determined. The results are shown in column 5 of Table 2.1 as the percentage of Ab present on the surface relative to monolayer coverage, assuming a 10^4 Å^2 footprint per antibody. The apparently low values most likely underrepresent the actual coverage of Ab for the following reasons. It was assumed that the kinetics of the HRP on the surface and in solution is the same, in spite of the fact that it is known that the kinetics of homogenous reactions are at least one order of magnitude faster than inhomogeneous ones.[2] Steric hindrances arising from the large size of the HRP labeled Ab (an average of 2.5 enzymes per antibody molecule) will also contribute to the lower apparent number of immobilized Ab. On account of such hindrances, the population of labeled Ab is likely to be smaller than the number of immobilized antibodies.

In the second method light microscope images were acquired of substrates on which the enzymatic reaction resulted in an insoluble metal enhanced DAB deposit. The reaction time was set at 7.5 minutes in order to prevent merging of the metal islands. An image of a slide with immobilized Ab and the corresponding control slide, both after the enzymatic reaction, are shown in Figure 2.2. Individual metal islands can clearly be seen in the former (Figure 2.2a) whereas no precipitate can be detected in the latter (Figure 2.2b). Based on these data, it was concluded that the Ab deposition is fairly uniform on the scale determined

by optical resolution. The absence of the precipitate in the control slide further confirms that nonspecific binding is negligible.

Finally, atomic force microscopy was employed to evaluate the density and uniformity of the immobilized Ab on glass. Because of the ambiguity involved with direct imaging of antibodies, DAB deposition was used to highlight the presence of immobilized Ab. AFM images were acquired in height and phase modes for several glass slides at various stages of modification. Figure 2.3 presents AFM images in height mode of a slide after surface modification with hydrazide, plain glass exposed to the BSA blocking solution, and slides with immobilized Ab before and after the enzymatic reaction. The same vertical scale (125 nm/div) was used in these images to facilitate the comparison of the surface morphology. As can be seen, the silanization procedure resulted in a fairly smooth surface; the bump in the uppermost corner corresponds to a dust particle (Figure 2.3a). On the other hand, treatment of glass slides with BSA, both plain (Figure 2.3b) and hydrazide modified (image not shown), produce characteristic features that can be attributed to the protein. Variations in size could be due to different aggregates of BSA on the surface. The image in Figure 2.3b illustrates the fact that BSA forms a uniform coating on glass surfaces, thereby reducing nonspecific binding of other proteins. When both immobilized and HRP labeled antibodies are present on the slide together with BSA (Figure 2.3c), the surface appeared noticeably different as compared to BSA alone. It can be inferred that surface features became larger due to the increased size of protein complexes and, consequently, increased surface roughness. An AFM image of DAB deposition shown in Figure 2.3d exhibits a very significant increase in height on account of metal islands. However, the DAB layer appeared less uniform than the layer of the protein complexes from Figure 2.3c. Variations in enzymatic activity of HRP labeled Ab as well as surface distribution of the complex can conceivably account for the apparent nonuniformity of the metal deposit. Also, the deposit could contain more small metal islands, which are indistinguishable in the height image from the protein layer.

Phase imaging is also a useful method for surface evaluation because it reflects a specific and nonspecific interaction between an AFM tip and surface species, thereby potentially providing information about the "chemical" uniformity of a substrate. Phase

images of substrates at different stages of modification are presented in Figure 2.4 where lighter features correspond to the phase lag that results from a higher level of affinity of the tip to the surface. A comparison of the images reveals relatively low contrast for the BSA and antibody modified surfaces (Figure 2.4a, 2.4b, and 2.4c) whereas the slide with DAB deposits (Figure 2.4d) exhibits distinct areas of low and high contrast suggesting a larger difference in affinity of the tip to metal islands compared to the rest of the surface. The phase contrast image in Figure 2.4d demonstrates better uniformity of the DAB deposit than the corresponding height image in Figure 2.3d. This fact can be attributed to the presence of small metal islands that are impossible to differentiate from the proteins in the height image, as was alluded to above. Overall, both height and phase measurements demonstrated that AFM imaging can be successfully applied to monitor different stages in antibody immobilization procedures on glass surfaces.

In conclusion, attaching antibodies to glass surfaces via the oxidized sugar moiety proved to be an adequate method providing uniform coverage of active antibodies on micron and submicron scales. Nonspecific binding can be minimized to undetectable levels by exposing the surface with attached Ab to BSA solution. The method will be further developed for patterning antibodies on surfaces for applications in multianalyte immunoassays.

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Table 2.1 OPD test results

| Substrate and Ab coverage: | Absorbance of solution containing: | | | % Coverage |
	Sample Slide	Control Slide	Uncatalyzed OPD substrate solution	
Quartz-Full	2.120	0.106	0.104	2.69
Quartz-Full	2.468	0.057	0.034	3.28
Quartz-2 dots	0.466	0.016	0.015	2.89
Glass-2 dots	0.505	0.017	0.015	3.13
ITO-2 dots	0.351	0.025	0.015	2.00

b = path length = 1cm

FIGURE CAPTIONS

Figure 2.1 Schematic for antibody immobilization on a glass surface through sugar residues.

Figure 2.2 Light microscope images of (a) DAB deposit on quartz slide with immobilized Ab and (b) quartz slide without immobilized Ab (control slide). Both slides were exposed to HRP labeled Ab and DAB substrate.

Figure 2.3 AFM images in height mode of (a) hydrazide activated surface, (b) plain glass with BSA, (c) hydrazide activated surface after treatment with immobilized Ab, HRP labeled Ab, and BSA, and (d) hydrazide activated surface treated with immobilized Ab, HRP labeled Ab, BSA, and DAB.

Figure 2.4 AFM images in phase mode of (a) plain glass with BSA, (b) hydrazide activated surface with BSA, (c) hydrazide activated surface after treatment with immobilized Ab, HRP labeled Ab, and BSA, and (d) hydrazide activated surface treated with immobilized Ab, HRP labeled Ab, BSA, and DAB. Each image corresponds to an ca. 0.5 x 0.5 μm sampling area.
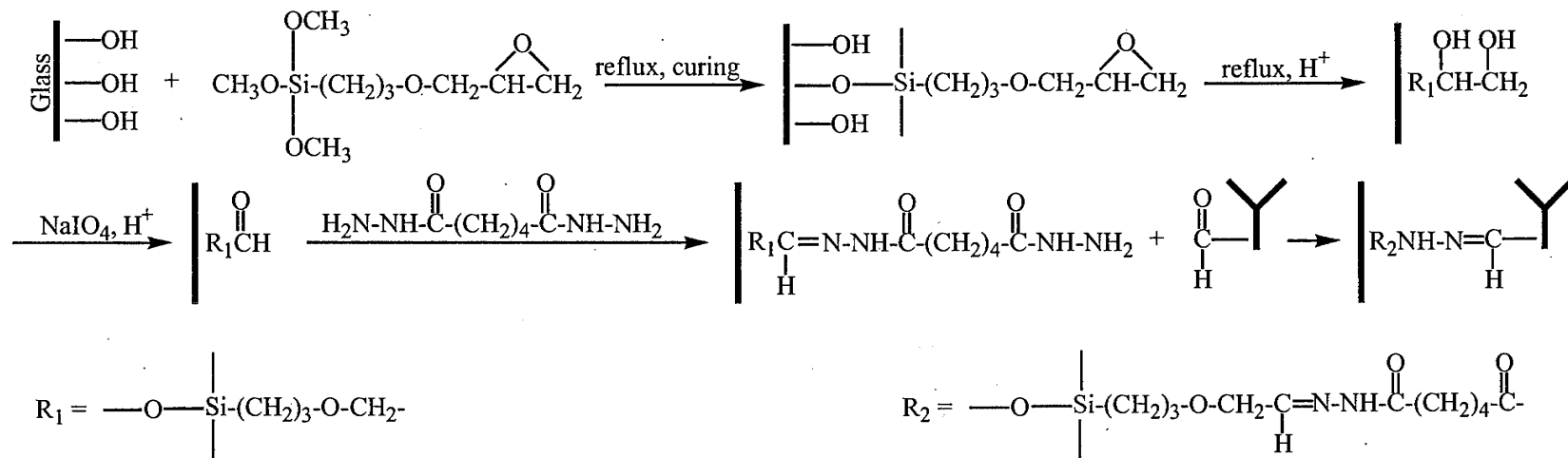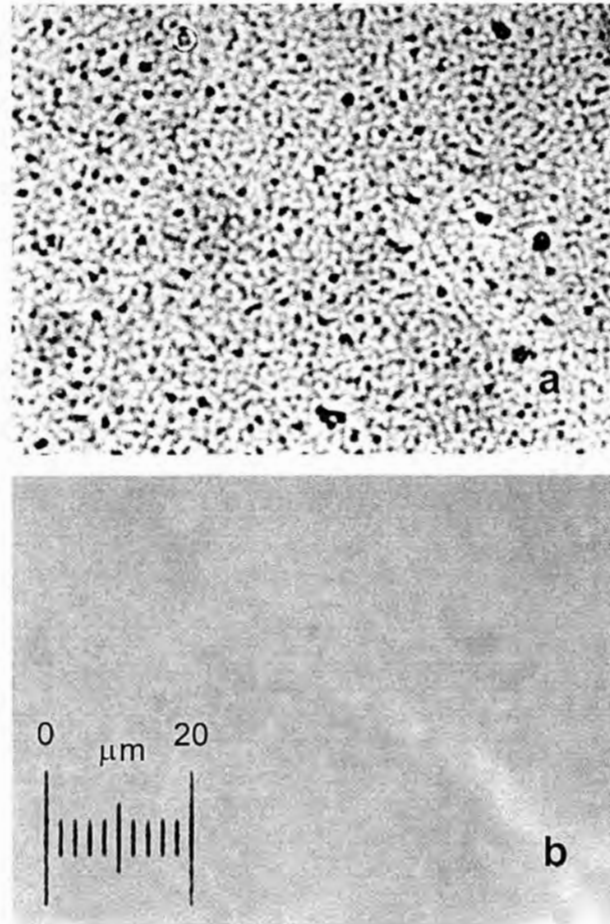
Glass —OH
—OH + CH₃O-Si-(CH₂)₃-O-CH₂-CH-CH₂ $\xrightarrow{\text{reflux, curing}}$ —OH Si-(CH₂)₃-O-CH₂-CH-CH₂ $\xrightarrow{\text{reflux, H}^+}$ R₁CH-CH₂
—OH

$\xrightarrow{\text{NaIO}_4,\ \text{H}^+}$ R₁CH   H₂N-NH-C-(CH₂)₄-C-NH-NH₂ $\longrightarrow$ R₁C=N-NH-C-(CH₂)₄-C-NH-NH₂ + $\longrightarrow$ R₂NH-N=C

$R_1 = $ —O—Si-(CH₂)₃-O-CH₂-

$R_2 = $ —O—Si-(CH₂)₃-O-CH₂-C=N-NH-C-(CH₂)₄-C-

Figure 2.1

0 μm 20

a

b

Figure 2.2
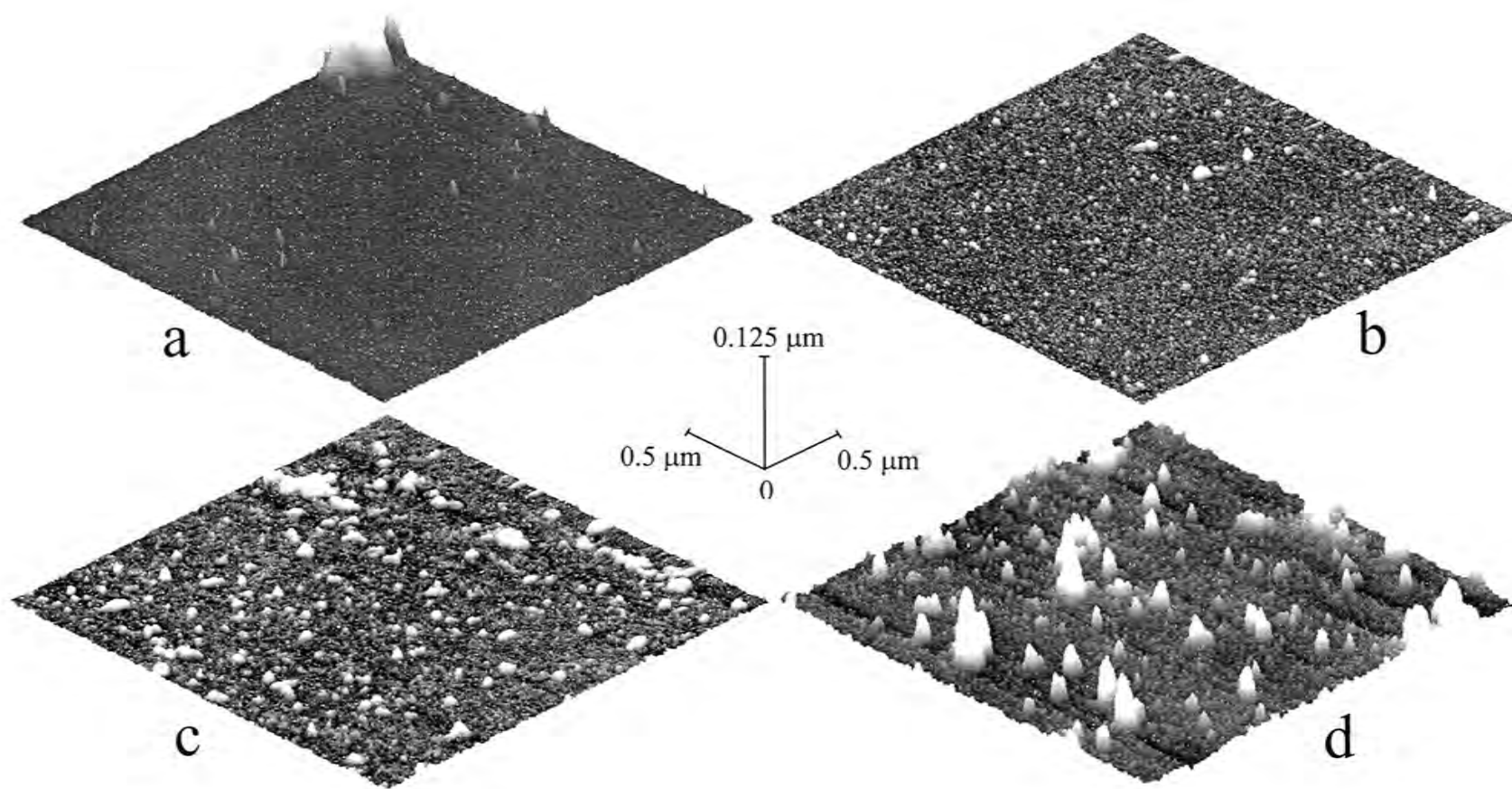
0.125 μm

0.5 μm   0.5 μm
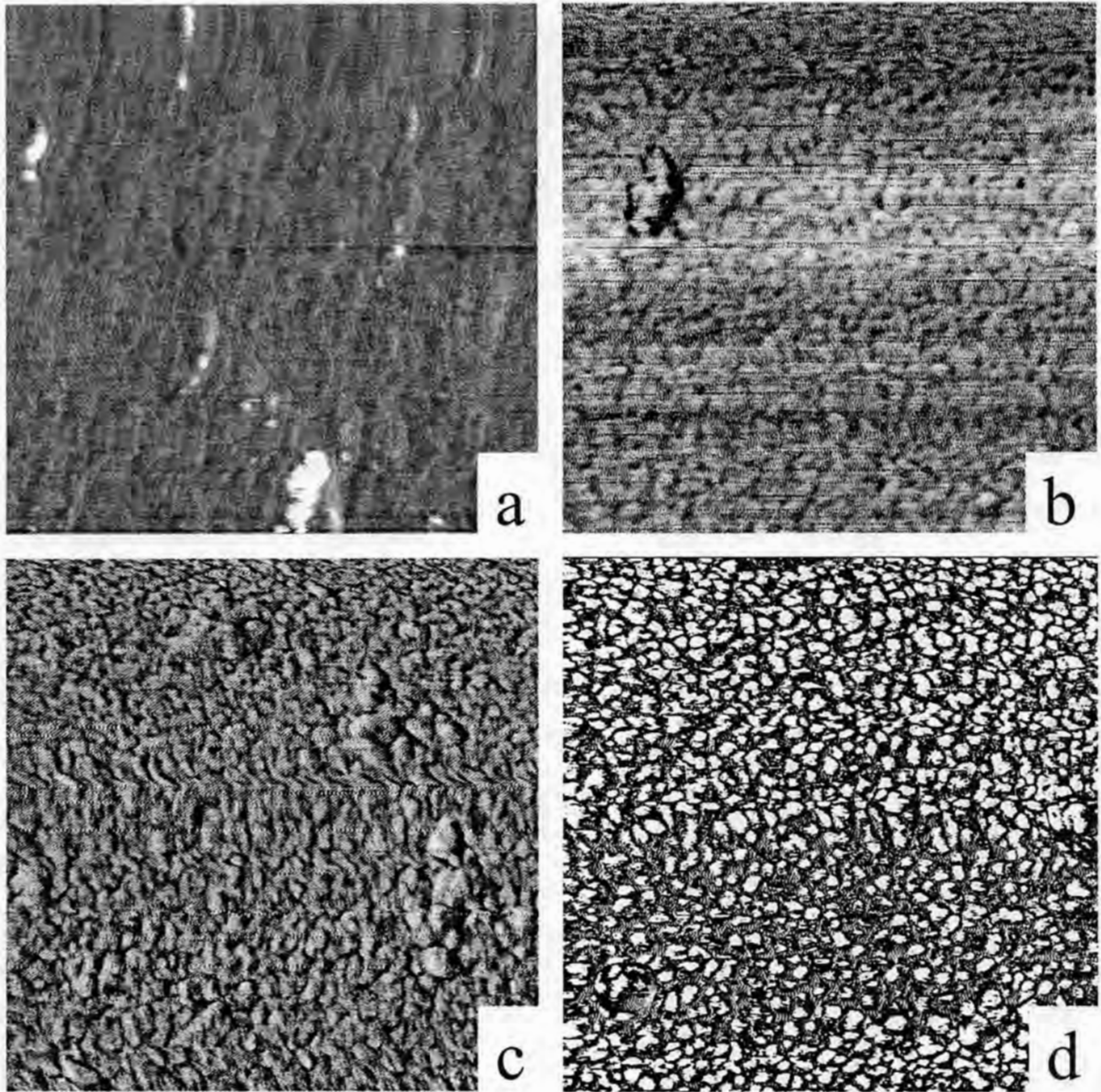
0

27

Figure 2.3

Figure 2.4

APPENDIX A. ILLUSTRATION OF AN ATTACHMENT METHOD USED BY WEIPING

The abstract and text of the article written by Weiping et al. claim that silicon wafers were silanized with aminopropyltriethoxysilane (APTES), however the figure in their article actually shows silanization with aminopropyltrimethoxysilane (APTMS). The difference between the two silanes is hardly significant because, in the course of forming the polymerized surface layer, the hydrocarbon portions of the alkoxy chains are eliminated anyway. The illustration provided below follows the text of Weiping's article (i.e. silanization with APTES rather than APTMS).
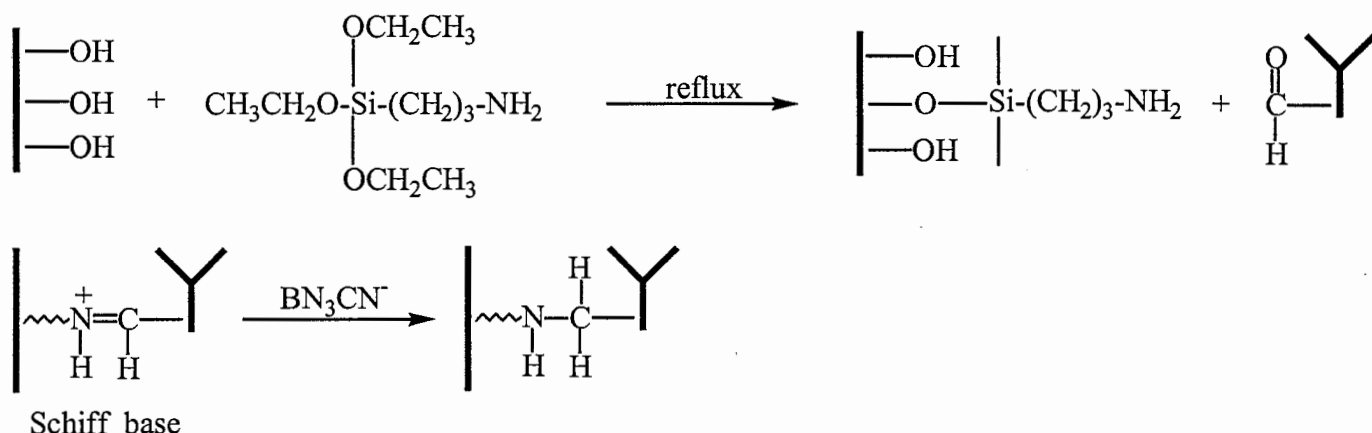

Figure Appendix A.1 Site selective immobilization is obtained by the reduction of a Schiff base, which results from the reaction between an amino activated support and the oxidized carbohydrate residue of an AB.

APPENDIX B. CALIBRATION CURVE AND CALCULATIONS FOR PERCENT COVERAGE OF SLIDES WITH IMMOBILIZED AB FROM OPD TESTS

The calibration curve used to determine the extent of coverage given in column 5 of Table 2.1 is presented on the following page. 0 to 100 µl volumes of HRP labeled AB solution were injected into 10ml aliquots of OPD substrate solution which were then treated as described in the experimental section of the journal article. The X axis represents percent coverage of a 1" by 0.5" area, assuming an average AB "footprint" of 10,000 square Angstroms and 150,000 grams/mole. Measured absorbance minus the absorbance of the uncatalyzed reaction served as the Y axis.

The Y values plugged into the regression line equation were obtained by subtracting the absorbance of OPD end product produced by a sample slide from that of a control slide. If only two ca. 6.6 mm spots were treated with primary AB, this difference was multiplied by a factor of 4.74, to obtain the equivalent absorbance for a 0.5 square inch area. The percent coverage, calculated on the basis of absorbance as well as the slope and Y intercept of the regression line, is reported in column 5 of Table 2.1.
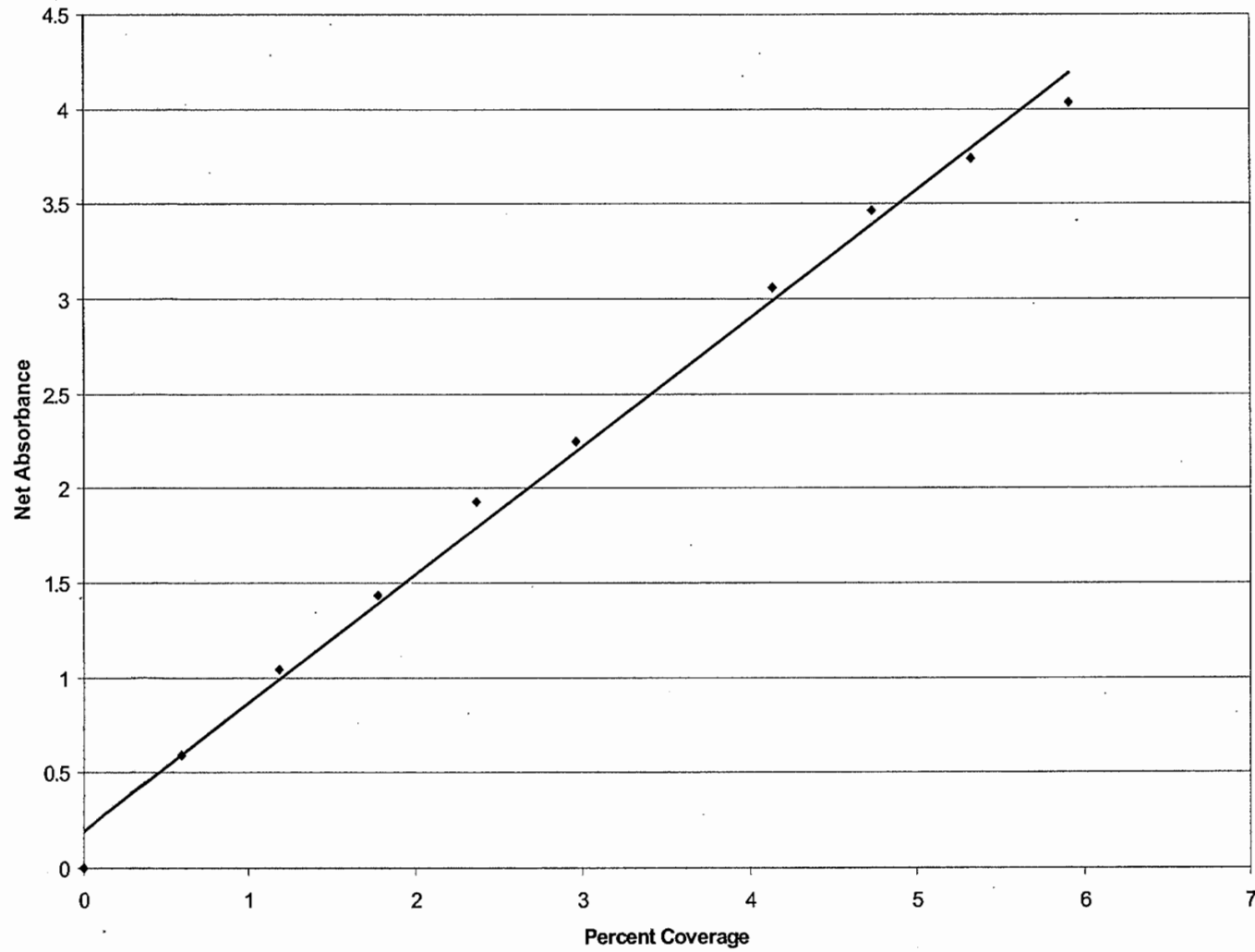
Figure Appendix B.1 Calibration curve used to determine percent coverage of slides with immobilized AB from OPD tests

CHAPTER 3. CONCLUSION
Optimization of Silanization

As observed in the preceding chapter, our data indicated that the most uniform depositions of silane are obtained by refluxing with a non-aqueous solvent (such as isopropanol), followed by curing at 107°C. In order to present the chemical rationale for this conclusion, it must pointed out that the process of silane deposition represented in the first step of Figure 2.1 actually involves two separate processes.[1] First, the methoxy-silicon bonds of 3-glycidoxyproplytrimethoxy silane are hydrolyzed to produce silanol groups:

$$2 \equiv Si\text{-}OCH_3 + 2\ H_2O \rightarrow 2 \equiv Si\text{-}OH + 2\ CH_3OH$$
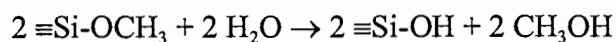
Then a condensation reaction occurs in which a siloxane linkage is formed between two silanol groups, one of which may be on the surface:

$$\equiv Si\text{-}OH + HO\text{-}Si \equiv\ \rightarrow\ \equiv Si\text{-}O\text{-}Si \equiv + H_2O$$
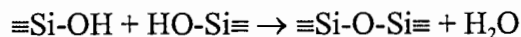
However prior to curing, the condensation reaction is mainly limited to the formation of siloxane bridges between molecules in solution.[1] The curing step facilitates the condensation reaction between silanol groups <u>on the surface</u> and the silanol groups of polymerized flakes. It has been reported that when depositions of aminopropyltriethoxy silane are carried out in aqueous solvents, irreproducible coatings result because of "uncontrollable hydrolysis and polymerization".[1] In other words, the polymerized flakes grow so rapidly that their attachment to the surface during curing is actually hindered by their size.

Ultrafiltration Versus Dialysis

It is also helpful for future researchers in this area to reflect carefully on the factors involved in choosing between ultrafiltration and dialysis as a technique for purifying antibodies and changing their buffers. Despite the disadvantage of slower speed, there are two reasons why dialysis is preferable to ultrafiltration as a method of reducing the concentration of species that compete with the oxidized biomolecules for surface coverage. First, centrifuging is less efficient in removing the ethylene glycol and formaldehyde. (Recall that the addition of excess ethylene glycol is used to consume the periodic acid after the antibodies have been oxidized to the desired extent. One mole of ethylene glycol reacts with one mole of periodic acid to produce two moles of formaldehyde.) Assume that 3ml of

oxidized antibodies are dialyzed against four 2L aliquots of buffer and that each dialysis cycle reaches equilibrium. Suppose also that in the course of four ultrafiltration cycles the volume of a 3 ml sample is reduced to 100 μl at the end of each cycle. In such a scenario the purification achieved by dialysis is 5 orders of magnitude greater:

$$[(100μl / 3,000μl)^4] / [(3.0ml / 2,000ml)^4] = 2.4 \times 10^5.$$

Secondly, ultrafiltration invariably raises the amount of Triton X-100 in the purified and concentrated antibodies far beyond the intended level of 0.1%. Once again, suppose that in the course of four ultrafiltration cycles the volume of a 3 ml sample is reduced to 100 μl at the end of each cycle. Even if the 3 ml volume at the start of the first cycle contains no Triton, 2900 μl of 0.1% Triton solution will be added at the start of each of the remaining three cycles. If it is assumed that (1) all of the Triton is in micelles, and (2) that none of the approximately 90kD micelles pass through the 30kD MWCO ultrafiltration membrane; then a total volume of 2900 x 4 μl of 0.1% Triton will be reduced to a 100 μl volume. This scenario will raise the Triton concentration to 11.6% - more than a hundred fold increase. On the other hand, if the dialysis of the oxidized antibodies is carried out in a 0.1% Triton buffer for only several hours, the Triton concentration after dialysis may even be somewhat less than 0.1%. This is the case because some of the Triton will be in the form of micelles, and thus unable to penetrate the 10kD MWCO pores of the dialysis membrane.

Summary of Work with Fluorescent Labeled AB

Although the first attempts at immobilization were carried out with fluorescein labeled AB, efforts to confirm the presence of bound antibodies using fluorescence were never successful. Reasons that account for the failure of these initial attempts fall into two categories. First of all, antibody coverage may have been minimal or non-existent. Secondly, even if antibody coverage was substantial, inherent difficulties in fluorescence as a detection method may have prevented their discovery.

With respect to the first class of factors, commercial sources almost always use BSA to stabilize fluorescent labeled antibodies, and the possible interference of BSA through non-specific as well as covalent binding has already been discussed in the previous chapter. Also, since the purification of the fluorescent labeled antibodies was done exclusively through ultrafiltration, the concentration of Triton X-100 was in fact much higher than the

intended 0.1% by volume. Although most of the slides treated with fluorescent labeled AB were silanized using an aqueous solvent without a curing step, attempts at detecting fluorescent labeled AB on slides treated with the improved method of silanization (i.e. a non-aqueous solvent for the silane followed by curing) were likewise inconclusive.

The intrinsic difficulties with using fluorescence as an analytical tool for the detection of antibody monolayers begin with extraneous peaks from the substrate itself that might obscure fluorescence coming from the antibody label. Although the emission peak for dichlorotriazinyl amino fluorescein in solution occurs at 517 nm,[2] analysis is complicated by the fact that proximity to a surface could cause the fluorescein emission peak to shift. For example, one study compared emission of the fluorescein dianion both in an aqueous solution and after its immobilization in ethyl cellulose. The emission was red-shifted from 513 nm to 536 nm.[3] The first attempts at detecting fluorescein labeled antibodies were done using a 488 nm laser for excitation and a triple monochromator normally used for the detection of Raman scattering. Hydrazide activated normal glass slides (which had never been exposed to fluorescent labeled AB) displayed a large but narrow peak at 516 nm and another broad peak centered at 535 nm.. Low alkali glass that had been hydrazide activated - but was not exposed to fluorescein labeled AB - showed little or no signal at 516 nm, but did have a broad peak centered at 530 nm.

It might be thought that one way of eliminating interference due to the fluorescence of the glass itself would be to establish the background using a control slide (i.e. a hydrazide activated slide that was not exposed to antibodies) and then simply subtract that background from measurements taken using the slide that was treated with AB. However, slight differences in the angle at which the laser beam hits the two slides, along with differences in the degree to which scattered and fluorescing light is focused on the entrance slit of the monochrometer, make this strategy very difficult. (The apparatus for restricting coverage to two 6.6 mm dots was not in use at this time.) Since plain quartz did not show any fluorescence in the vicinity of 535 nm, it was used as the substrate in all the remaining work with fluorescent labeled antibodies.

Partly for convenience, but also in order reduce the possibility of bleaching due to the intensity of the laser beam, the work was continued on a Fluro-Max 2 fluorometer. Using

this instrument, it was established that the process of oxidation with sodium periodate did not significantly affect the emission of fluorescein in solution. It was also shown that a drop of fluorescein labeled antibodies deposited on a plain quartz slide maintained the same intensity of fluorescent emission fifteen minutes and one hour after the drop was placed on the slide. Hence drying, or at least a certain degree of drying, did not affect the fluorescence quantum yield.

When analyzing surfaces with the Fluro-Max 2 reflections from the long wavelength shoulder of the 492 nm excitation beam became a problem. (Recall that fluorescence was expected to occur at 517 nm.) In addition to reducing the bandwidth of the excitation and emission monochromator slits, several strategies were adopted to minimize this problem. The Fluro-Max 2 emission collecting slit is located at an angle normal to the path of the excitation beam. In the following discussion about how the signal to noise ratio was optimized, the angle of the excitation beam is always given relative to a line normal to the surface being analyzed. Although the apparent "signal" of a fluorescent surface can be maximized by turning it so that the excitation beam is at 45 degrees, the signal to background ratio was optimized by using an excitation angle between 45 and 90 degrees. This result is not surprising. Reflection does increase with the angle of incidence (especially after 60 degrees).[4] However, a smaller percentage of the reflected light enters the emission monochromator as the angle of incidence increases, since the angle of the incident and reflected beam relative to a line normal to the fluorescing surface is equal.[5]

The factory holder that came with the Fluro-Max 2 had a solid block of metal (painted black) positioned on the side of the slide that was opposite the excitation source. Removal of this metal block decreased the intensity at 505 nm more than 10 fold when exciting at 492 nm. A final tactic for reducing interference from the long wavelength shoulder of the excitation beam was to change its wavelength from 492 nm to 465 nm. Because internal conversion across overlapping vibrational levels of two different electronic states is more likely than fluorescence from the higher electronic state, the fluorescence peak (unlike a Raman peak) will generally not shift as the energy of the incident photon is increased.[6] A square glass filter with maximum transmission centered around 465 nm was placed between the exit slit of the monochromator and the slide. Even when all these

strategies to reduce reflection of the excitation beam were employed, immobilized fluorescent labeled AB could not be detected on a slide which had been silanized with the improved (Goss) method.

Differences in the Concentration of Oxidized AB for the Two Approaches Used in Covalent Attachment

As described in chapter 2, two approaches were used to attach oxidized AB to the slides. In the first approach, a small drop (35 µl) of oxidized AB was "sandwiched" in between two slides in such a way that the entire area of each 1 x 0.5 inch slide was exposed to the AB. In the second approach, only one slide was used and its exposure to oxidized AB was limited to two 6.6 mm spots. Future researchers should be aware of the fact that, although the area of an entire slide is 4.74 times bigger than the area of two small dots, the difference between these two approaches is not merely a difference of the area covered. Assume that in each approach the excess of antibody over and above the amount needed for monolayer coverage of the available surface is equal. If both holes above the dots are filled to a volume of 80 µl, it follows that the volume of the solution used per slide in second approach will be 9.1 times greater than the volume used in the first or "sandwich" approach [i.e. (80 µl * 2)/(35 µl * .5)]. Since the area covered in the first method is 4.74 times greater than the area of the two dots, it follows that - all other factors being equal - the concentration of the oxidized antibodies used for making a "whole slide sandwich" will be about 43 (4.74 * 9.1) times greater than that which is used to cover two small dots. If less than 80 µl is used per hole in the second approach, evaporation may become a problem.

Future Work Towards the Development of a Multianalyte Immunoassay

As was pointed out earlier, slides coated with BSA and immobilized antibodies remain fully transparent. Future research will be directed towards the development of spectroscopic methods of detection for multicomponent immunoassays. Our group did preliminary work on a detection method which utilizes the ability of colloidal silver to scatter an evanescent wave produced under conditions of total internal reflection.

Suppose a beam of light propagates towards the interface between two mediums of differing refractive indices, and the angle of incidence (Θ_i) is measured relative to a line perpendicular to the interface (see Figure 3.1). If n_1 is used to specify the refractive index in

the medium that contains the incident beam, and n_2 the refractive index of the other medium, an angle known as the critical angle (Θ_c) will be given by the following formula: $\Theta_c = \sin^{-1}(n_2/n_1)$. Note that Θ_c exists (is a real number) only when $n_1 \geq n_2$. When $\Theta_i = 0$, the amount of light transmitted through the interface will be at a maximum, and the amount of reflected light will be at a minimum. As Θ_i approaches Θ_c transmission through the interface decreases and reflection of the incident beam increases. When Θ_i is greater than Θ_c, total internal reflection occurs - in other words the percent transmitted is zero and all of the light is reflected.[5]

However, even under the conditions of total internal reflection, an evanescent wave extends into medium with the lower refractive index (medium$_2$) for a few hundred nm, provided that medium$_2$ is not a perfect electrical conductor. The electric field amplitude of the evanescent wave decays exponentially along a line perpendicular to the interface, and is a function of Θ_i, the wavelength of the incident light, and the difference in refractive indices of the two mediums. If an absorbing or scattering species resides within several hundred nm of the interface, part of the energy of the evanescent wave can be captured before it returns the medium of higher refractive index.[5] Figure 3.1 portrays the scattering of an evanescent wave by a particle of colloidal silver that has been coated with antibodies that recognize an antigen immobilized by antibodies attached to the glass surface. Although the drawing is not to scale, the silver particles would have a diameter of about 100 nm. While using a patterned array of different immobilized antibodies, several analytes of interest could be detected and quantified by measuring the amount of scattered light emanating from each region of the array.
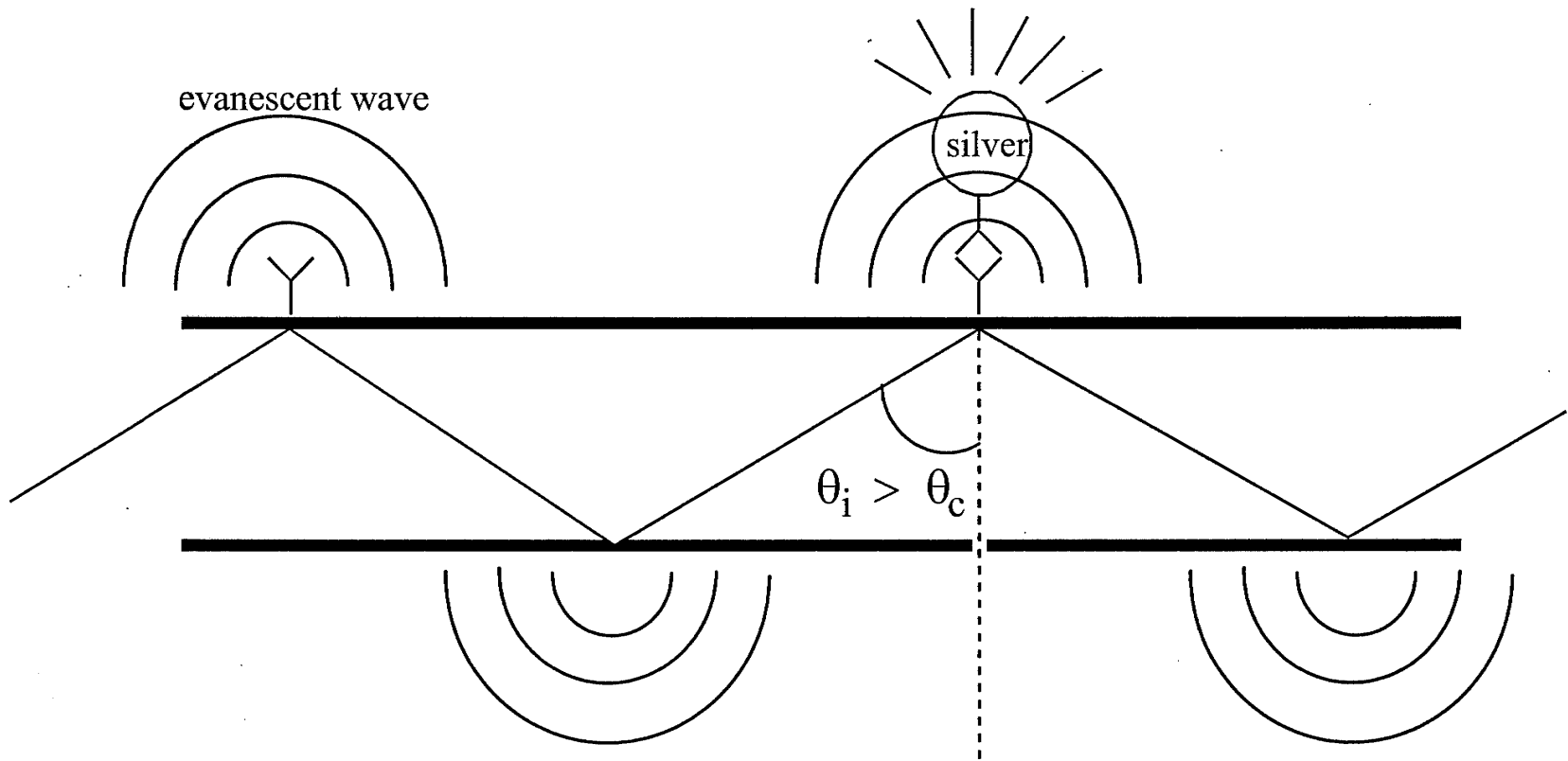
$$\theta_i > \theta_c$$

Figure 3.1 Total internal reflection produces an evanescent wave that can be scattered by an AB labeled silver particle. The antigen which binds the two antibodies on the right is not shown.

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