CHAPTER 4
RESULTS AND DISCUSSION

The work stated in this thesis was mainly focussed towards the development of immobilization strategies for coating antibodies on the silicon chip surface. Antibodies were immobilized by various protocols using non-covalent and covalent interactions. Several immobilization chemistries like silanes (7-9), crosslinkers (6), biotin-avidin interactions (19-21) and different polymers (10, 11) have been used by researchers for immobilizing antibodies for immunosensor applications. Although many of these chemistries provide greater stability and binding of human IgG but there is loss in the specificity and sensitivity of the immunosensor as some of the antigen binding sites of human IgG are oriented in such a way that they are inaccessible to the antigen molecules.

A key component in an immunodiagnostic kit is the recognition molecule i.e antibodies and its immobilization. To achieve maximum sensitivity, it is essential that antibody molecules are immobilized in such a manner that occlusion of their antigen binding sites present in the Fab region does not occur.

The major emphasis of my doctoral research was on the oriented-immobilization of human IgG antibodies on the bare as well as gold-coated silicon surfaces. Silicon chips (area: 1 cm x 1 cm) were employed for immobilization studies as they were easy to handle. The upper surfaces of silicon chips were coated with gold using vacuum coating unit. The Gold coating had an advantage in the sense that antibodies would be selectively coated on the gold-coated surface. For the immobilization of antibodies on gold-coated surfaces, various methodologies were used:

(a) Protein A,
(b) Protein G,
(c) 3-APTES + glutaraldehyde + protein A,
(d) Neuravidin

For the immobilization of antibodies on bare silicon surface (i.e. not coated with gold) human IgG immobilization was carried out using 3-APTES + Glutaraldehyde + Protein A. Other methodologies (a, b and d) were not used as human IgG does not bind
effectively to bare silicon. In addition, we wanted directed immobilization of human IgGs as it was essential for our experiments.

4.1 HUMAN IgG IMMobilIZATION ON GOLD-COATED SILICON

To immobilize horse radish peroxidase (HRP) conjugated human IgGs on to gold-coated silicon (area: 1 cm x 1 cm), the latter was coated with various agents i.e. Protein A, Protein G, Neutravidin and 3-APTES + Glutaraldehyde + Protein A. Different concentrations of each treatment were used. All the treatments resulted in maximal immobilization of IgGs when Protein A/Protein G/Neutravidin was present in the concentration around 0.1 mg/ml. Thereafter, each human IgG-HRP coated silicon was dipped, with the coated surface facing up, in 5 ml capacity glass beakers containing TMB substrate assay solution i.e. 250 µl of TMB + 250 µl of H₂O₂ + 499 µl of TBS + 1 µl of 0.1% BSA. The immobilization of the HRP conjugated human IgGs was confirmed by the conversion of colorless solution to blue color. This happens because the enzyme peroxidase (conjugated to IgG molecules) in the presence of H₂O₂ catalyses the oxidation of colorless TMB substrate to a blue colored product. The extent of immobilization of human IgG varied depending on the type of treatment given (table 3). The O.D. at 450 nm of TMB substrate assay solution after TMB substrate assay with human IgG-HRP samples were 0.76 for protein A procedure which corresponded to the immobilization density of 610 ng/cm²; 0.73 for protein G procedure which corresponded to the immobilization density of 590 ng/cm²; 0.69 for Neutravidin procedure which corresponded to the immobilization density of 530 ng/cm² and 0.63 for 3-APTES + Glutaraldehyde + protein A which corresponded to the immobilization density of 490 ng/cm². The immobilization density of human IgG (610 ng/cm²) was maximum when protein A immobilization procedure was followed. The immobilization density was 3.28 percent less w.r.t. protein A procedure when protein G procedure was used; 13.11 percent less w.r.t. protein A procedure when Neutravidin procedure was used and 19.67 percent less w.r.t. protein A procedure when 3-APTES + glutaraldehyde + protein A procedure was used. Based on these results, it was concluded that ‘protein A treatment’ for the oriented immobilization of antibodies was most suitable for immobilizing antibodies on gold-coated silicon. Therefore, this procedure was assessed in detail. The qualitative and
quantitative analysis of antibodies immobilized on the gold-coated silicon was done by inverted fluorescence microscopy, fluorescence spectroscopy, TMB substrate assay and AFM.

**Table 3.** Immobilization levels of human IgG bound on gold-coated silicon chip (1 cm x 1 cm) by different immobilization procedures.

<table>
<thead>
<tr>
<th>Immobilization procedures</th>
<th>Immobilization levels of human IgG followed (as analysed by TMB substrate assay)</th>
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</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>610 ng/cm² (24.3 x 10¹¹ molecules/cm²)</td>
</tr>
<tr>
<td>Protein G</td>
<td>590 ng/cm² (23.5 x 10¹¹ molecules/cm²)</td>
</tr>
<tr>
<td>Neutravidin</td>
<td>530 ng/cm² (21.1 x 10¹¹ molecules/cm²)</td>
</tr>
<tr>
<td>3-APTES + Glutaraldehyde</td>
<td>490 ng/cm² (19.6 x 10¹¹ molecules/cm²)</td>
</tr>
<tr>
<td>Protein A</td>
<td></td>
</tr>
</tbody>
</table>

3-APTES + Glutaraldehyde procedure and Protein A-gold procedure have been used by others (116) for the immobilization of antibodies on the gold-coated quartz crystal. Protein A immobilized on gold surface has been successfully used to orient the antibody with its antigen binding sites free to bind antigen molecules. The best results in terms of long-term sensitivity and stability were obtained when the antibody was covalently coupled to the surface via protein A-gold immobilization method. Glutaraldehyde used in 3-APTES immobilization method adversely affected the gold surface because of its high reactivity with the surface leading to an overall reduction in the stability of the system (118). But protein A-gold complexes were very stable and have an association constant of 10⁹ M⁻¹ as reported by Roth (119). The immobilization density results obtained by Suri and co-workers at Institute of Microbial technology, Chandigarh, India (120) for the IgG binding on Protein G were comparable with those obtained by Muramatsu and co-workers at Research and Development Department, Seiko Instruments Inc., Chiba, Japan (121) to determine IgG concentrations using protein A. Similarly
comparable results were achieved by us with Protein A and Protein G procedure as shown in Table 3.

4.1.1 Qualitative Analysis

For the qualitative analysis of the selectivity of protein A towards gold, gold electrodes patterned on the silicon substrate were employed. Protein A-FITC labeled was immobilized on the substrate and from the inverted fluorescent microscope image as shown in figure 11, it was concluded that human IgG antibodies were bound specifically on gold electrodes and had only little affinity towards silicon surface.

Similarly Protein A-FITC was immobilized on gold-coated silicon and visualized by inverted fluorescent microscopy. It was apparent from figure 12 that protein A bound uniformly on the gold-coated silicon.

Goat anti-human IgG-FITC labeled antibodies were immobilized on human IgG coated silicon. It was found from the inverted fluorescent image that goat anti-human IgG was coated uniformly on silicon surface (figure 13). Thus, the uniform coating of goat anti-human IgG directly correlates with the uniform coating of human IgG on the protein A coated silicon.

Fluorescence microscopy had been used to give information on the distribution of the antibody coating on the gold-coated silicon nitride cantilever by Grogan and co-workers at Department of Physics, National University of Ireland, Galway, Ireland (65). Thus, the immobilization of antibodies on the solid substrate can be better visualized qualitatively by fluorescent imaging and information can be drawn about the uniformness of the immobilized antibodies.

4.1.2 Quantitative Analysis

The quantitative analysis of immobilized protein A and goat anti-human IgG on the silicon chip surface was done by eluting the immobilized protein A-FITC and goat anti-human IgG-FITC from the substrate, and taking the reading of the eluted samples in fluorescent spectrophotometer. Goat anti-human IgG-FITC was eluted from the substrate by 50 mM Glycine-HCl buffer (pH 2.2) whereas protein A-FITC was eluted from the
Figure 11. Fluorescent image (under 10X objective) of protein A-FITC specifically immobilized on gold electrodes patterned on silicon. The right image is the enlarged view of the section of left image.

Figure 12. Fluorescent image (under 10X objective) of Protein A-FITC bound to gold-coated silicon.

Figure 13. Fluorescent image (under 10X objective) of goat anti-human IgG-FITC bound to protein A-coated silicon.
surface by 0.1 N dilute HCl. Calibration assays (appendix I) were performed with known concentrations of protein A-FITC and goat anti-human IgG-FITC. The fluorescence intensity of the eluted protein A-FITC and goat anti-human IgG-FITC samples were 0.0 and 0.23 respectively which corresponded to the immobilization density of 0.1 µg/cm² (12.0 x 10¹¹ molecules/cm²) for protein A and 0.6 µg/cm² (24.0 x 10¹¹ molecules/cm²) for goat anti-human IgG as determined from their calibration curves.

Fluorescent spectroscopy had been employed by Suri and co-workers (120) as quantitative method to determine the amount of 3-APTES bound to the solid substrate in gold-coated quartz crystals. Therefore, this technique can be used for quantitation of molecules immobilized on the solid substrate. But more reliable quantitative analysis of amount of molecules immobilized on the solid substrate can be done by TMB substrate assay employing HRP labelled molecules as discussed below.

The quantitative analysis of protein A and human IgG immobilized on silicon was done by employing protein A-HRP and human IgG-HRP. Gold-coated silicon having protein A-HRP immobilized on its surface was put in a 5 ml beaker, with the coated surface facing up. The amount of HRP labeled protein A immobilized on the gold-coated silicon was determined by a colorimetric assay using TMB. The peroxidase enzyme, in the presence of H₂O₂, catalyses the oxidation of colorless TMB substrate to a bluish colored product. After 30 min, the reaction was stopped by adding 100 µl of 2M H₂SO₄ and the absorbance of the solution was measured at 450 nm. Calibration assays (appendix I) were performed with known volumes of protein A-HRP. The O.D. at 450 nm of TMB substrate assay solution after TMB substrate assay with protein A-HRP and human IgG-HRP samples were 0.11 and 0.76 respectively which corresponded to the immobilization density of 0.14 µg/cm² (16.9 x 10¹¹ molecules/cm²) for protein A and 0.61 µg/cm² (24.3 x 10¹¹ molecules/cm²) for human IgG as determined from their calibration curves.

Similar quantitation procedure based on TMB substrate assay was followed by Theegala and co-workers at Department of Chemistry, Southeastern University and A. M College, Baton Rouge, USA (117) for the quantification of antibody bound on the biosensing surface. The solid substrates were cut in small pieces of 1 cm² and HRP labeled E. coli antibody was bound on the surface using different immobilization procedures. Each of the antibody-coated and washed crystal/membrane was placed in a *It is a known fact that a single molecule of protein A has five antibody binding domains located in different directions.*
ml beaker, with the coated surface facing up and TMB substrate assay was done to determine the amount of HRP labeled antibody bound to the substrate. Based on the absorbance value of the sample and pre-determined calibration curves, the percent-immobilized antibody was determined.

The quantitation of antibodies was done by TMB substrate assay using HRP-labeled analytes by Kandimalla and co-workers at Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore, India (122). Grogan and co-workers (65) also employed HRP labeled antibodies and quantified the antibodies immobilized on the cantilever by TMB substrate assay.

Anti-hapten antibody was immobilized on gold-coated glass using a primary layer of protein A by Kaur and co-workers (123). Using a labeled antibody, the density of immobilized IgG molecules was estimated to be between 250 and 500/µm² i.e. 2.5-5 x 10^{10} molecules/cm². However, the immobilization density of human IgG on the gold-coated silicon, as determined by TMV substrate assay in our experiment, was 610 ng/cm² i.e. 2.43 x 10^{11} molecules/cm² using protein A-gold immobilization procedure. The variation may be due to difference in the substrates used (gold-coated glass and gold-coated silicon) or the different antibodies used for immobilization.

4.1.3 Atomic force microscopy (AFM)

The structural morphology of immobilized biomolecules was studied using contact-mode AFM. Imaging was done in air using 0.7 micron AFM head. AFM images of bare silicon, gold-coated silicon, protein A-coated silicon and human IgG-coated silicon were taken as shown in figures 14-17. The AFM image in figure 16 showed that the gold-coated silicon was uniformly coated with protein A. The size of the protein A molecule as determined by AFM was found to be 2.8 nm in height by 4.9 nm in width. The spatial distribution of protein A molecules on the silicon surface was limited by several factors like steric repulsion between protein A molecules. Similarly, the surface morphology of the human IgG immobilized on gold-coated silicon (figure 17) was analyzed by AFM. The height of the human IgG molecule was about 12 nm and its width was about 8.4 nm. The dimensions of protein A and human IgG were in agreement with the dimensions of protein A and IgG reported by Murata and co-workers at Surface.
The length and width of IgG was found to be 14.3 nm and 10.6 nm respectively as reported by You and co-workers at Institute of Biotechnology, University of Cambridge, UK (124).

The size of protein A and IgG molecules preadsorbed on mica was investigated by Murata and co-workers (8) by surface force measurements. Pure protein A binds at least two IgG molecules. It contains five homologous IgG binding units named E, D, A, B, and C, each containing 56-61 amino acids, in the order from the N terminus. Recombinant protein A was found to be a rod-like molecule, where each (globular) IgG binding unit with a diameter of about 2-2.5 nm and a length of about 4.5 nm was linked by the flexible polypeptide segments. An immunoglobulin (IgG) molecule was found to be arranged in three discrete domains, two Fab segments and one Fc segment. From the X-ray crystallographic data, the dimensions of the Fc and Fab segments were assumed as a first approximation to be 8 x 5 x 4 nm³ and 7 x 5 x 4 nm³, respectively (125, 126). The thickness of the Fc and Fab segments was 4-5 nm. The adsorbed amount of recombinant protein A was about 5 x 10¹⁵ molecules per m². The adsorbed amount of IgG was about 1 x 10¹⁶ molecules per m². The occupied area for one IgG molecule was about 100 nm².

Allowing for the experimental errors involved, the above value was compatible with the estimated values of 50-100 nm². AFM was also employed by Grogan and co-workers (65) to determine the presence of the biological coating on the cantilever and to obtain information on the surface morphology of biological element of the sensor.

4.1.4 Surface coverage by pixel count method

A software was developed (using Microsoft Visual Basic 6.0) to analyse the fluorescent images of the fluorescent dye labelled biomolecules immobilized on gold-coated silicon. In this method, a particular fluorescent image file in 24-bit bitmap picture (BMP) format was taken as input for image processing to calculate the surface coverage of fluorescent dye labelled biomolecules immobilized on gold-coated silicon. After separating header information from the input image, the image was read byte-by-byte and
compared with the values considered to be of immobilized pixel values. The matching pixels are counted and given different color values i.e. white (any color can be taken).

(a)

Figure 14. AFM image of bare silicon. (a) Top View (b) 60° view (c) 30° view.
Figure 15. AFM image of gold-coated silicon. (a) Top View (b) 60° view (c) 30° view.
Figure 16. AFM image of protein A immobilized on gold-coated silicon. (a) Top View (b) 60° view (c) 30° view.
Figure 17. AFM image of human IgG molecules immobilized on gold-coated silica employing protein A. (a) Top View (b) 60° view (c) 30° view.
Thus, white pixels in the processed binary image represent the fluorescent biomolecules immobilized on gold-coated silicon and the green pixels represented the background. The amount of surface coverage of gold-coated silicon by fluorescent biomolecules was calculated by dividing the no. of white pixels in the image with the total no. of pixels in the image.

The method is meant for calculating percentage coverage as per the image available, on the basis of color values of the pixels involved. Further studies would be required to take into account this particular fact so that the method could be used more reliably to calculate the surface coverage of biomolecules on the solid substrate. We were unable to find any report in literature pertaining to calculation of surface coverage by fluorescent molecules.

The logic of the program is stated in the flowchart (figure 18). Surface coverage of fluorescein isothiocyanate (FITC) labelled protein A and human IgG immobilized on gold-coated silicon was determined with the pixel count method as illustrated in figures 19 & 20.

Steps involved in the program were:

*Step 1.* Fluorescent image file (in 24-bit BMP format) was fed into the program for image processing.

*Step 2.* The whole image was taken for processing.

*Step 3.* The image was read byte-by-byte and compared with the values of interest (having immobilized fluorescent biomolecules) and matching pixels were converted to white colored pixels, keeping background green

*Step 4.* Calculations done by the software were:

1. Total no. of pixels of positive image
2. Number of white pixels (corresponding to the region having immobilized fluorescent biomolecules)
3. Number of green pixels (corresponding to background)
4. Total surface coverage by fluorescent biomolecules = (No. of white pixels / Total no. of pixels) x 100
Figure 18. Estimation of surface coverage of fluorescent bimolecules immobilized on gold-coated silicon by pixel count method.
Protein A-FITC
Total no. of pixels = 397854.00

Protein A-FITC (G > 100)
No. of fluorescent pixels read by software = 237916.7
Percentage surface coverage by biomolecules = 59.8 %

Protein A-FITC (G > 90)
No. of fluorescent pixels read by software = 288046.296
Percentage surface coverage by biomolecules = 72.4 %

Protein A-FITC (G > 80)
No. of fluorescent pixels read by software = 325444.572
Percentage surface coverage by biomolecules = 81.8 %
Protein A-FITC (G > 70)
No. of fluorescent pixels read by software = 352896.5
Percentage surface coverage by biomolecules = 88.7%

Figure 19. Surface coverage of protein A-FITC immobilized on gold-coated silicon by pixel count method. (a) Original inverted fluorescent image (10 X) of protein A-FITC biomolecules immobilized on gold-coated silicon. (b) Image processed with software taking value of green, red and blue colours as G >100, R<50 and B<50 respectively. (c) Image processed with software taking value of green, red and blue colours as G >90, R<50 and B<50 respectively. (d) Image processed with software taking value of green, red and blue colours as G >80, R<50 and B<50 respectively. (e) Image processed with software taking value of green, blue and red colours as G >70, R<50 and B<50 respectively.

Sensitive high-resolution fluorescence imaging with accurate calibration had been employed by Hinterdorfer and co-workers at Institute for Biophysics, University of Linz, Austria (127) for the determination of surface density of antibody bound to silicon nitride tips of AFM cantilever.

4.1.5 Regeneration of human IgG coated silicon

An effective dissociation of the antigen and regeneration of antibody are most important in the reuse of antibody in immunosensor applications. As the binding
The chemistries of antigen-antibody complexes is not well understood, care has to be taken while choosing the dissociation agents as they may affect the affinity of antibodies and lead to leaching of antibody from the immunosensor surface. The dissociation agent should not affect the association bonds between the antibody and the immunosensor surface.

**Figure 20.** Surface coverage of goat anti-human IgG-FITC immobilized on gold-coated silicon by pixel count method. (a) Original inverted fluorescent image (10 X) of protein A-FITC biomolecules immobilized on gold-coated silicon. (b) Image processed with software taking value of green, blue and red colours as $G > 100$, $R < 50$ and $B < 50$ respectively.

Antibody regeneration using acidic or alkaline solutions or high ionic strength are potentially harmful to the binding ability of antibodies and sometimes may lead to a short life, which limits the repeated use of immobilized antibodies, a prerequisite for diagnostic economics. Use of organic solvents, surfactants, chaotropic ions, organic solvents and strong acid buffers are the most common methods. Glycine-HCl buffer at a pH of 2.8 is the most commonly used dissociating agent. But it is difficult to determine rules for the
selection of a good dissociating agent as the antigen and antibody binding efficiencies and interactions will vary for each antigen-antibody system (128).

The regeneration of biosensing human IgG immobilized on gold-coated silicon, once it has been bound to goat anti-human IgG, was studied. This required efficient dissociation of the immune complex and regeneration of the human IgG immunosensing surface with maximum retention of its activity and specificity.

0.1 M Glycine-HCl, pH 2.8 and immunopure gentle antibody-antigen (Ab/Ag) eluting buffer, pH 6.9 from Pierce Co., USA were used by Suri and co-workers (116) to remove bound insulin from antibody-coated crystal. But immunopure gentle Ab/Ag eluting buffer gave better results in their case and successfully eluted bound insulin from antibody-coated matrix without affecting the antibody. Regeneration of coated crystal for at least 9 to 10 assays was demonstrated. But in our studies, Glycine-HCl buffer (50 mM, pH 2.2) was used to dissociate the human IgG-goat anti-human IgG complex and regenerate the functional human IgG surface. It effectively dissociated the immune complex without affecting the association bonds between silicon and human IgG. The percentage regeneration of the surface was monitored by TMB substrate assay based quantification of functional HRP labelled antibodies left immobilized on gold-coated silicon after treatment with Glycine-HCl buffer (50 mM, pH 2.2). Similar procedure was followed by Grogan and co-workers (65) for monitoring the activity of immobilized antibody after multiple regeneration steps. In our studies, the regenerated human IgG surface retained sufficient activity which was greater than 90 percent. The regeneration was 95 percent after the first regeneration cycle and decreased to 74 percent after fourth regeneration cycle. Thus the regeneration procedure is suitable to carry out four reproducible assays (figure 21) after which the activity of the human IgG immobilized on gold-coated silicon decreased due to unknown phenomenon.*

Similarly, different dissociating agents (dimethyl sulphoxide (DMSO); Tween 20; cetyl trimethylammonium bromide (CTAB); methanol; chloroform; guanidium chloride (GdmCl); glycine-HCl buffer in the pH range of 1.5-3.0; pierce buffer; combination of DMSO and methanol in phosphate buffer, Glycine-HCl buffer and salts like NaCl and MgCl2) have been used (122) for dissociation of ethyl parathion (EP) pesticide from the EP antibodies immobilized on the solid substrate. 1 percent DMSO in

* The loss of binding of anti-human IgG to matrix bound human IgG upon repeated cycles of absorption/desorption is a practical phenomenon seen in all cases of protein immobilization studies.
Regeneration Profile

Figure 21. Regeneration profile of the human IgG immobilized on gold-coated silicon after treatment with Glycine-HCl buffer (50 mM, pH 2.2).

4.1.6 SPR Analysis

The interactions between human IgG and goat anti-human IgG were visualized by surface plasmon resonance based AUTOLAB ESPRIT. Human IgG (0.1 mg/ml) was immobilized on the modified gold disk employing protein A-gold immobilization procedure. Different concentrations of goat anti-human IgG were then employed to visualize the interactions between human IgG immobilized on the gold disk and goat anti-human IgG. Determination of kinetic parameters of biomolecular interactions for different concentrations of goat anti-human IgG was done at different positions on human IgG coated gold disk (by manual rotation). The double channel design of AUTOLAB
ESPRIT had the advantage to run a reference measurement in the second channel to exclude systematic errors and correct the measurement in the first channel. Overlay plot of kinetic analysis for different concentrations of goat anti-human IgG were then plotted and the data was analysed for determining the various parameters of biomolecular interactions. Table 4 shows the experimental results obtained.

**Table 4. SPR analysis of biomolecular interactions between human IgG and goat anti-human IgG.**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Conc. of Goat anti-human IgG employed</th>
<th>Dissociation constant ($k_d$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 mg/ml</td>
<td>2.378 x 10^{-4}</td>
</tr>
<tr>
<td>2</td>
<td>0.1 mg/ml</td>
<td>2.375 x 10^{-4}</td>
</tr>
<tr>
<td>3</td>
<td>10 µg/ml</td>
<td>1.816 x 10^{-4}</td>
</tr>
<tr>
<td>4</td>
<td>1 µg/ml</td>
<td>9.258 x 10^{-5}</td>
</tr>
<tr>
<td>5</td>
<td>0.1 µg/ml</td>
<td>7.708 x 10^{-6}</td>
</tr>
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</table>

The surface plasmon resonance measured angle versus time. There was a linear relationship between the amount of goat anti-human IgG bound to human IgG coated gold disk and shift in SPR angle. The SPR angle split, in millidegrees, was used as a response unit to quantify the binding of goat anti-human IgG to human IgG coated gold disk. A change of 120 millidegrees represented a change in surface protein of approximately 1 ng/mm^2. The reference channel was used to correct for any unspecific interaction factors. After the association phase, the dissociation phase was performed by washing the sample away with association buffer i.e. PBS (pH 7.4) in which the goat anti-human IgG was diluted. The dissociation phase was used for determining the strength of the interaction between goat anti-human IgG and human IgG. After the dissociation phase, all goat anti-human IgG was removed from human IgG coated gold disk by 0.1 N diluted hydrochloric acid (regeneration phase) and the gold disk was restored to the baseline position by diluting with PBS buffer (pH 7.4).

The dissociation constant became constant at 0.1 mg/ml when increasing concentrations of goat anti-human IgG was provided to human IgG coated gold disk. This
showed that the saturation limit of human IgG coated gold disk for goat anti-human IgG binding had reached. The dissociation constant was $2.375 \times 10^{-4}$. The SPR angle split was 991.30 millidegrees which corresponds to a change in surface protein of approximately 8.26 ng/mm$^2$ or 826.0 ng/cm$^2$. Thus the amount of goat anti-human IgG bound to human IgG coated gold disk was 826.0 ng/cm$^2$. As one human IgG binds to one goat anti-human IgG, the amount of human IgG bound to the gold disk was taken as 826.0 ng/cm$^2$. The value was in direct agreement with the immobilization density of human IgG i.e. 610 ng/cm$^2$ on gold-coated silicon as determined by TMB substrate assay. The difference between the immobilization density as determined by SPR on gold disk and by TMB substrate assay on gold-coated silicon may be due to difference between the base substrates or variations in the gold-coating procedures i.e. as done by ECOCHEMIE on glass disk and by CSIO on silicon. But the immobilization results obtained with SPR demonstrated greater amount of functional human IgG antibody bound to the gold surface by protein A-gold immobilization procedure. The SPR response curve is shown in figure 22.

SPR analysis to confirm active biosensor surface was done by Kaur and co-workers (123) to analyse antigen binding to antibody immobilized on the biosensor surface. The shift (increase) in angle of incidence for antibody-coated surfaces, upon incubation with antigen indicated an increase in mass upon the sensor surface. This clearly demonstrated that the coated antibodies retain their capacity to recognize and capture their respective antigens even after being immobilized to form the active biosensor surface. SPR measurements of the surface coverage of IgG on gold by Moulin and co-workers at Nanoscale Science Group, Department of Engineering, Cambridge, UK (63) have shown that maximum coverage is achieved after about 10 min. Therefore, association time of 30 minutes was taken in our measurements. SPR analysis provided a useful technique for the simultaneous determination of biomolecular interaction parameters between human IgG coated gold disk and goat anti-human IgG and the amount of functional human IgG antibody immobilized on gold disk.
Figure 22. SPR response curve showing the change in SPR angle (in millidegrees) w.r.t. time (in sec) when human IgG-coated gold disk was provided with 0.1 mg/ml goat anti-human IgG.

4.2 ANTIBODY IMMOBILIZATION ON BARE SILICON BY 3-APTES + GLUTARALDEHYDE + PROTEIN A METHOD

3-APTES was coated on cleaned bare silicon. Homobifunctional crosslinker glutaraldehyde was employed for the immobilization of human IgG on the silanized silicon. 3-APTES reacts with the free hydroxyl groups of silicon surface to generate silane-modified surface. The amine groups of the silane modified surface (figure 23) react with glutaraldehyde forming an aldehyde derivatived surface, which further reacts with the free amino groups of protein A. Direct covalent binding of antibodies to silicon via primary amines without employing protein A would result in their random orientation. Some of the antibodies would be partially inactivated as their antigen binding sites on the variable antigen binding Fab region face the immunosensing silicon surface. Therefore, amine groups of the silane modified silicon substrate were crosslinked to protein A, which provided an orientation to the human IgG molecules with their antigen binding sites free to interact with the antigens. This immobilization procedure obviated the need of gold and could be employed for the oriented immobilization of antibodies on silicon. The cleaning of the silicon surface to generate reactive hydroxyl groups was
critical for the effective immobilization of biomolecules. There were several types of Si-OH groups (figure 24) that could form on the silicon surface. If the silicon surface was not properly cleaned of oils, dirt and detergents, the reactive hydroxyl groups would not be formed and the silane would not be deposited in a uniform manner.

The quantitative analysis of protein A and human IgG was done by TMB substrate assay employing protein A-HRP and human IgG-HRP. The O.D. at 450 nm of TMB substrate assay solution after TMB substrate assay with protein A-HRP and human IgG-HRP samples were 0.09 and 0.70 respectively which corresponded to the immobilization density of 0.12 µg/cm² (14.5 x 10¹¹ molecules/cm²) for protein A and 0.550 µg/cm² (22.0 x 10¹¹ molecules/cm²) for human IgG as determined from their calibration curves. These immobilization density values of amount of protein A and human IgG immobilized on bare silicon surface were lesser than the values obtained by the direct specific adsorption of protein A molecules on gold-coated silicon. The amount of protein A immobilized on bare silicon i.e. 120 ng/cm² was 14.3 percent less than the amount of protein A bound to gold-coated silicon i.e. 140 ng/cm². The amount of human IgG immobilized on bare silicon i.e. 550 ng/cm² was 9.84 percent less than the amount of human IgG bound to gold-coated silicon i.e. 610 ng/cm² employing protein A-gold immobilization procedure.

3-APTES immobilization chemistry was used for the site directed immobilization of IgG on 3-APTES modified silicon by Weiping and co-workers at National Laboratory of Molecular and Biomolecular Electronics, Southeast University, Nanjing, China (7).

Ahluwalia and co-workers at Center E. Piaggio, Pisa, Italy (129) employed covalent binding with 3-APTES and glutaraldehyde to immobilize anti-dinitrophenyl (DNP) antibodies on quartz slides. The amount of anti-DNP antibodies immobilized on quartz slides was 320 ng/cm². But the amount of human IgG immobilized on bare silicon using 3-APTES-glutaraldehyde-protein A method by us i.e. 550 ng/cm² was greater than most of the immobilization procedures followed by various researchers on different substrates. This may be due to the greater amount of functional sites made available by the specific binding of protein A to the Fc portion of human IgG which resulted in their oriented immobilization with antigen binding sites free to interact with the antigens. But it is difficult to compare the two results as the substrate employed i.e. bare silicon in our
case and quartz slides in the above mentioned case has different affinity to 3-APTES. Similarly the antibodies employed i.e. anti-DNP and human IgG may have different biomolecular interactions on the basis of their different specificities.

The same 3-APTES-Glutaraldehyde-Protein A procedure was employed for immobilizing antibodies on gold-coated silicon chip surface. The quantitative analysis of protein A and human IgG was done by TMB substrate assay employing HRP labelled protein A and human IgG. The O.D. at 450 nm of TMB substrate assay solution after TMB substrate assay with protein A-HRP and human IgG-HRP samples were 0.08 and 0.64 respectively which corresponded to the immobilization density of 0.1 µg/cm² (12 x 10¹¹ molecules/cm²) for protein A and 0.49 µg/cm² (19.6 x 10¹¹ molecules/cm²) for human IgG as determined from their calibration curves. The amount of protein A immobilized on gold-coated silicon i.e. 100 ng/cm² was 16.67 percent less than the amount of protein A immobilized on bare silicon i.e. 120 ng/cm². Whereas the average immobilization density of human IgG immobilized on the gold-coated silicon i.e. 490 ng/cm² was 10.9 percent less than the amount of human IgG immobilized on bare silicon i.e. 550 ng/cm². The difference in values between the amount of protein A and human IgG immobilized on the gold-coated silicon and bare silicon may be due to greater affinity of 3-APTES towards the bare silicon as compared to gold-coated silicon.

4.3 ANTIBODY IMMOBILIZATION ON GOLD-COATED SILICON EMPLOYING BIOTIN-NEUTRAVIDIN INTERACTIONS

Biotin-Neutravidin interactions were employed for the effective immobilization of human IgG antibodies on gold-coated silicon. Cleaned gold-coated silicon chips were taken and silanized with 3-APTES. Neutravidin was coated on the surface with the help of EDC crosslinker which crosslinked the amino groups of silanized silicon surface to the carboxyl groups of neutravidin employing EDC. The biotinylated human IgG was bound to the neutravidin coated silicon surface by the very strong interactions between the biotin and neutravidin molecules. The biotinylation of human IgG was done with EZ-Link Sulfo-NHS-Biotinylation kit. HABA assay of the biotinylated human IgG sample was done to determine the number of moles of biotin bound per mole of the human IgG.
The results of the HABA assay of biotinylated human IgG were as shown below in table 5.

![Chemical structures and reaction scheme](image)

Figure 23. Schematic view of immobilization chemistry of protein A binding to bare silicon employing 3-APTES and glutaraldehyde.
Figure 24. Types of hydroxyl groups on silicon surfaces. Only isolated and germinal silanols are reactive while vicinal silanol and siloxane are not reactive.

Table 5. HABA assay of biotinylated human IgG.

<table>
<thead>
<tr>
<th>Different 1 ml aliquots</th>
<th>O.D. at 280 nm</th>
<th>O.D. of HABA-Avidin at 500 nm</th>
<th>O.D. of HABA-Biotin-Avidin at 500 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.1643</td>
<td>1.238</td>
<td>0.663</td>
</tr>
<tr>
<td>5</td>
<td>3.132</td>
<td>1.238</td>
<td>1.692</td>
</tr>
<tr>
<td>6</td>
<td>1.806</td>
<td>1.238</td>
<td>0.714</td>
</tr>
<tr>
<td>7</td>
<td>3.356</td>
<td>1.238</td>
<td>0.802</td>
</tr>
<tr>
<td>8</td>
<td><strong>4.3408</strong></td>
<td><strong>1.238</strong></td>
<td><strong>1.0822</strong></td>
</tr>
<tr>
<td>9</td>
<td>2.694</td>
<td>1.238</td>
<td>0.901</td>
</tr>
<tr>
<td>10</td>
<td>2.121</td>
<td>1.238</td>
<td>0.873</td>
</tr>
</tbody>
</table>

Eighth aliquot was selected for biotin-avidin immobilization method.
4.3.1 Calculation of number of moles of bound biotin per mole of human IgG

Concentration of human IgG employed for biotinylation = 5 mg/ml
Mol. Wt. of the human IgG = 150,000 Da
Absorbance at 500 nm for HABA/Avidin solution (A_{500} HABA/Avidin) = 1.238
Absorbance at 500 nm for HABA/Avidin/Biotin sample mixture (A_{500} HABA/Avidin/Biotin) = 1.0822
Dilution factor (sample was diluted before addition to the HABA/Avidin solution) = 10

Calculation 1:

Biotinylated Mouse IgG concentration in mmoles/ml:

\[
\text{mmoles biotinylated Mouse IgG per ml} = \frac{\text{Mouse IgG conc. (mg/ml)}}{\text{MW of protein (Da)}}
\]

\[
= \frac{5}{150,000}
\]

Calculation 2:

Change in absorbance at 500 nm:

\[
\Delta A_{500} = (0.9 \times A_{500} \text{ of HABA/Avidin}) - (A_{500} \text{ of HABA/Avidin/Biotin sample})
\]

\[
= (0.9 \times 1.238) - (1.0822)
\]

\[
= 0.032
\]

= Calc # 2

Calculation 3:

Conc of biotin in mmoles/ml:

\[
\text{mmoles Biotin} = \frac{\Delta A_{500}}{34000 \times b} = \frac{\text{Calc # 2}}{34000 \times b} = \frac{0.032}{34000 \times 1}
\]

Here \( b \) is the cell path length expressed in cm. Path length of 1.0 cm is to be used for the cuvette format and 0.5 cm is to be used when using the microplate format with the recommended reaction volumes. As the cuvette format was used, therefore path length of 1 cm was taken.
Calculation 4:
The mmoles of biotin per mmole of protein:

\[
\text{mmoles Biotin} = \text{mmoles Biotin per ml reaction mixture x 10 x Dilution factor} \\
\text{mmoles protein} \quad \text{mmoles protein per ml} \\
= \text{(Calc # 3) x 10 x Dilution factor} \\
\text{Calc # 1} \\
= 0.032 \times 10 \times 10 \times 150,000 \\
34000 \times 1 \times 5 \\
= 2.823
\]

Therefore, the amount of biotinylation achieved was 2.823 moles of biotin per mole of human IgG.

(It was necessary to account for the dilution of biotin sample with HABA/Avidin solution in the calculation. With the addition of the biotinylated compound as described, 90 percent of the solution was HABA/Avidin solution and 10 percent was sample. Therefore, a multiplier of 10 was used.)

The quantitative analysis of human IgG was done by TMB substrate assay employing biotinylated human IgG-HRP. The O.D. at 450 nm of TMB substrate assay solution after TMB substrate assay with human IgG-HRP samples was 0.68 which corresponded to the immobilization density of 0.530 µg/cm² for human IgG as determined from its calibration curve (appendix I). The amount of human IgG bound to gold-coated silicon using NeutrAvidin-biotin interactions i.e. 530 ng/cm² was 13.11 percent less than the amount of human IgG bound to gold-coated silicon using protein A immobilization method i.e. 610 ng/cm². To the best of our knowledge, there was not any literature pertaining to the immobilization density of biotinylated antibodies on neutravidin-coated substrate with which we could compare our results.

4.4 BIOMECHANICS OF MICROCANTILEVER BASED DIAGNOSIS

A software has been developed to calculate the various biomechanical parameters of the microcantilever based devices which include force on the cantilever, stress change,
strain change at the anchor point of the microcantilever, deflection of the microcantilever, angle of deflection, radius of curvature, relative change in resistance of the piezoresistive substance i.e. boron doped at the anchor point of the microcantilever. The software was based on the microcantilever mechanics as discussed in the review of literature and took into account only the gravitational aspects of the microcantilever based diagnosis of disease biomarkers (antigens/antibodies/ligands). But it has been proved by several researchers that several other factors such as the balance between compressive and tensile stress contribute to the actual deflection response of the microcantilever. Several studies are going on in our group to take into account all these factors so that the software would provide more reliable values taking into consideration all the possible factors which account for the microcantilever deflection in addition to the gravitational stress.

Assumptions made

(i) The software has been made only for rectangular solid microcantilevers. But it can be readily employed for triangular and double-legged microcantilevers by slight modifications.
(ii) The percentage coverage of microcantilever surface by immobilized Protein A molecules was assumed to be 50%.

Input

(i) Material of microcantilever with material properties i.e. Young's modulus, density.
(ii) Dimensions of microcantilever (Length, width and thickness).
(iii) Coatings on cantilever (e.g. gold, Au and chromium, Cr).
(iv) Thickness of coatings.
(v) Types of immobilized antibodies (IgG, IgM, IgA, IgD, IgE).
(vi) Molecular mass and area occupied by single molecule of protein A, antibody and antigen.
(vii) No. of immobilized biomolecules of protein A, antibody and antigen.

Calculations

(i) Force constant/Stiffness of microcantilever.
(ii) Resonance frequency of microcantilever.
(iii) Mass of Protein A, Antibody and Antigen layers.
(iv) Force of Protein A, Antibody and Antigen layers.
(v) Moment of Inertia of microcantilever.
(vi) Deflection of microcantilever with and without Antigen.
(vii) Angle of deflection of the microcantilever with and without Antigen.
(viii) Radius of curvature of the microcantilever with and without Antigen.
(ix) Strain on microcantilever with and without Antigen.
(x) Change in relative resistance with and without Antigen.

Output

(i) Comparison between theoretical and experimental results.
(ii) Total deflection of cantilever and change in relative resistance before and after antigen binding.

The software program for calculating the deflection of the microcantilever and the various biomechanical parameters is given in appendix II.

The various steps involved in the simulation are as described below:

1. Values fed into the software:

Material properties of Silicon as provided by S.C.L., Mohali, India:
   
   Young’s modulus (E) = 106.8 x 10^9 Pa
   Density (ρ) = 2328.0 kg/m^3
   Poisson’s ratio = 0
   Thermal expansion coefficient (/degree Kelvin) = 24.992 x 10^-7

Material properties of Polysilicon fed as provided by S.C.L., Mohali, India:
   
   Young’s modulus (E) = 175 x 10^9 Pa
   Density (ρ) = 2300 kg/m^3
   Poisson’s ratio = 0.226
   Thermal expansion coefficient (/degree Kelvin) = 20 x 10^-7

Mass of single molecule of Protein A = 8.35 x 10^-22 kg (50 kDa)
Mass of single molecule of Protein G = 3.674 x 10^-21 kg (22 kDa)
Mass of single molecule of IgG = 2.505 x 10^-22 kg (150 kDa)
Mass of single molecule of Antigen 85 complex = 1.42 x 10^-22 kg (85 kDa)
Radius of single molecule of Protein A = 4.9 nm
Radius of single molecule of Protein G = 3.53 nm

2. Force Constant of Cantilever (k) (or Stiffness) is calculated by
   \[ k = \frac{(Et^3w)}{(4L^3)} \]
   where E is the Young's modulus of the material of the cantilever, and L, w and t are
   the length, width and thickness of the cantilever.

3. Number of immobilized biomolecules on the cantilever (In Simulation I, number of
   immobilized protein A molecules was calculated by dividing total area of the upper
   surface of cantilever by area occupied by single protein A molecule and assuming 50
   percent surface coverage of cantilever by protein A. No. of immobilized antibody
   molecules were assumed to be double of no. of protein A molecules and no. of
   antigen molecules were assumed to be double of no. of antibody molecules. In
   Simulation II, no. of immobilized biomolecules was calculated by first calculating the
   mass of immobilized biomolecules on the cantilever as given in step 4 and then
   dividing it by the mass of single biomolecule. No. of antigen molecules were assumed
   to be double of no. of immobilized antibody molecules).

4. Mass of immobilized biomolecules on the cantilever (In Simulation I, it was
   calculated by multiplying mass of single biomolecule with no. of biomolecules
   immobilized on the cantilever. In Simulation II, it was determined on the basis of
   immobilization values of protein A and human IgG determined by experiments on
   gold-coated silicon. Mass of bound antigen molecules was calculated by multiplying
   mass of single antigen molecule with no. of antigen molecules immobilized on the
   cantilever).

5. Force (F) on cantilever due to immobilized biomolecules (by multiplying mass of
   biomolecule with acceleration due to gravity, g).

6. Moment of inertia (I) of the rectangular cantilever is calculated by
   \[ I = \frac{(wt^3)}{12} \]

7. Deflection (Z) of the cantilever due to immobilized biomolecules is calculated by
   \[ Z = \frac{(L^3F)}{(8EI)} \]

8. Angle of Deflection (θ) of the cantilever due to immobilized biomolecules was
   calculated by
\( \theta = \text{Deflection} / \text{Length of the cantilever} \)

9. Radius of curvature (R) of the cantilever due to immobilized biomolecules was calculated by
   \[ R = \frac{L}{\theta} \]

10. Strain on the cantilever due to immobilized biomolecules was calculated by
    \[ \text{Strain} = t \theta \]

11. Change in relative resistance (\(\Delta R/R\)) of integrated boron at the anchor point due to immobilized biomolecules was calculated by
    \[ (\Delta R/R) = \text{Gage Factor} \times \text{Strain} \times \text{Strain Enhancement} \]
    Gage factor of boron was taken as 100 and strain enhancement which is normally 5-10 times was taken as 5.

An example of the simulation done with the software for the diagnosis of tuberculosis disease biomarker i.e. antigen 85 complex employing silicon cantilever of dimensions (L x w x t) 500 x 150 x 0.5 \(\mu\text{m}\) is shown in table 6:

Table 6. Simulation of microcantilever based diagnosis of tuberculosis disease biomarker i.e. antigen 85 complex.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Details</th>
<th>Simulation I (assuming 50 percent surface coverage by protein A)</th>
<th>Simulation II (incorporating the immobilization values determined by experiments*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Material of cantilever</td>
<td>Silicon</td>
<td>Silicon</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>Chromium layer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Gold layer</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Force constant/ Stiffness of cantilever (N/m)</td>
<td>0.01068</td>
<td>0.01068</td>
<td></td>
</tr>
<tr>
<td>Mass (x 10^{-15} kg)</td>
<td>Protein A</td>
<td>41.5333</td>
<td>75.15</td>
</tr>
<tr>
<td></td>
<td>Antibody</td>
<td>249.2</td>
<td>433.991</td>
</tr>
<tr>
<td></td>
<td>Total mass on cantilever without antigen</td>
<td>290.733</td>
<td>509.141</td>
</tr>
<tr>
<td></td>
<td>Protein A</td>
<td>Antibody</td>
<td>Antigen</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>Mass per unit area</td>
<td>4.97404</td>
<td>9.94808</td>
<td>19.8962</td>
</tr>
<tr>
<td>(x 10(^{-5}) kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of molecules</td>
<td>4.07607</td>
<td>0.407607</td>
<td>2.44564</td>
</tr>
<tr>
<td>(x 10(^{8}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Force (x 10(^{-12}) N)</td>
<td>2.85325</td>
<td>2.77173</td>
<td>5.62498</td>
</tr>
<tr>
<td>Total force on cantilever</td>
<td>4.99671</td>
<td>4.82708</td>
<td>9.82379</td>
</tr>
<tr>
<td>without antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen</td>
<td>2.77173</td>
<td>4.82708</td>
<td>9.82379</td>
</tr>
<tr>
<td>Total force on cantilever</td>
<td>5.62498</td>
<td>9.82379</td>
<td></td>
</tr>
<tr>
<td>with antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Force per unit area</td>
<td>7.49997</td>
<td>13.0984</td>
<td></td>
</tr>
<tr>
<td>(x 10(^{-5}) N)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deflection (nm)</td>
<td>0.0381655</td>
<td>0.228993</td>
<td>0.259525</td>
</tr>
<tr>
<td>Total deflection of</td>
<td>0.267158</td>
<td>0.467857</td>
<td>0.451974</td>
</tr>
<tr>
<td>cantilever without</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antigen</td>
<td>0.259525</td>
<td>0.451974</td>
<td></td>
</tr>
<tr>
<td>Total deflection of</td>
<td>0.526684</td>
<td>0.919831</td>
<td></td>
</tr>
<tr>
<td>cantilever with antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angle of deflection</td>
<td>0.76331</td>
<td>4.57986</td>
<td>5.19051</td>
</tr>
<tr>
<td>(x 10(^{-7}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total angle of deflection</td>
<td>5.34317</td>
<td>9.35714</td>
<td>9.03948</td>
</tr>
<tr>
<td>of cantilever without</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antigen</td>
<td>5.34317</td>
<td>9.03948</td>
<td></td>
</tr>
<tr>
<td>Total angle of deflection</td>
<td>10.5337</td>
<td>18.3966</td>
<td></td>
</tr>
<tr>
<td>of cantilever with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antigen</td>
<td>10.5337</td>
<td>18.3966</td>
<td></td>
</tr>
<tr>
<td>Radius of curvature</td>
<td>6.55042</td>
<td>1.09174</td>
<td></td>
</tr>
<tr>
<td>(km)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total radius of curvature</td>
<td>0.935775</td>
<td>0.534351</td>
<td></td>
</tr>
<tr>
<td>Antigen</td>
<td>0.963297</td>
<td>0.553129</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Total radius of curvature of cantilever with antigen</td>
<td>0.474668</td>
<td>0.271789</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain (x 10^16)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>0.76331</td>
<td>1.38113</td>
</tr>
<tr>
<td>Antibody</td>
<td>4.57986</td>
<td>7.97601</td>
</tr>
<tr>
<td>Total strain of cantilever without antigen</td>
<td>5.34317</td>
<td>9.35714</td>
</tr>
<tr>
<td>Antigen</td>
<td>5.19051</td>
<td>9.03948</td>
</tr>
<tr>
<td>Total strain of cantilever with antigen</td>
<td>10.5337</td>
<td>18.3966</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative Resistance change (x 10^-7)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>0.381655</td>
<td>0.690564</td>
</tr>
<tr>
<td>Antibody</td>
<td>2.28993</td>
<td>3.98801</td>
</tr>
<tr>
<td>Total relative resistance change of integrated piezoresistor without antigen</td>
<td>2.67158</td>
<td>4.67857</td>
</tr>
<tr>
<td>Antigen</td>
<td>2.59525</td>
<td>4.51974</td>
</tr>
<tr>
<td>Total relative resistance change of integrated piezoresistor with antigen</td>
<td>5.26684</td>
<td>9.19831</td>
</tr>
</tbody>
</table>

* The average immobilization densities, as determined by protein A-gold immobilization procedure, were (i) 140 ng/cm² (16.8 x 10^{11} molecules/cm²) for protein A and (ii) 610 ng/cm² (24.3 x 10^{11} molecules/cm²) for human IgG. These immobilization densities of protein A and human IgG were used to determine the biomechanical parameters of the microcantilever.

Based on the simulation studies being conducted with the help of the software and detailed analysis of review of literature, three dimensions of the rectangular silicon microcantilevers (L x w x t, in µm) had been finalized by our research group for our ongoing project pertaining to the diagnosis of tuberculosis employing microcantilever.

1. 200 x 50 x 0.5
2. 300 x 100 x 0.5
3. 500 x 150 x 0.5
The polysilicon cantilevers of different dimensions fabricated by S.C.L., Mohali, India and employed for experimental analysis are given in figure 25. The fabrication procedure used by S.C.L., Mohali, India for the manufacture of piezoresistive polysilicon microcantilever is described in appendix III. The simulation of microcantilever based diagnosis of hepatitis B biomarker i.e. hepatitis B surface antigen (HbsAg) is given in appendix IV.

![Figure 25. Polysilicon microcantilevers fabricated by S.C.L., Mohali, India.](image)

**4.5 FLOW CELL SET UP FOR MICROCANTILEVER**

A teflon flow cell arrangement, as shown in figure 26, was made in the form of a cube with side length of 16 mm. The teflon block contains a central circular cavity of diameter 6.8 mm and depth of 11 mm. There is an inlet having diameter of 2.4 mm, whose centre is below 6 mm from the top surface of the block and an outlet having diameter of 2.4 mm directly below the centre of the circular cavity. There is an opening for inserting microcantilever into the teflon block in the side of the cube, which is at 90° angle from the side having inlet. This opening is in the form of square with side of length...
4 mm and its centre is 7 mm from the top surface of the block. Initially the top surface of the teflon block was left open (figure 27). But thereafter (figure 28), the upper surface was closed with a teflon cover in the form of a square with side of length 16 mm. The height of the cover was 5.5 mm. A circular cavity having threading M14 x 1 was made for tightening the bottom teflon block with top cover having O-ring with inner diameter of 8 mm and outer diameter of 13 mm.

Figure 26. Flow cell set up for microcantilever based diagnostic kit.

4.6 CHARGE TRANSFER IN PROTEINS IMMOBILIZED ON POLYLYSINE COATED GLASS SLIDE

During the solid substrate immobilization of proteins, suddenly the concept of charge transfer in solid substrate immobilized proteins was tried and studied for its potential applications. It was observed that the conductance of antibody immobilized on polylysine glass slide (area: 1 cm x 1 cm) decreased upon the formation of immune complex between antibodies and their specific antigens. An experimental observation involving a change in surface conductance of antibody coated on polylysine glass slide due to the formation of immune complex may have a bearing in future on immunodiagnosis and may have wide ranging ramifications in disease diagnosis, biomolecular engineering and nanobiotechnology. Using the aforementioned dogma, it
may be possible to develop next generation of miniaturized, simple, cost effective and rapid diagnostic kits with greater sensitivity, specificity, stability and reproducibility.

Figure 27. Flow cell set up employed initially for microcantilever experiments.

The stable and uniform immobilization of mouse IgG antibodies on the polylysine-coated glass slide was done employing EDC and protein A as shown schematically in figure 29. The amino groups of the polylysine-coated glass slide were cross-linked to the carboxyl groups of protein A employing EDC as a crosslinker. The mouse IgG molecules immobilized employing protein A were oriented with their antigen binding sites free to bind to the analytes to be detected. The binding and distribution of EDC, protein A and mouse IgG immobilized on polylysine-coated glass slide was analyzed by atomic force microscopy (Figure 30).
Figure 28. Flow cell set up with top teflon cover employed in the later stage for microcantilever experiments.
Mouse IgG
Polylysine coated glass slide

**Figure 29.** Schematic of the experimental set-up for studying charge transfer in mouse IgG antibodies immobilized on polylysine coated glass slide. The antibodies were immobilized employing protein A, which was crosslinked to polylysine-coated glass slide with the help of EDC.

From the current-voltage (I-V) curve (figure 31), the values of conductance of the biomolecules at 10 V (table 7) were calculated. The conductance increased from $2.2 \times 10^{-11} \, \Omega^{-1}$ to $2.07 \times 10^{-8} \, \Omega^{-1}$ at 10 V upon the binding of protein A to EDC-coated polylysine glass slide and it decreased from $2.07 \times 10^{-8} \, \Omega^{-1}$ to $1.02 \times 10^{-8} \, \Omega^{-1}$ at 10 V upon the binding of mouse IgG to protein A. When the substrate immobilized mouse IgG was provided with rabbit anti-mouse IgG F(ab')₂, the conductance showed a decrease from $1.02 \times 10^{-8} \, \Omega^{-1}$ to $1.41 \times 10^{-11} \, \Omega^{-1}$ at 10 V.

Various voltage ranges ranging from -1 to 1 V to -40 to 40 V were employed for measuring the conductance of biomolecules immobilized on polylysine coated glass slide. But maximum stable readings of solid substrate immobilized biomolecules were found to be at 10 V. Varying the distance between the probes of SIGNATONE probing station in the range of 200-400 μm did not cause significant change in the conductance of immobilized biomolecules. Therefore, the I/V measurements were taken in the voltage range from -10 to 10 V keeping the distance between the probes fixed at 200 μm. A reference slide for the exact measurement of distance between the probes of the SIGNATONE probing station was made by taking gold-coated microscopic glass slide.
and making different reference line lengths (from 200 to 400 μm) on it by removing the gold with the help of laser used in optical tweezer cum microdissection system.

**Figure 30.** AFM topographic images in contact mode (a) polylysine coated glass slide. (b) EDC coated polylysine glass substrate. (c) Protein A coated polylysine glass substrate. (d) Mouse IgG coated polylysine glass substrate.
Figure 31. Current-Voltage measurements of biomolecules immobilized on polylysine coated glass slide at 10 V.

Table 7. Conductance of biomolecules immobilized on polylysine coated glass slide at 10 V.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Coated surface</th>
<th>Conductance at 10 V (in $\text{S/m}^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>$9.70 \times 10^{-12}$</td>
</tr>
<tr>
<td>2</td>
<td>Blank + EDC</td>
<td>$2.20 \times 10^{-11}$</td>
</tr>
<tr>
<td>3</td>
<td>Blank + EDC + Protein A</td>
<td>$2.07 \times 10^{-9}$</td>
</tr>
<tr>
<td>4</td>
<td>Blank + EDC + Protein A + Mouse IgG</td>
<td>$1.02 \times 10^{-9}$</td>
</tr>
<tr>
<td>5</td>
<td>Blank + EDC + Protein A + Mouse IgG + Rabbit anti-mouse IgG</td>
<td>$1.41 \times 10^{-11}$</td>
</tr>
<tr>
<td>6</td>
<td>Difference between EDC and Protein A</td>
<td>$2.07 \times 10^{-9}$</td>
</tr>
<tr>
<td>7</td>
<td>Difference between Protein A and Mouse IgG</td>
<td>$1.05 \times 10^{-9}$</td>
</tr>
<tr>
<td>8</td>
<td>Difference between Mouse IgG and Rabbit anti-mouse IgG</td>
<td>$1.02 \times 10^{-9}$</td>
</tr>
</tbody>
</table>
4.6.1 Effect of Temperature

The temperature at the time of immune complex formation has a major impact on the electronics of biomolecules immobilized on polylysine coated glass slide as shown in figure 32. The temperature was varied from 20° C to 50° C and it was observed that the biomolecules immobilized on polylysine coated glass slide showed maximum conductance characteristics at 30° C during measurements with SIGNATONE probing station. Therefore, this temperature was employed during the immobilization of biomolecules on the polylysine coated glass slide and the formation of immune complex between mouse IgG coated polylysine glass slide and rabbit anti-mouse IgG F(ab')2.

The temperature after immune complex formation also has a major impact on the electronics of biomolecules immobilized on polylysine coated glass slide as shown in figure 33. The temperature was varied from 20° C to 50° C and it was observed that the biomolecules immobilized on polylysine coated glass slide showed maximum conductance characteristics when measurements were taken with SIGNATONE probing station at 30° C. Therefore, this temperature was employed for the conductance measurements of biomolecules immobilized on polylysine coated glass slide.

![Figure 32. Effect of temperature at the time of immune complex formation on the I-V measurements at 10 V.](image-url)
Figure 33. Effect of temperature after immune complex formation on the I-V measurements at 10 V.

4.6.2 Stability

The stability of protein A, mouse IgG and rabbit anti-mouse IgG F(ab)₂ coated polylysine glass slides was observed for five weeks and it was found that the electrical properties of all the solid substrate immobilized biomolecules were stable for one week at room temperature as shown in figures 34-36. After one week, the electrical properties of biomolecules decreased gradually due to unknown mechanisms. A detailed study is required to understand the mechanisms of charge transfer in biomolecules immobilized on polylysine coated glass slide.
Figure 34. Stability of protein A biomolecules immobilized on polylysine glass slide at 10 V.
Figure 35. Stability of mouse IgG biomolecules immobilized on polylysine glass slide at 10 V.
Figure 36. Stability of rabbit anti-mouse IgG F(ab\(^\)2\) biomolecules immobilized on polylysine substrate at 10 V.

4.6.3 Kala Azar positive patient serum and Leishmania promastigote specific antigen

The proposed concept was practically tested in field on two samples. In one case, polyclonal antibodies against Leishmania promastigote specific antigen from Kala Azar positive patient (0.1 mg/ml in 50 mM PBS, pH 7.4) were immobilized on the polylysine-coated glass slide. When the immobilized antibodies against Leishmania antigen were
provided with Leishmania antigen (10 μg/ml in 50 mM PBS, pH 7.4), the conductance showed a decrease from $4.27 \times 10^{-10}$ to $2.18 \times 10^{-11}$ at 10 V (figure 37).

**Figure 37.** Current-Voltage curve at 10 V for detecting biomolecular interactions between Leishmania antigen and polyclonal antibodies against Leishmania antigen (taken from Kala Azar positive patient) immobilized on polylysine coated glass slide.

**4.6.4 Anti-p21 and p21**

In the second case, antibodies against p21 (a membrane marker) (0.1 mg/ml in 50 mM PBS, pH 7.4) were immobilized on the polylysine-coated glass slides. When the immobilized anti-p21 antibodies were provided with p21 (10 μg/ml in mM PBS, pH 7.4), the conductance showed a decrease from $4.35 \times 10^{-10}$ to $8.19 \times 10^{-11}$ at 10 V (figure 38).
Figure 38. Current-Voltage curve at 10 V for detecting biomolecular interactions between p21 antigen and anti-p21 antibodies immobilized on polylysine coated glass slide.

4.6.5 Mechanism of charge transport in proteins

Charge conductivity in biomolecules and other large polymeric systems has become a topic of substantial current research. Schlag and co-workers at Institut fur Physikalische and Theoretische Chemie, Technische Universitat Munchen, Germany (130) demonstrated that charge migration in proteins is highly efficient but the mechanistic origin is still debated and largely unknown even though various models have been proposed by Weinkauf and co-workers at Institut fur Physikalische and Theoretische Chemie, Technische Universitat Munchen, Germany (131-133). In one of the experiments, Schlag and co-workers (134) took 20 synthetic peptides and arrived at a mechanism that each amino acid in zero order contributes semi-independently to the
The electronic surface of the total polypeptide which could be approximated to a first order by the values of the ionization potential. The charge transfer between amino acids takes place by hole hopping between local amino acid sites assisted or driven by the torsional motions of the floppy backbones. The charge transfer occurs as a vibronically induced hole hopping between local sites of lowest ionization potential in the amino acid chain i.e. the highest occupied molecular orbital (HOMO) of peptide groups, -CONH-. The charge flow can be blocked by local barrier as small as 0.2-0.3 eV which is the difference in ionization potentials between neighboring amino acids depending on their R groups. This model was called “rest and fire” or bifunctional model in which individual amino acids are weakly coupled and undergo alignment to reach the special strong coupling as assisted by torsional motion of the floppy backbones.

Study of structure of proteins reveals that the carbamide group of each amino acid is stiff and very loosely hinged to the next amino acid. At this hinge carbon atom, the angles $\phi$ and $\psi$ define the orientation of these amino acids with respect to each other. Over a large range of angles, this hinge motion is nearly free rotation with virtually no potential energy restrictions except at their respective limits, the range of which is given in Ramachandran plot.

Ab initio calculations show that even a pair of two identical amino acids will have a general asymmetry of about 0.6 eV because of the natural asymmetry of the C side and the N side of each amino acid. Baranov and Schlag (135) observed that any such energy difference is not constant but rather strongly dependent on the angle between the adjacent carbamide groups. For the ionized species in a small range of $\phi$ and $\psi$, the electronic energy difference reaches a minimum when the carbonyl groups of the neighboring amino acids are only about 2.87 apart. At this stage, the initial and final states for the charge are isoenergetic with little or no energy barrier between them. The two states are strongly correlated at this stage which is referred to as firing state for charge hopping as it facilitates the charge movement to the next amino acid site in the chain and so on.

In view of this, it was felt that conductance of antibody coated surface would change due to immune complex formation.

One of the most important applications of the present study is in the detection of specific biomolecular interactions employing rapid and economical detection technique.
with minimized sample volumes. The studies required highly interdisciplinary approach with researchers from biology, medicine, physics, chemistry, computer science and engineering. The demonstrated concept is of extreme importance for fast and reliable clinical diagnosis and is a direct application of the much debated charge transfer in proteins in the field of medicine.

4.6.6 Proposed mechanism for decrease in conductance after the formation of immune complex

It was considered that if charge would be introduced at one polypeptide chain of the Y-shaped antibody, it would move along the polypeptide chain through amino acid hopping and then go to the other polypeptide chain through disulfide bond and reach the free site of the antibody where due to spatial hopping this charge would jump to next antibody molecule as shown in figure 39. When the mouse IgG functionalized polylsine glass substrate was provided with anti mouse IgG F(ab')2, some of the sites on antibodies would have clogged or inactivated resulting in the decrease in conductivity after the formation of immune complex between mouse IgG and rabbit anti mouse IgG F(ab')2.

Figure 39. Proposed charge transfer mechanism in Mouse IgG antibodies immobilized on polylsine coated glass slide.
4.6.7 Advantages of the proposed concept

Conventional diagnostic assays such as enzyme linked immunosorbent assay (ELISA), radio immunoassay (RIA) and enzyme immunoassay (EIA) require enzyme labeling or radio tagging, more duration and sophisticated instruments. On the other hand, the proposed concept based on the precept of change in conductance is less time consuming, cost effective, sensitive and reliable.

The studies demonstrated the concept of change in conductance upon specific biomolecular interactions of solid substrate immobilized antibodies with specific antigens. It would find innumerable biosensing applications in healthcare, environmental monitoring, food analysis, biochemical warfare and, biomolecular electronics. The development of simple, efficient, easy to use and cost effective detection technique for analytes is one such application, which is of paramount importance. Further research is required to study the charge transfer mechanisms in solid substrate immobilized biomolecules. These fundamental studies at the nanoscale level of biomolecules would be the platform for the next generation of biosensing devices in nanobiotechnology.