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Quantification of human immunoglobulin G immobilized on gold-coated silicon chip for biosensing applications

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Abstract. The most important aspect of biosensor development with high sensitivity is the oriented immobilization of antibodies on the solid substrate. Human IgG was immobilized on gold-coated silicon employing protein A, protein G and neutravidin immobilization procedures. The amount of human IgG immobilized was analyzed by 3, 3’-tetramethyl benzidine (TMB) substrate assay and was maximum when protein A immobilization procedure was followed. Human IgG coated biosensing surface was regenerated by treatment with glycine-HCl buffer (50 mM, pH 2.2). Atomic force microscopy was used to analyze the distribution of biomolecules immobilized on gold-coated silicon. Our study indicates that human IgG molecules were uniformly bound to gold-coated silicon by protein A immobilization procedure.

1. Introduction

Antibodies can be immobilized on the surface of a sensor transducer covalently employing various crosslinkers [1], silanes [2-4], polymers [5-6] and thiols [7-8] etc. or noncovalently employing protein A [9-10], protein G [11-12], protein L [13], streptavidin [14-16]. Each of these methods has its own advantages and disadvantages. No single universally acceptable immobilization method exists. As various immobilization strategies are already available for a particular solid substrate and new ones are constantly being devised, scientists and researchers are faced with the challenge of choosing the best immobilization technique that best suits their application. Scientific assessment of antibody attachment with different methods is a tedious task. In this paper we employed the horse radish peroxidase enzyme substrate (TMB) colorimetric reaction to quantify the amount of antibody immobilised on a solid substrate as already proposed by Theegala and Suleiman [17] and by Kandimalla et al. [18]. Based on the absorbance value of the sample and pre-determined calibration curves, the percent-immobilized antibody can be determined. Grogan et al. [19] also employed HRP labeled antibodies and quantified directly the antibodies immobilized on the cantilever by TMB substrate assay.

As a part of our research oriented towards the development of silicon based biosensors, we investigated various human IgG immobilization procedures for their efficiency in binding human IgG to gold-coated silicon chip surface. The immobilization procedures were based, respectively, on the use of protein A, protein G and neutravidin. Oriented immobilization of human IgG was carried out to create active immunobiosensor surface. The amount of human IgG antibodies bound to gold-coated silicon was determined by TMB substrate assay. Based on the quantitative estimation of human IgG immobilized by the different immobilization procedures, it was concluded that protein A immobilization procedure was the most suitable for immobilizing human IgG on gold-coated silicon. Qualitative analysis of distribution of immobilized human IgG immobilized by protein A procedure was done by inverted fluorescence microscopy at low resolution and atomic force microscopy (AFM) at high resolution. The possibility of regeneration of human IgG coated surface was done by treatment with glycine-HCl buffer was also tested.

2. Materials and methods

2.1. Materials. Immunopure recombinant Protein A horse radish peroxidase (HRP) conjugates, purified recombinant Protein A, purified recombinant protein G, neutravidin biotin binding protein, N-ethyl-N’-(3-diethylaminopropyl) carbodiimide (EDC) and 3, 3’, 4, 4’-tetramethyl benzidine (TMB) substrate kit were
procured from Pierce Co., USA. Protein A-FITC, Goat anti-human IgG-FITC, Human IgG and Human IgG-HRP were obtained from Bangalore Genei, India. 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde were procured from Sigma-Aldrich, USA. The buffers used were: 50mM phosphate buffered saline (PBS), pH 7.4; 50mM phosphate buffer (PB), pH 7.2 and 50 mM glycine-HCl buffer, pH 2.2 for regeneration. All buffers and solutions were made up of pyrogen-free deionized water. All other reagents and solvents used were of best analytical grade. Gold substrates were obtained by coating silicon chips (area: 1cm by 1 cm) with 30 nm of gold by thermal evaporation.

2.1.2. Cleaning of gold-coated silicon. Prior to immobilization of biomolecules on the gold-coated silicon, the surface was thoroughly cleaned sequentially in methanol, acetone and isopropanol-2 for 10 minutes each. This was followed by a quick 1 minute dip in piranha solution (H₂SO₄ 70%:H₂O₂ 30%≈ 3:1 v/v). Gold-coated silicon surface was then rinsed with deionized water for about 10 minutes. Finally it was washed with ethanol and dried at 70°C for 30 minutes.

2.1.3. Immobilization of human IgG on gold by protein A/protein G procedure. The immobilization procedure described by Suri et al. [20] was followed. Clean gold surface was dipped in protein A/protein G solution (0.1 mg/ml in PB) and incubated overnight at room temperature. After washing with PB, the substrates were immersed in BSA solution (1 mg/ml in PB) to block all non-specific protein binding sites. They were further washed with PB and subsequently, 50 μl of human IgG (0.1 mg/ml in PBS) was added to the gold-coated substrates and left overnight at 4°C. The excess antibody was removed by washing with PBS. It was then immersed in BSA solution (1 mg/ml in PB). The substrate was dried in clean air and the amount of human IgG bound to gold-coated silicon was determined by TMB substrate assay.

2.1.4. Immobilization of human IgG on gold-coated silicon by neutravidin procedure. Biotinylation of human IgG was done by EZ-link sulfo-NHS-biotinylation kit procured from Pierce Co., USA. Clean gold surface was immersed in 2% 3-APTES in deionized water for 30 minutes at room temperature. It was then rinsed sequentially with deionized water and absolute ethanol. The substrate was then cured in a vacuum oven at 80°C overnight. 50 μl of EDC (10 mg/ml) and 50 μl of neutravidin solution (0.1 mg/ml in PBS) were then provided simultaneously to the substrate and incubated for two hours at room temperature. After washing with PBS, the substrate was immersed in BSA solution (1 mg/ml in PB) to block all non-specific protein binding sites. The substrate was further washed with PBS and, subsequently, human IgG was immobilized by the procedure as described in the previous section.

2.1.5. Quantitative analysis by TMB substrate assay. The procedure employed by Theegala and Suleiman [17] for the quantification of HRP labeled biomolecules bound to the solid substrate was followed. Gold-coated silicon chips having HRP labeled human IgG immobilized on the gold surface by the various immobilization procedures were put in different 5 ml beakers, with their coated surfaces facing up. 250 μl of TMB was added and the beaker was immediately placed on an orbital shaker (100 rev./min). Thereafter, 250 μl of H₂O₂, 499 μl of TBS with 1 μl of 0.1% BSA were added. The peroxidase enzyme, in the presence of H₂O₂, catalyses the oxidation of colorless TMB substrate to a blue colored product. After a fixed reaction time (30 min), the reaction was stopped with 100 μl of 2M H₂SO₄ and the absorbance of the solution was measured at 450 nm. Calibration assays were performed with known concentration of human IgG-HRP. Thus the amounts of human IgG-HRP bound to the gold-coated silicon substrate by various immobilization procedures were determined. Similarly, the amount of protein A-HRP immobilized on gold was determined.

2.1.6. Qualitative analysis by Fluorescence Microscopy. Protein A-FITC labeled (0.1 mg/ml in PB) was immobilized on gold-coated silicon by the previously described procedure and washed with PB. The binding of the protein A-FITC was determined by taking fluorescence images with an Axiovert 25 CFL inverted fluorescent microscope (Carl Zeiss, Jena, Germany) under darkroom conditions. The binding of human IgG to protein A was analyzed by incubating human IgG bound gold-coated silicon with goat anti-human IgG-FITC (0.1 mg/ml in PBS) at room temperature for 2 hours. Excess of goat anti-human IgG-FITC labeled bound to the human IgG was removed by washing with deionized water and PBS. The binding of goat anti-human IgG-FITC (0.1 mg/ml in PBS) was detected by taking fluorescence images.
2.1.7. **Regeneration of immuno sensing human IgG surface.** The human IgG coated surface was regenerated by treatment with glycine-HCl buffer. The regeneration profile of the immuno sensing human IgG surface was found by monitoring the activity of HRP labeled human IgG by TMB substrate assay after multiple regeneration steps.

2.1.8. **AFM measurements.** Silicon nitride triangular cantilevers with nominal spring constant 0.12 N/m (Veeco, USA) were used for imaging using PicoPlus AFM (Molecular Imaging, Tempe, AZ, USA). The cantilevers were washed in ethanol and deionized water and dried at 70°C for 30 minutes. They were finally cleaned by UV treatment for 20 minutes. The imaging of samples was done in contact mode in air.

3. **Results and discussion**

3.1.1. **Quantification of human IgG by TMB substrate assay.** Horse radish peroxidase (HRP) conjugated human IgG antibodies were immobilized on different gold-coated silicon chips by protein A, protein G and neutravidin immobilization procedures. Thereafter, each human IgG-HRP coated gold surface was dipped in different 5 ml capacity glass beakers each containing TMB substrate assay solution i.e. 250 μl of TMB, 250 μl of H2O2, 499 μl of TBS and 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 happened because the enzyme peroxidase in the presence of H2O2 catalysed the oxidation of colorless TMB substrate to a blue colored product. The extent of immobilization of human IgG varied depending on the type of immobilization procedure followed (table 1). The immobilization densities of human IgG bound to gold-coated silicon as determined by TMB substrate assay were 610 ng/cm² (24.3 x 10¹¹ molecules/cm²), 590 ng/cm² (23.5 x 10¹¹ molecules/cm²), and 530 ng/cm² (21.1 x 10¹¹ molecules/cm²) for protein A, protein G and neutravidin procedures respectively. The immobilization density of human IgG was maximum i.e. 610 ng/cm² when protein A immobilization procedure was followed. The immobilization density was 3.28 percent and 13.11 percent less w.r.t. protein A procedure when protein G and neutravidin procedures were employed respectively. Based on these results, it was concluded that protein A procedure for immobilizing human IgG was the most suitable for immobilizing antibodies on gold-coated silicon. Therefore, this procedure was assessed in detail.

| Immobilization procedures followed | Amount of human IgG immobilized\(^a\) (ng/cm²) |
|----------------------------------|-----------------------------------------------|
| Protein A                        | 610 ± 30.2 ng/cm²                              |
| Protein G                        | 590 ± 36.5 ng/cm²                              |
| Neutravidin                      | 530 ± 41.2 ng/cm²                              |

\(^a\) Results are mean values of all recordings ± standard deviation

3.1.2. **Qualitative analysis of immobilized human IgG by fluorescence microscopy and AFM.** The qualitative analysis of human IgG immobilized on gold by protein A procedure was done by inverted fluorescence microscopy and AFM. It was apparent from the fluorescence images (as shown in figure 1a) that protein A-FITC bound uniformly on gold-coated silicon. Similarly, goat anti-human IgG-FITC labeled antibodies were bound to human IgG immobilized on gold-coated silicon. Goat anti-human IgG was also coated uniformly as shown in figure 1b. The uniform coating of goat anti-human IgG directly correlates with the uniform coating of human IgG on protein A treated gold.

The quantification of protein A bound to gold-coated silicon was done by employing protein A-HRP. Gold-coated silicon chip having protein A-HRP immobilized on its surface was put in 5 ml beaker, with its coated surface facing up. The amount of protein A-HRP immobilized on gold-coated silicon as determined by a colorimetric assay using TMB was 140 ng/cm² (16.9 x 10¹¹ molecules/cm²).

AFM was employed to assess the uniformness of biomolecules immobilized on gold-coated silicon at high spatial resolution. AFM images of clean gold surface is shown in figures 2 (a). The binding of protein A to gold-coated silicon was highly uniform as shown in figure 2 (b). It was further confirmed by AFM imaging (data not shown) that two subsequent PBS washings with little shaking were enough for removing unspecifically bound and getting uniform coating of human IgG as shown in figure 2 (c).
3.1.3. Regeneration of immunosensing human IgG surface. The regeneration of human IgG immobilized on gold, once it has been bound to goat anti-human IgG, was studied. As the binding chemistries of antigen-antibody complexes is not well understood, care has to be taken while choosing the dissociation agents as they may affect the activity and specificity of antibodies and lead to leaching of antibody from the immunosensor surface. The dissociation agent should not affect the antigen affinity and the stability of the bonds between the antibody and the transducer surface.

![Figure 1](image1.png)

**Figure 1.** Fluorescent image (magnification 200X) of (a) protein A-FITC bound to gold-coated silicon, (b) goat anti-human IgG-FITC bound to human IgG immobilized on gold-coated silicon by protein A procedure.

![Figure 2](image2.png)

**Figure 2.** AFM images (a) gold-coated silicon after sequential treatment with piranha solution, ethanol and ultraviolet light (b) protein A immobilized on gold-coated silicon (c) human IgG immobilized by protein A procedure on gold-coated silicon. The root mean square(RMS) roughness increased sequentially from 1.06 nm in case of cleaned gold-coated silicon to 10.6 nm when protein A was coated and then to 17.3 nm when human IgG was bound to protein A. The roughness was calculated after carrying out a slope correction by a 2nd order polynomial plane fit.
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. Use of organic solvents, surfactants, chaotropic ions 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 [21].

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 gold-coated silicon and human IgG. The percentage regeneration of human IgG bound surface was monitored by TMB substrate assay based on the quantification of functional human IgG-HRP left immobilized on gold after treatment with Glycine-HCl buffer. Similar procedure was followed by Grogan et al. [19] for monitoring the activity of immobilized antibody after multiple regeneration steps. In our studies, the regenerated human IgG surface retained sufficient activity. The regeneration decreased from 95 percent after first regeneration cycle to 74 percent after fourth regeneration cycle as shown in figure 3.

Figure 3. Regeneration profile of the human IgG immobilized on gold-coated silicon after treatment with Glycine-HCl buffer (50 mM, pH 2.2).

4. Conclusions

The present study describes a technique for the scientific quantification of antibody immobilization on solid substrates which can be of help in screening for the best scientific biosensor/transducer surface and immobilization strategies in terms of maximization of bound antibody concentration and efficiency. Protein A, protein G and neutravidin immobilization procedures were employed in order to immobilize human IgG on gold-coated silicon. Based on the experimental results, it was concluded that protein A immobilization procedure was the most appropriate. The immobilization density as determined by TMB substrate assay was 140 ng/cm² (16.9 x 10¹¹ molecules/cm²) and 610 ng/cm² (24.3 x 10¹¹ molecules/cm²) for protein A and human IgG respectively. The functional human IgG surface was effectively regenerated up to four times by treatment with Glycine-HCl buffer (50 mM, pH 2.2).

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