CHAPTER 3

IMMUNOGLOBULIN IMMOBILISATION ON MODIFIED POROUS SILICON SURFACE

INTRODUCTION

Porous silicon (PS) can be used as smart transducer material in sensing application particularly in the detection of vapors, liquids and biochemical molecules. In fact, on exposure of chemical substances, several physical quantities such as refractive index, photoluminescence, and electrical conductivity change drastically [1, 2]. A key feature of a physical transducer, being sensitive to organic and biological molecules either in vapor or liquid state is a large surface area of the order of 200–500 m² cm⁻³, thus ensuring an effective interaction with several adsorbates [3]. PS technology has shown great capability in detecting biological molecules with high selectivity using specific linker agent and probe molecules. For the biomedical applications of PS, biomolecules have to be first immobilized on its surface through functional groups deposited on it. The common approach is to create a covalent bond between the PS surface and the biomolecules which specifically recognize the target analytes [4]. The aim of the present study is to demonstrate the covalent bonding between organic molecules (immunoglobulin) and modified inorganic surface (PS) which can be used for the detection of protein signals. PS surface is biofunctionalized by depositing on to its surface a thin biocompatible film with a large density of amine groups, using APTS. The choice of APTS as a functionalization material is based upon the fact that amine groups are present in proteins and their chemical interaction with other functional groups is well documented [2, 5]. Glutaraldehyde is used as a homobifunctional cross-linker to bridge the APTS functionalised PS surface and the antibody. The amino groups on the surface can be used for Schiff base formation with glutaraldehyde, then covalent binding of the antibody can easily be achieved. Antibody is selectively and specifically binds to its complimentary antigen. To test for immunocomplex specificity, the antibody-functionalized PS is treated with complimentary FITC labelled antigen.
The biofunctionalized PS surface was characterized by different techniques like X-ray photoelectron spectroscopy (XPS) and fourier transform infrared spectroscopy (FTIR), respectively. The present work shows a potential approach to PS-based biosensors.

MATERIALS AND METHODS

Porous silicon formation

In the present study PS prepared at current density 50 mAcm\(^{-2}\) (Pore size ~50-60 nm) has been utilized for immunoglobulin attachment. Details of formation of PS are given in chapter 1 Part A (page no. 42-44).

Chemical modification of silicon surfaces

The description of silanization process used for PS surface has been given in Chapter 2 (page no. 79). The APTS functionalized flat silicon and PS samples were immersed into a solution of glutaraldehyde (2.5%, pH 7), which was used as a cross linker. The wafers were kept in the solution for 1 hr at room temperature. Subsequently, the samples were rinsed with DI water in order to remove unbound glutaraldehyde.

Immunoglobulin immobilization

The antibody (Human IgG) (5 µg/ml in acetic/acetate buffers, pH 5) was immobilized on glutaraldehyde treated PS surface for ~1 hr. The unoccupied sites of the functionalized samples were blocked by incubating with blocking solution bovine serum albumin (0.1%) at room temperature for 15 min. The substrates were cleaned with 0.05% Tween 20 in phosphate buffered saline (PBS). To verify the activity of the covalently bonded antibodies, antigen–antibody incubation was carried out. For this, antibodies immobilised on PS surface were incubated with the antigen (goat anti human IgG, fluorascien isothiocyanate (FITC) labeled, 5 µg/ml in 0.05M PBS at pH 7.2) for 1 h at room temperature in dark. After rinsing with deionized water thrice, the
FITC conjugate was avoided to light exposure and examined under UV light using a fluorescence microscope.

Sample characterization

Various steps involved in immobilization were monitored and characterized. Samples were characterized using X-ray photoelectron spectroscopy (XPS) and fourier transform infrared spectroscopy (FTIR). Antigen–antibody binding was examined using Nikon Fluorophot (Axioskope 40) microscope fitted with digital camera (USA). The pictures were scanned using 10 x objectives with apple green fluorescence.

RESULTS AND DISCUSSION

Scheme 1 Antibody-antigen binding on modified PS surface
Scheme 1 shows the antibody-antigen chemical attachment to the modified PS surface. Initially, freshly prepared PS is SiH₂ terminated which is then treated with a solution of hydrogen peroxide, deionized water and hydrochloric acid (SC2) to obtain a layer of hydroxyl-terminated silicon oxide about 1.0-1.5 nm thick [6] (A). Secondly, these hydroxyl-terminated PS surfaces are then silanized with APTS in toluene resulting in amine-terminated surfaces (B); thirdly, this APTS-modified surface is reacted with glutaraldehyde. Glutaraldehyde have been commonly applied for biomolecular immobilisation the two aldehyde groups of glutaraldehyde are used to bridge amine-reactive PS surface (APTS modified) with biomolecules bearing primary amines (Immunoglobulin). The reaction is through a nucleophilic attack of amine on C=O group of glutaraldehyde. (C); fourthly, antibody (Human IgG) is bound to one of the aldehyde group of glutaraldehyde (D) and finally which bind with FITC labelled antigen (goat anti human IgG) (E), respectively. The size of antibody is generally in the range 10-15 nm. The conformation of the immunoglobulin (Ig) antibody is Y-shape, composed of an upper Fab region with active sites (paratopes) at its tips and a lower Fc region with no specific binding capability. Antigen binds specifically at antibody binding sites. Only complementary (or conjugate) partners can bind at complementary antibody spots. All other unbound antigen are rinsed away. Specific binding is a 3-D conformational fit between the antigen binding region (epitope) and antibody binding region (paratope). The specific interaction at this interface largely consists of hydrogen bonding between protein resides and through interstitial water molecules.

FTIR studies

Covalent attachment of a biomolecule on any substrate depends on the efficiency of the surface functionalization. A biomolecule, such as protein (immunoglobulin, enzyme etc) may be attached to a surface via a spacer group having reactive end groups like amine, carboxylic acid and hydroxyl groups. These spacer groups reduce steric hindrance and provide better freedom of movement to the immobilized biomolecules for increased activity [6]. A silane reaction or silanization provides necessary spacer groups for the purpose of immobilizing a biomolecule [7]. The IR
spectra for fresh PS and APTS treated samples have been described earlier in (Chapter 4, page no. 80-81) The APTS modified surface is then reacted with glutaraldehyde which can form linkage with the primary amine group on proteins [8, 9].

IR spectrum of APTS modified PS surface obtained after exposure to a glutaraldehyde solution (Fig. 5.1) contains a band at ~1737 cm$^{-1}$ assigned to the stretching vibration of the aldehyde (C=O) group and another band at ~1670 cm$^{-1}$ corresponds to the imine (C-N) bond. These bands reveal the Schiff’s base formation with glutaraldehyde. The presence of these two peaks indicates the reaction between the amine groups of APTS and the aldehyde group of glutaraldehyde. Glutaraldehyde treated PS samples do not show any bands corresponding to –NH bonds. After silanization, the PS sample has bands corresponding to –NH bonds and –CH bonds. The absence of –NH bonds also corroborate the formation of imine bond. Hence, it confirms the formation of Schiff’s base as a result of glutaraldehyde treatment [9, 10].

![Figure 5.1 FTIR spectrum of glutaraldehyde PS surface](image)

**Figure 5.1 FTIR spectrum of glutaraldehyde PS surface**
Figure 5.2 shows the FTIR spectrum of modified PS after antibody binding; two bands are observed at ~ 1650 and 1540 cm\(^{-1}\), indicative of protein attachment. Antibody is attached to one of the available aldehyde group of glutaraldehyde, most likely through the random amines of lysine residues at the surface of the Fab fragment [11]. The spectrum (Fig. 5.2) explain the typical characteristics of the amide backbone of all proteins namely, the amide bands near 1650 (C=O stretch; amide I) and 1540 cm\(^{-1}\) (both C-N and C-N-H stretching mode; amide II) bands which can be readily assigned to the amide functionalities of a peptide group, providing evidence of chemical bonding of antibody to the APTS modified PS surface [12-14]. The above results show that antibody is covalently bound to the amine-functionalized PS surface using glutaraldehyde as a crosslinking agent.

Figure 5.2 FTIR studies of PS film after Ab immobilization
XPS studies

A comparison of the XPS survey spectra of APTS modified PS, glutaraldehyde treated PS and antibody (Human IgG) attached PS surfaces illustrated changes in the intensities of carbon (C1s), oxygen (O1s), nitrogen (N1s) and silicon (Si 2s, 2p) composition of the surface before and after each chemical modification. Fig. 5.3 shows the general scan XPS survey spectra of carbon, oxygen, nitrogen and silicon after silanization, glutaraldehyde treatment, and antibody attachment. Table 1 shows the percentage elemental composition present after surface modification and antibody immobilisation.

Figure 5.3 XPS spectra of (a) APTS modified (b) glutaraldehyde treated and (c) antibody immobilised PS surface
**Table 1: Atomic concentration of elements C, N, O and Si**

<table>
<thead>
<tr>
<th>Element (%)</th>
<th>APTS modified PS</th>
<th>Glutaraldehyde treated PS</th>
<th>Antibody immobilised PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>44.7</td>
<td>53.8</td>
<td>54.4</td>
</tr>
<tr>
<td>Oxygen</td>
<td>38.3</td>
<td>34.1</td>
<td>32.9</td>
</tr>
<tr>
<td>Silicon</td>
<td>10.1</td>
<td>8.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>6.8</td>
<td>3.2</td>
<td>10.4</td>
</tr>
</tbody>
</table>

There is an increase in carbon (C 1s peak) concentration as well as in nitrogen (N 1s peak) content with antibody immobilised PS sample (Fig. 5.3) as compared to glutaraldehyde and APTS treated PS. After silanization, N1s and Si 2p signals of nitrogen and silicon are detected with peak intensity (area %) ~6.8 % and 10.1 %, respectively. When antibodies are immobilized onto PS, the N1s peak intensity (area %) increased from 6.8 to 10.4 %, and the Si2p peak intensity is decreased to about 2.3%. Enhancement of the content of nitrogen (N 1s) and reduction in Si2p signals after immobilization of the antibodies suggest that the immobilization of antibodies onto the APTS modified PS surface is complete [15]. The APTS functionalised PS indicate the presence of NH$_2$ group uniformly distributed over the surface. The thickness of the silane layer deposited on PS is approximately quantified with XPS spectra using formula $I_{Si}/I_{\infty Si} = \exp \left( -d / \lambda_{APTS Si} \right)$ where $I_{Si}/I_{\infty Si}$ is the Si2p intensity ratio for silylated and blank substrates, respectively, $d$ is the thickness of film, and $\lambda_{APTS Si}$ is the attenuation length, assumed to be equal to 43 Å (density for APTS=0.946 g cm$^{-3}$ [16]. The average thickness of APTS film (d) is approximately equal to 16 Å [4, 17].

Figure 5.4 shows a detailed deconvolution analysis of C (1s) spectra of PS surfaces to identify the contributions of different types of covalent bond formation after surface functionalisation and antibody immobilisation. C (1s) spectra of APTS modified PS surface indicates presence of hydrocarbon species (C1), which is higher (~78%) as compared to glutaraldehyde treated (60%) and antibody attached (47%) PS. Upon glutaraldehyde treatment another carbon species (C$_3$) appeared in the C1s
spectrum (Fig. 5.4.b) which can be attributed to oxygenated carbon species (C=O, O–C–O) thus confirming reaction with glutaraldehyde. A new carbon component consisting of contributions from C–N and C–O (C2), which is much higher in case of PS sample immobilized with antibody. Table 2 elucidates the percentage contribution of carbon bonds formed during different chemical modification of PS surface [4].
Figure 5.4 C (1s) XPS spectra of (a) APTS modified (b) glutaraldehyde treated and (c) antibody immobilised PS surface

Table 2 Explains the binding energies and percentage area of deconvoluted C (1s) core level peak for different chemical states of carbon.

<table>
<thead>
<tr>
<th>Species</th>
<th>C1 (C-C, C-H) % Binding Energy (eV)</th>
<th>C2 (C-N, C-O) % Binding Energy (eV)</th>
<th>C3 (C=O, O-C-O) % Binding Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTS</td>
<td>78 (284.4)</td>
<td>22 (285.4)</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>60 (284.6)</td>
<td>26 (285.5)</td>
<td>14 (287.3)</td>
</tr>
<tr>
<td>Immobilised Antibody</td>
<td>47 (284.6)</td>
<td>24 (285.2) C-N 14 (286) C-O</td>
<td>15 (287.7)</td>
</tr>
</tbody>
</table>

To quantify the nitrogen contribution after surface functionalisation and immunoglobulin attachment on PS, N (1s) peakfit was attempted by using three curve components, which are shaped as a convolution of Gaussian curve. Deconvoluted N (1s) spectra explicate the occurrence of three nitrogenous species (Fig. 5.5.a-c). The nitrogen component (N1) with the lowest binding energy, ~399.1 eV (Fig. 5.5) are
assigned to unsaturated chemical bonds (-N=), another peak (N2) ~ 400 eV of binding energy shows the nitrogen contribution due to -NH- and finally, the peak at ~ 401 eV (N3) agrees well with NH₂ nitrogen. Appearance of the N1s signal is evidenced that the APTS silanized substrate anchoring NH₂ groups to the PS surface. The percentage of nitrogen components is compared to the relative intensities of their contributions (Table 3).
Figure 5.5 \( N (1s) \) XPS spectra of APTS modified (a) glutaraldehyde treated (b) and antibody immobilised (c) PS surface.

Table 3 Deconvolution of \( N (1s) \) spectra after surface modification and antibody immobilised on PS

<table>
<thead>
<tr>
<th>Species</th>
<th>N1 (( -N= )) %</th>
<th>N2 (( -NH- )) %</th>
<th>N3 (( NH_2 )) %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding Energy (eV)</td>
<td>Binding Energy (eV)</td>
<td>Binding Energy (eV)</td>
</tr>
<tr>
<td>APTS</td>
<td>73 (399.1)</td>
<td>20 (399.9)</td>
<td>7 (401)</td>
</tr>
<tr>
<td>GA</td>
<td>64 (399.2)</td>
<td>23 (400.3)</td>
<td>11 (401.5)</td>
</tr>
<tr>
<td>Antibody</td>
<td>56 (399.1)</td>
<td>30 (400.3)</td>
<td>14 (401.7)</td>
</tr>
</tbody>
</table>

Antibody–antigen immunocomplex formation can be used as an essential tool to diagnose and profile diseases rapidly and reliably [18]. The immunocomplex can easily be detected and quantified by optical, electrical or mass measurement methods using surface immobilized biomolecules [19-22]. The bioactivity of functionalized PS film which shows great promise for immunoassay has been assessed by the detection of a FITC-labelled antigen (secondary antibody) binding with its primary antibody. Fluorescence micrograph (Figure 5.6 b) shows PS surface after antigen binding, show a apple green colour which indicates binding of secondary FTIC labeled antibody.
(goat anti human IgG) uniformly with primary anitbody (Human IgG). Figure 5.6a corresponds to biofunctionalized PS with antibodies immobilized on its surface. It is observed that FITC labelled antigen reacts with antibodies immobilized on functionalised PS surface. It is evident from XPS and FTIR that antibodies immobilized on PS surface keep their functionality since they are recognized by their correspondent antigens.

**Figure 5.6** Fluorescent image (a) antibody (Human IgG) immobilised PS (b) FITC labelled antigen (Goat anti-human IgG) binding with antibody

**SUMMARY**

The above study demonstrates that antibody (Human IgG) binds to the APTS derived PS surface by covalent bonds between the reactive amine group of the antibody and aldehyde group of the glutaraldehyde. This also demonstrates that antibody immobilised on PS surface binds selectively to its complimentary antigen (goat anti-human IgG). The results from XPS, FTIR and fluorescence microscopy has convincingly proved the antibody-antigen binding on PS surface. Therefore, it can be concluded that PS surface can be used for bionanotechnology applications in general, and immunosensing in particular.
REFERENCES


