Covalent immobilization of *Pseudomonas cepacia* lipase on semiconducting materials

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Received 18 September 2007; received in revised form 9 January 2008; accepted 10 January 2008
Available online 31 January 2008

Abstract

Lipase from *Pseudomonas cepacia* was covalently immobilized on crystalline silicon, porous silicon and silicon nitride surfaces. The various stages of immobilization were characterized using FTIR (Fourier transform infrared) spectroscopy. The surface topography of the enzyme immobilized surfaces was investigated using scanning electron microscopy (SEM). The quantity of the immobilized active enzyme was estimated by the para-nitrophenyl palmitate (pNPP) assay. The immobilized lipase was used for triglyceride hydrolysis and the acid produced was detected by a pH sensitive silicon nitride surface as a shift in the C–V (capacitance–voltage) characteristics of an electrolyte–insulator–semiconductor capacitor (EISCAP) thus validating the immobilization method for use as a biosensor.
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Keywords: Lipase; Immobilization; Silanization; Glutaraldehyde; EISCAP; Triglyceride biosensor

1. Introduction

Lipase (triacylglycerol ester hydrolase, E.C.-3.1.1.3) is an enzyme which catalyses the hydrolysis of triacylglycerols. These enzymes are commercially important and have many applications in food industry such as the synthesis of esters and peptides. Another application, which is still unexplored to a large extent, is the integration of lipase with the semiconductor based systems in order to develop an accurate and sensitive triglyceride biosensor. However, many studies have been done on the integration of lipase on various other supports like glass beads and polypropylene powder [1,2]. The potential of enzyme mediated reactions in semiconductor devices was identified by Bergveld in 1981 [3,4]. Enzyme immobilized porous/crystalline silicon wafers were also used as microreactors which are an essential component of wafer integrated chemical analyzers [5,6]. Enzymes are either covalently immobilized or are allowed to adsorb on the surface of the support. Enzyme immobilization by covalent binding has many advantages viz. tighter binding during the reaction and systems with immobilized enzymes are reported to have more heat stability [7]. Another type of immobilization is encapsulation and an enzyme encapsulated membrane matrix, integrated in the gate region, was the pH sensing agent in an enzyme field-effect transistor (EnFET) introduced by Caras and Janata [8]. Since then many research efforts have been made on the immobilization of enzymes/proteins on semiconductors but most of these studies do not emphasize the characterization of the enzyme immobilized semiconductor surfaces. In our constant efforts to improve the sensitivity of the biosensor developed by us [9,10], this study reports an immobilization procedure for covalent binding of lipase on various semiconducting materials and its detailed characterization, at different stages of immobilization. The functionalized surfaces were characterized using FTIR at each stage and the morphology of the enzyme immobilized surface was characterized using SEM (scanning electron microscopy). The amount of active enzyme was estimated using pNPP assay. Lipase mediated hydrolysis of tributyrin produces butyric acid and the resulting change in pH causes a shift in the flat-band voltage of an electrolyte–insulator–semiconductor capacitor (EISCAP) that shows a shift in the measured capacitance–voltage (C–V) characteristics with changes in the pH of the electrolyte. The EISCAP sensor device is analogous to a metal-oxide semiconductor (MOS) capacitor,
with the metal replaced by an electrolyte. An EIS-CAP structure contains a stack of pH sensitive dielectric layers deposited on silicon. The electrochemical relation between the flat-band shift of the CV curve and the pH of the electrolyte is given by the “Nernst response” [11]. SiO₂ and Si₃N₄ are good options for the of the silicon. The electrochemical relation between the flat-band shift contains a stack of pH sensitive dielectric layers deposited on with the metal replaced by an electrolyte. An EIS-CAP structure

2. Materials and methods

Aminopropyltriethoxysilane (APTES) was purchased from Sigma–Aldrich, USA, and all the other chemicals of electronic grade were purchased from Qualigens Fine Chemicals, Mumbai, India. Buffers were made from analytical grade reagents and deionised water. Crystalline silicon, p-type, (1 0 0) oriented wafers of resistivity 1–10 Ω cm were used. Lipase (Pseudomonas cepacia) with an activity of 26 μm/mg/min was bought from Amano, Japan. C–V measurements were done with an HP 4275A LCR meter at 4 kHz with 15 mV signal amplitude by sweeping the dc bias from −7 to 0.5 V in steps of 100 mV.

2.1. FTIR study

The Fourier transform infrared (FTIR) spectra were collected on a JASCO 550 operating in transmission mode. All spectra were recorded at 2 cm⁻¹ resolution and averaged using 32 scans. The samples were dried in nitrogen ambient and were mounted on a small holder for measurements.

2.2. SEM study

A high resolution scanning electron microscope (FEI Quanta 200, USA), operating at 30 kV at high vacuum (HV), was used to observe the morphological changes on the surface due to enzyme immobilization.

2.3. pNPP assay

The para-nitrophenyl palmitate (pNPP) assay was done as described in reported methods [13,14] except for the usage of enzyme immobilized samples instead of free enzymes. The release of p-nitrophenol at 37 °C, and pH 7.4 (0.05 M phosphate buffer) was detected at 412 nm (Jasco V-530 spectrophotometer).

2.4. Contact angle measurement

To determine the hydrophilicity/hydrophobicity of the modified surfaces, contact angles with deionized water are measured with a video contact angle meter (AST Products, Inc.). Three measurements were done on each sample. Samples were dried with nitrogen at room temperature before measurements.

2.5. Wafer cleaning procedure

The silicon wafers were boiled in trichloroethylene (TCE) in order to remove organic impurities. After that the wafers were rinsed in acetone for 2 min and then treated in warm HNO₃ for 5 min to oxidize the metallic impurities. This was followed by etching the wafers in 1:10 hydrofluoric acid in water to remove the primary layer of oxide formed due to the HNO₃ treatment. Finally, the wafers were rinsed in deionized water and dried with a nitrogen gun.

3. Sample preparation

The silicon wafers were cleaned using the cleaning procedure, as discussed in Section 2.5, and cut into 2.5 cm × 2.5 cm size pieces. Porous silicon (PS) was made by the anodic etching of silicon in a 1:1 mixture of HF and isopropyl alcohol at a constant current density of 10 mA cm⁻² for 10 min in a custom made teflon cell. The etched surfaces were washed in isopropanol and n-pentane and were kept in an oven for 5 min at 80 °C for drying. Silicon nitride was deposited by plasma enhanced chemical vapor deposition technique (PECVD) on thermally oxidized silicon samples in order to obtain a good interface between the insulator layer and semiconductor since the interface state density is negligible at the Si–SiO₂ interface. Crystalline silicon and porous silicon were oxidized for 2 h at 1000 and 500 °C, respectively. PECVD nitride deposition was done for 10 min at 300 °C to obtain a thickness of 80 nm. The samples were annealed at 800 °C for 20 min in nitrogen ambient. The annealing step is essential because untreated silicon nitride when kept exposed to ambient conditions may degrade the pH sensitivity of silicon nitride [15].

3.1. Surface functionalization and lipase immobilization

The samples were treated with conc. HNO₃ for 30 min at 80 °C to make the surface hydrophilic. This was followed by treating the samples in 3% APTES in toluene (3 ml APTES + 97 ml of toluene) for 3 h at 120 °C. The silanized samples were treated in a 10% solution of glutaraldehyde in DI water for 2 h at room temperature [16]. The surfaces are modified as in step 1 and step 2 in Fig. 1. Lipase (0.8 mg/ml) in phosphate buffer solution (10 mM, pH 7) was used for immobilization. The glutaraldehyde activated samples were immersed in 2 ml of 0.8 mg/ml lipase solution for 24 h. The samples were washed thoroughly with DI water for 40 min to remove any adsorbed enzyme particles. The samples were heated in an oven at 120 °C for 10 min after every step of immobilization except for the lipase immobilization lest the enzymes would be denatured.

4. Results and discussion

4.1. FTIR study

Permanent attachment of a biomolecule on any support depends on the efficiency of the surface functionalization. A
biomolecule, such as an enzyme, 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 greater activity [17]. A silane reaction or silanization provides necessary spacer groups for the purpose of immobilizing a biomolecule. [18]. The surface was modified with 3-aminopropyltriethoxysilane, then the amine group of APTES modified surface was reacted with glutaraldehyde to yield an aldehyde which can form an imine linkage with the primary amine group on proteins [16]. The various steps involved in immobilization were monitored and characterized. The plain porous silicon samples and the silicon nitride deposited porous silicon samples showed distinct bands at various stages of immobilization as discussed below.

4.1.1. Blank porous silicon and silicon nitride deposited porous silicon
Blank porous silicon samples, in Fig. 2A and B, did not show any particular bands except for the Si–H bonds around 2100 cm\(^{-1}\) [19,20]. Blank silicon nitride deposited porous silicon sample, in Fig. 3A had bands around 840 and 1100 cm\(^{-1}\) which are attributed to the Si–N [21] bonding and Si–O [8] formation, respectively. The Si–O bond formed is due to the thermal oxide grown prior to nitride deposition. The partially masked Si–N band at 840 cm\(^{-1}\) was clearly revealed when spectral deconvolution was performed (Fig. 3C).

4.1.2. HNO\(_3\) treated samples
The broad band around 3500 cm\(^{-1}\), found in both samples, is due to the Si–OH bonds on the surface [18]. The chemically oxidized blank porous silicon nitride had a band around 1100 cm\(^{-1}\) due to Si–O bond formation [22].

4.1.3. Silanized samples
Both the porous silicon samples and silicon nitride deposited porous silicon samples showed similar bands after silanization. The silanized wafers showed two small bands at 3294 cm\(^{-1}\) and near 3380 cm\(^{-1}\) (Figs. 2A and 3A), which are attributed to –NH bonds [23,24]. A small band at 1602 cm\(^{-1}\) also can be attributed to –NH bonds as seen in 2B and 3B [23]. Two bands around 2850–2950 cm\(^{-1}\) are due to the presence of –CH bonds [18].

4.1.4. Glutaraldehyde activated samples
Glutaraldehyde activated samples did not show any bands corresponding to –NH bonds. 2B and 3B show the distinct bands corresponding to a glutaraldehyde activated sample. Bands at 1580 cm\(^{-1}\) [imine –C=N] and 1710 cm\(^{-1}\) [–C=O of aldehyde] revealed the Schiff’s base formation with glutaraldehyde as expected [25].
4.1.5. Lipase immobilized samples
The lipase treated samples showed a higher absorption peak at 1580 cm\(^{-1}\) [imine –C\(^\equiv\)N] due to the formation of imine bonds during enzyme immobilization by displacing the 1710 cm\(^{-1}\) [–C\(^\equiv\)O bond of glutaraldehyde activated sample].

As discussed above, after the step 1, in Fig. 1, it is evident that –OH bonds (3500 cm\(^{-1}\)) were formed on the sample surface after HNO\(_3\) treatment. After silanization, the samples (step 2 in Fig. 1) had bands corresponding to –NH bonds (3294, 3380 and 1602 cm\(^{-1}\)) and –CH bonds. The glutaraldehyde activated sample (step 3 in Fig. 1) had the bands corresponding to aldehyde (\(^{1710}\) cm\(^{-1}\) [–C\(^\equiv\)O]) and imine (–C\(^\equiv\)N at 1580 cm\(^{-1}\)). The absence of –NH bonds also corroborate the formation of imine bond. The lipase immobilized samples showed an enhanced absorbance at the bands assigned to imine bonds. This is due to the formation of additional imine bonds as a result of the reaction between the amine group of lipase and the aldehyde groups in the glutaraldehyde activated sample (step 4 in Fig. 1).

Further confirmation of the Schiff’s base formation was done by treating a glutaraldehyde activated sample in a mild acid solution which hydrolyses the Schiff’s base formed. From Fig. 4 it can be seen that the acid treated samples did not possess the bands near 1710 cm\(^{-1}\) [–C\(^\equiv\)O of aldehyde] nor at 1580 cm\(^{-1}\) [imine –C\(^\equiv\)N] which are exclusive to glutaraldehyde activated samples. On the other hand, it had bands which are specific to silanized samples like 1602 cm\(^{-1}\) (–NH bond). Hence it confirms the formation of Schiff’s base as a result of glutaraldehyde activation. The SEM pictures of blank PS and silicon nitride deposited PS surfaces are shown in Fig. 5a and b. The blank samples did not show any change in the morphology of the surface. The pictures (magnification: 10,000\(\times\)) of the enzyme immobilized samples are shown in Fig. 5c–f. Lipase attaches itself on to the surfaces in a very interesting fashion. The pictures show the pattern in which lipase is immobilized on the surfaces which is quite uniform on a small surface area. It can be seen that the particles are packed in a regular order and in repeating pattern regardless of the fact that the surfaces are crystalline or not.

4.2. Enzyme assay
The pNPP assay is operationally very simple and was used in this study to compare the amount of enzyme on the surface.
modified and unmodified substrates. The assay was found to be accurate enough to determine enzyme amounts as low as 10 μg. The results are presented in Table 1 with corresponding error bars. On the whole the porous silicon based samples (entries 3, 4, 7, 8) had more amounts of enzymes on them than crystalline silicon based samples (entries 1, 2, 5, 6) because of the enhanced surface area to accommodate more enzymes. However, we can see that the surface modified porous based samples (4 and 8) had more amounts of enzyme than unmodified samples (3 and 7). The unmodified porous silicon sample (3) had approximately 72 μg of enzyme, whereas the surface modified porous silicon samples (4) showed ~208 μg which is almost thrice as much as that in the unmodified. Surface modified silicon (2) had ~79 μg of enzyme which is almost four times more than unmodified silicon (1) which had only ~19 μg of enzyme. It was observed that the silicon nitride deposited samples (5, 6, 7, and 8) showed greater amount of enzymes than crystalline silicon based samples (1, 2, 3, and 4). The greater affinity of silicon nitride for enzymes can be due to its amine rich surface [26]. Unmodified silicon nitride deposited silicon (5) had ~30 μg of enzyme on its surface where its surface modified counterpart had ~90 μg. The surface porous silicon nitride based sample (8) also showed twice as much enzyme (~224 μg) than an unmodified sample (7), which had only ~100 μg of enzyme.

Table 1

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Material</th>
<th>Amount of enzyme (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unmodified silicon</td>
<td>78.66 ± 0.57</td>
</tr>
<tr>
<td>2</td>
<td>Surface modified silicon</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>Unmodified porous silicon</td>
<td>71.66 ± 0.57</td>
</tr>
<tr>
<td>4</td>
<td>Surface modified porous silicon</td>
<td>207.66 ± 0.57</td>
</tr>
<tr>
<td>5</td>
<td>Unmodified silicon nitride</td>
<td>30 ± 1</td>
</tr>
<tr>
<td></td>
<td>deposited silicon</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Surface modified silicon</td>
<td>89.66 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>nitride deposited silicon</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Unmodified silicon nitride</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td>deposited porous silicon</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Surface modified silicon</td>
<td>224.33 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>nitride deposited porous silicon</td>
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</table>

Fig. 5. (a) Blank porous silicon surface. (b) Blank silicon nitride deposited porous silicon surface. (c) Lipase immobilized porous silicon surface. (d) Lipase immobilized silicon nitride deposited porous silicon surface. (e) Lipase immobilized crystalline silicon surface. (f) Lipase immobilized silicon nitride deposited crystalline silicon surface.
4.3. Contact angle measurement

The contact angle of DI water on these samples was measured and is shown in Table 2. To begin with all the unmodified samples were hydrophobic however crystalline silicon (80°) and porous silicon samples (85°) were more hydrophobic than its silicon nitride deposited counterparts (60–65°). All the samples became hydrophilic in nature (10–25°) when treated with nitric acid due to the formation of –OH bonds. The silanized samples were found to be more hydrophobic (45–55°) as it displaced most of the –OH bonds with the reactive groups of silane [28]. The hydrophobicity of the silanized surface can also be ascribed to –NH2 bonds present on the silanized surface [26]. Glutaraldehyde activated samples did not show much change in the contact angle readings from the silanized samples as it did not contribute much to the hydrophilicity/hydrophobicity of the samples. The enzyme immobilized samples showed a slight reduction in the contact angles (40–50°) as the hydrophobic groups on the enzymes, like amines, would anchor itself to the aldehyde of the glutaraldehyde activated surface. This may expose the hydrophilic groups to the outside thus reducing the contact angle [27].

4.4. Capacitance–voltage (CV) measurements

Tributyrin hydrolyses, in the presence of lipase, to form butyric acid which causes a change in pH. The change in pH can be detected using an EISCAP which shows a shift in the measured C–V, with changes in the pH of the electrolyte as in Fig. 6. An EISCAP sensor for tributyrin detection, with silicon nitride as the insulator, has been reported earlier [9,11], where enzyme was allowed to adsorb itself on the sensor surfaces. In order to detect the activity of the immobilized enzymes on the samples the lipase immobilized silicon nitride deposited crystalline silicon sample and porous silicon samples were used as EISCAPs. The lipase immobilized silicon nitride surface will act as the insulator in the EISCAP structure. The samples were loaded on to the custom made teflon cell which can contain 2 ml of 5 mM tributyrin solution in 10 mM phosphate buffer (pH 7), to which 1 M KCl was added as an ionic strength adjuster. The contact is taken from a platinum wire dipped in the electrolyte and the semiconductor contact was taken by providing an aluminum base at the bottom of the cell as shown in Fig. 7. As a result of tributyrin hydrolysis the lipase immobilized silicon nitride samples (both crystalline and porous silicon based) showed a left shift in the depletion region over a period of time (Fig. 8a and b). The CV characteristics of an EISCAP critically depend on the charges at the semiconductor–insulator interface. Porous silicon with its much larger surface area has a very different interface with oxide as compared to crystalline silicon which has a much superior interface with oxide. Hence, although the insulator is same, the shape and where the depletion region occurs in the CV are very different for porous silicon based EISCAPs from that on crystalline silicon as seen in Fig. 8a and b. The measurements were taken at regular intervals till there is no more shift in the C–V. After a period of one hour the C–V characteristics did not show any considerable shift. The reduction in the shift in the C–V was due to the reduction in the hydrolysis reaction rate. The CV measurements showed a negative shift for both crystalline and porous silicon based samples as expected. Porous silicon based samples showed a higher negative shift than the crystalline silicon samples as they could contain more enzymes than the crystalline silicon based.

![Fig. 6. Shift in C–V curves due to change in pH of a solution.](image)

![Fig. 7. Experimental setup to characterize the sensor.](image)
samples The porous silicon based samples showed a shift of 59–62 mV, for a pH change from 6.98 to 4.81 whereas the crystalline silicon based samples showed a shift of 30–35 mV for a pH change of 6.97–5.54 after 60 min.

5. Conclusion

Lipase from *P. cepacia* was successfully immobilized on semiconducting materials like porous silicon, crystalline silicon, silicon nitride deposited porous silicon and crystalline silicon surfaces. The various steps of immobilization were monitored using FTIR spectrometry. FTIR studies on acid treated glutaraldehyde activated porous silicon confirmed the formation of Schiff’s base. The enzyme immobilized porous based samples showed a higher absorption peak at 1580 cm\(^{-1}\) due to the formation of imine bonds (Schiff’s base) during enzyme immobilization by displacing the aldehyde bonds at 1710 cm\(^{-1}\) of glutaraldehyde activated sample. The SEM images revealed the presence of immobilized enzymes on the surfaces. The amount of immobilized active enzymes on the surfaces was estimated using pNPP assay and it was found that the porous silicon based samples, due to its higher surface area, had more than twice the amount of protein on the surface than crystalline silicon based samples. The nitride deposited surfaces, in general, bound more enzymes than its silicon counterparts. The activity of the enzymes, immobilized on silicon nitride based samples, was confirmed by employing the samples as EISCAPs. The silicon nitride deposited porous silicon showed a greater negative shift than the silicon nitride deposited crystalline silicon as expected. Hence the potential of the lipase immobilized silicon nitride surfaces as EISCAP based triglyceride sensors was demonstrated.

Acknowledgements

This work was partially supported by IIT Madras through the IDRP project on MEMS. We also thank Departments of Science and Technology (DST), and Biotechnology (DBT), Government of India for funding this project. We are indebted to B. Senthil Kumar, Department of Biotechnology for many helpful discussions.

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