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Formation, topography and reactivity of *Candida antarctica* lipase B immobilized on silicon surface

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Abstract

*Candida antarctica* lipase B (CaLB) was immobilized on silicon wafers previously modified with aminopropyltriethoxysilane (APTES) and activated with glutaraldehyde (GLA). The various steps of immobilization were characterized using transmission FTIR, AFM, contact angle measurements and XPS. Furthermore, the formation of APTES films during the initial immobilization step was additionally analyzed by ellipsometry and an 'island' monolayer film formation was revealed. When the concentration of APTES was increased, the amount of immobilized lipase also increased. On the other hand, while the activity of immobilized enzyme in lipase-catalyzed transesterification of 6,8-difluoro-4-methylumbelliferyl octanoate initially increased, showing the highest value when 0.00050% w/v APTES solution was used for the initial immobilization step, it subsequently decreased. Comparison of enzyme activity and surface filling results indicate that there has to be multilayer formation in the enzyme layer, as revealed by AFM images and determination of enzyme loading.

Keywords: Enzyme immobilization, Candida antarctica lipase B, silanization, glutaraldehyde activation, silicon surface

Introduction

Enzymes have excellent features (activity, selectivity, specificity) for designing synthetic processes to obtain a wide range of products under mild and environmentally friendly conditions. However, most enzymes show none of their profound characteristics in organic solvents and can easily denature under industrial conditions (e.g. high temperature, solvent effect, mechanical shear). Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are generally difficult.

An important approach for improving enzyme performance in non-natural environments is to immobilize them. Enzyme immobilization involves the localization of an enzyme, i.e. restricting the enzyme's mobility. Many immobilized lipase catalysts have been developed and their performances reported (Gross et al. 2001; Castro & Knubovets 2003; Miletić et al. 2009a). Increased stability reflects the fact that an enzyme's activity will change as a function of its local environment (e.g. solvent polarity, surface chemistry).

Among lipases, *Candida antarctica* lipase B (CaLB) is one of the most commonly utilized biocatalysts because of its high degree of selectivity in a broad range of synthetic applications of industrial importance, including kinetic resolutions, aminolysis, esterification and transesterification (Gross et al. 2001; Castro & Knubovets 2003; Miletić et al. 2009a). By enzyme immobilization, catalysts can be developed with significant advantages relative to the free enzyme (Wang & Jin 2004; Laurell et al. 1996; Miletić et al. 2009b). Many literature reports describe the high utility of immobilized CaLB for chemical transformations of low-molar-mass compounds (Bhushan et al. 2005; Libertino et al. 2007) and polymerization reactions (Subramanian et al. 1999; Libertino et al. 2003a; Wu et al. 2004).

CaLB has mainly been immobilized into porous materials in order to prevent 'leaching out' of the enzyme from the matrix and the production of enzyme-contaminated products. However, this restricts gaining a better understanding on a molecular level of how immobilization on surfaces can stabilize and
activate protein catalysts. Enzyme immobilization on solid surfaces is a powerful tool for this purpose, as the surface topography of the immobilized enzyme can be evaluated and full characterization of the architecture down to the molecular level can be described.

Biological molecules can be anchored on various substrates using different methods (Subramanian et al. 1999; Libertino et al. 2003a,b, 2007; Wang & Jin 2004; Wu et al. 2004; Bhushan et al. 2005; Laurell et al. 1996) depending on the biological molecule requiring immobilization. The most frequently used approach is the formation of covalent bonds between the molecule and the solid surface, often using linker molecules (Subramanian et al. 1999; Libertino et al. 2003a, 2007; Wang & Jin 2004; Bhushan et al. 2005; Laurell et al. 1996). The immobilization procedure must be optimized to obtain the maximum surface coverage and to prevent denaturation of the biological molecule and/or the loss of its specific properties (e.g. for an enzyme, its enzymatic activity).

Enzyme immobilization on flat surfaces has been studied over decades by various groups (Subramanian et al. 1999; Libertino et al. 2007, 2008a; Fernandez et al. 2008). Subramanian et al. (1999) immobilized glucose oxidase on Si substrates by five different coupling procedures (covalent coupling through a metal link reagent or silane reagents containing pendant amino (APTES) or epoxide linkers, an entrapment technique using a thin layer of gelatin, or an adsorption technique using poly-L-lysine). It was concluded that amine treatment was the simplest of the covalent methods, but it caused irregularities on the Si surface that may lead to inconsistent silanization.

In the present study, CaLB was immobilized on Si surfaces using an aminopropyltriethoxysilane (APTES)–glutaraldehyde (GLA) modification approach. First, APTES dissolved in chloroform was used to silanize the Si wafers in order to generate surface amine groups; the amine groups were then activated into aldehyde groups with GLA; and finally enzyme was immobilized on these surfaces through Schiff’s base condensation between free amine groups on the enzyme and aldehyde groups on the modified surfaces. The modification of the surfaces after each step was confirmed by transmission FTIR measurements, contact angle measurements, AFM visualization and XPS. The APTES–GLA modification approach has been used previously for attachment of enzymes (Saal et al. 2002; Lee et al. 2003; Blasi et al. 2005; Fernandez et al. 2008; Libertino et al. 2008b). Libertino et al. (2008b) performed immobilization of glucose oxidase on SiO2 surfaces using the APTES–GLA modification approach. Using XPS measurements they showed a uniform layer after activation with GLA and full surface coverage after enzyme immobilization, due to the absence of uncovered surface regions. The same group performed immobilization of glucose oxidase on Si-based material. Every step of the APTES–GLA protocol was tested by AFM measurements on bulk SiO2 samples (Libertino et al. 2007). The various stages of covalent immobilization of Pseudomonas cepacia lipase on crystalline Si, porous Si and SiN surfaces by the APTES–GLA method were characterized using FTIR spectroscopy and formation of a Schiff’s base was confirmed (Fernandez et al. 2008).

However, the present report is the first one of CaLB immobilization on a Si substrate using APTES–GLA as the modifying agent. Detailed characterization of each immobilization step enabled full control of the immobilization protocol and optimization in order to obtain the maximum enzyme activity.

It has been already shown by Blasi et al. (2005) that the silanization process is the crucial step in the covalent immobilization of glutamate dehydrogenase on an activated Si/SiO2 support and that the properties of the subsequently immobilized enzyme are strictly determined by APTES film formation (Kim et al. 2009). The complexity of the APTES silanization reaction mainly originates from the presence of reactive amino groups in APTES and its inherent tendency to enter into competing reactions (Chiang et al. 1980). Therefore we chose the reaction conditions that will favor monolayer formation (chloroform as solvent and short reaction time) and minimize both the interaction of amino groups in APTES and silanols present on the Si surface and the polymerization of APTES that will give rise to multilayer formation (White & Tripp 2000; Loos et al. 2005; Kamisetty et al. 2006).

Formation of APTES films has been analyzed in comparison with the activity of the subsequently immobilized enzyme. Since the amount of immobilized enzyme is very low (up to 13.2 μg cm⁻²), a fluorescence activity assay based on the lipase-catalyzed transesterification of 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU octanoate) in acetonitrile to 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) (Weigel & Kellner 1989) was chosen to assess the enzyme activity on the Si surface.

Materials and methods

Chemicals

CaLB in the form of a dried powder was purchased from Codexis® (Jülich, Germany) (assay conditions: 2% v/v tributyrin, 2% v/v tricaprylin, 0.3% v/v poly(vinyl alcohol), emulsion in 10 mM potassium phosphate buffer, pH 7.0, 22°C. Acetonitrile (dry), 1-hexanol (≥99.9% pure), APTES (99% pure), GLA (50% w/w solution in water) and chloroform...
were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA) and were used without further purification. DiFMU octanoate and DiFMU were obtained from Molecular Probes® (Carlsbad, CA, USA). Single-side polished Si wafers were purchased from MEMC Electronic Materials, Inc. (St. Peters, MO, USA) and double-sided polished Si wafers from TOPSIL (Frederikssund, Denmark). Water was purified via a Millipore Milli-Q system (Billerica, MA, USA).

**Surface treatment and enzyme immobilization**

Si wafers were cut manually using a diamond-tip scribe, and the Si chips then cleaned sequentially with: (1) a mixture of hydrogen peroxide–ammonia–water (1:1:5 v/v, 60°C for 30 min); (2) a mixture of hydrochloric acid–water (1:6 v/v, ultrasonication for 15 min); (3) methanol (ultrasonication for 15 min); (4) a mixture of methanol–chloroform (3:1 v/v, ultrasonication for 15 min); (5) a mixture methanol–chloroform (1:3 v/v, ultrasonication for 15 min); and (6) chloroform (ultrasonication for 15 min). Cleaned Si chips were kept in chloroform until measurement.

The enzyme immobilization procedure consists of three steps: (1) silanization; (2) linker molecule deposition; and (3) enzyme coupling (Figure 1) (Subramanian et al. 1999; Blasi et al. 2005). In the first step the Si chips were immersed in a freshly prepared solution of APTES in chloroform at different concentrations (0.000010, 0.00050, 0.0010 and 1.0% w/v) and incubated at room temperature for 3 min. The silanized cover chips were then thoroughly rinsed with abundant chloroform in order to remove excess APTES, dried under N₂ and cured under vacuum at 110°C for 2 h.

The linker molecule deposition was carried out by placing silanized cover chips in 10.0% v/v GLA solution in phosphate buffer (pH 8.0) for 17 h at room temperature. Activated Si wafers were then meticulously rinsed with deionized water, kept in phosphate buffer for 30 min, rinsed again with deionized water and dried under N₂.

The aldehydic groups on the Si surface were coupled to free amino groups on the enzyme through Schiff’s base formation, by incubating the activated chips in CaLB solution, 2.0 mg mL⁻¹, in phosphate buffer (pH 8.0) overnight at room temperature. All samples were rinsed with deionized water and stored in phosphate buffer (pH 8.0) at 4°C until measured, which was always performed within 2 days of preparation. Just before measurement, samples were rinsed with deionized water in order to remove buffer residues and dried under a gentle N₂ flow.

Finally, the protocol steps were labeled as up-to-APTES (after the first step), up-to-GLA (after the second step) and up-to-CaLB (after the third step).

**Fourier transform infrared spectroscopy**

The transmission FTIR analysis of CaLB immobilized on double-sided polished Si wafer was done on a Bruker (Billerica, MA, USA) IFS66 V/S FTIR spectrometer equipped with a MIR DTGS detector. A sample shuttle accessory was used for interleaved sample and background scans. A clean Si substrate was used as the reference. Each spectrum is an average of 15 cycles of 120 scans. Due to the very low intensity of the signal, the spectrum measured at a resolution of 2 cm⁻¹ contained a lot of noise. Instead, a resolution of 3 cm⁻¹ was used. The areas under the amide I bands, that originate from the enzyme immobilized on the Si wafer, were calculated using a peak integration function of the OPUS 4.0 system software (Bruker).

In order to quantify the area under the amide I bands a calibration was performed. KBr pellets with known amount of enzyme, ranging from 0.50 to 7.0 mg, were prepared. Enzymes were homogeneously dispersed throughout the pellets. The transmission FTIR measurements of these pellets were carried out (using the same instrument and keeping the same measurement settings), obtaining the calibration curve (amount of enzyme versus area under the amide I band). Taking into consideration the fact...
that enzyme was immobilized on both sides, the amount of deposited enzyme per area of the Si surface could be determined.

**Contact angle measurements**

The contact angle measurements were carried out at room temperature using a custom-built microscope-goniometer system. A 1.25 μL drop of deionized water was placed on a freshly prepared Si wafer using a Hamilton micro-syringe and the contact angle was measured after 60 s. The measurements involve the fitting of a drop picture with home-built software. Six measurements were carried out in different regions on the surface.

**Atomic force microscopy**

AFM measurements were carried out on a Digital Instruments EnviroScope AFM (Plainview, NY, USA), equipped with a Nanoscope IIIa controller in tapping mode using Veeco RTESPW Si cantilevers ($f_0=240–296$ kHz and $k=20–80$ N m$^{-1}$ as specified by the manufacturer). Samples subjected to different steps of the immobilization protocol were characterized. For each sample, AFM scans were carried out on several surface positions in order to check the surface uniformity. Surface roughness ($R_q$) was evaluated after flattening the images (second order) to correct for image ‘bow’, which is due to non-linearity of the piezoelectric scanner, using the Nanoscope software. Surface roughness is defined as the root-mean-square average of height deviations taken from the mean data plane.

**X-ray photoelectron spectroscopy**

The samples were introduced through a load lock system into an SSX-100 (Surface Science Instruments, St Leonards-on-Sea, UK) Photoemission Spectrometer with a monochromatic Al Kα X-ray source ($h\nu = 1486.6$ eV). The base pressure in the spectrometer during the measurements was 10$^{-10}$ mbar. The photoelectron takeoff angle was 37°. The energy resolution was set to 1.3 eV to minimize measuring time.

**Ellipsometry**

The film thickness was measured with a VASE VB-400 spectroscopic ellipsometer (Lincoln, NE, USA). The wavelength of the laser beam was from 400 to 1000 nm and the angle of incidence was set from 74 to 76°. The incident laser beam covered an area of approximately 1 mm$^2$. A model based on three organic layers (Si, SiO$_2$ and APTES layer) with characteristic values for the refractive indices (outlet values) and thicknesses (thickness of the Si layer was granted as 1 mm, while the thickness of the SiO$_2$ layer was found to be 3.368 nm) was used to calculate the thickness of the APTES films.

**Fluorescence activity**

For the fluorescence activity assay, 50 μL of a solution of DiFMU octanoate in dry acetonitrile (1 mmol L$^{-1}$) was mixed with 50 μL of a solution of 1-hexanol in dry acetonitrile (1 mmol L$^{-1}$) and 2.9 mL of dry acetonitrile (Figure 2). The 1 mmol L$^{-1}$ stock solution of DiFMU octanoate in dry

![Figure 2. Hydrolysis of DiFMU octanoate with 1-hexanol in dry acetonitrile.](image-url)
acetonitrile could be stored for about 1 week in the freezer, whereas the activity assay starts to develop fluorescent activity by itself about 10–15 h after mixing. Hydrolysis of DiFMU octanoate started with addition of CaLB immobilized on a single-sided polished Si wafer (Loos et al. 2005). Released DiFMU is fluorescence active and thus alcohol formation can be followed in a fluorescence spectrometer. The assay reaction was carried out for 60 min at room temperature (20 °C) and subsequently an aliquot (50 μL) was taken, mixed with 200 μL of dry acetonitrile in the cuvette and the fluorescence spectrum was immediately recorded. Dry acetonitrile was used as the reference. The reference reaction was carried out without enzyme in the system and no fluorescence activity was observed. Enzyme fluorescence activity is defined herein as picomoles of DiFMU released in dry acetonitrile per unit time per surface area of silicon wafer (pmol DiFMU min⁻¹ cm⁻²) or per unit of weight of enzyme (pmol DiFMU min⁻¹ mg⁻¹).

Fluorescence measurements were conducted on an MDS SpectraMax M2 spectrometer (Sunnyvale, CA, USA).

Results and discussion

The Si surface is an ideal surface for CaLB immobilization, offering the possibility of covalent enzyme immobilization. Therefore, a series of Si wafers with various concentrations of APTES used for silanization (0.000010–1.0% w/v), but with identical concentrations of GLA used for activation (10.0% v/v) and concentrations of CaLB used for enzyme coupling (2.0 mg mL⁻¹), was prepared to assess how the APTES concentration affected surface topography, enzyme loading and enzymatic activity.

Aminopropyltriethoxysilane-modified silicon surfaces

In order to investigate the formation of APTES films using different silane concentrations, transmission FTIR measurements were performed. The FTIR spectra of all up-to-APTES films showed similar features between 3000 and 2800 cm⁻¹ which stem from several C–H stretching modes of the APTES backbone and ethoxy groups. The most important structural information regarding APTES films was found between 1800 and 1200 cm⁻¹. This part of the spectrum is dominated by peaks that mainly originate from amino groups (N–H bending vibration at 1570 cm⁻¹) and overlapping peaks that originate from alkyl groups and the methylene group adjacent to silicon (C–H scissor vibration and –Si–CH₂– bending vibration, respectively, at 1449 cm⁻¹) (Chiang et al. 1980; Weigel & Kellner 1989; Peña-Alonso et al. 2007). The intensity of these peaks is proportional to the amount of APTES deposited on the Si wafer, and decreases from 1 to 4 in Figure 3.

According to the previously reported literature and proposed mechanism of APTES film formation (Kim et al. 2009), it can be concluded in our case that an APTES monolayer was formed. Such a conclusion can be drawn since the intensities of the peaks in Figure 3 are quite low and the conclusion is further confirmed by the ellipsometric data below. Previous studies by AFM and SEM have observed that thinner APTES films are generally flat and homogeneous, but aggregates are found on thicker APTES films produced for extended reaction times (Vandenberg et al. 1991; Howarter & Youngblood 2006; Gu & Cheng 2008; Mo et al. 2008).

Table I shows the static water contact angles measured on films prepared after each immobilization step. Contact angle data indicated that the wettability of up-to-APTES films depends primarily on the number of APTES molecules attached to the Si surface. As expected, the surface becomes more hydrophobic as the number of APTES molecules increases (Kim et al. 2009). For up-to-APTES films prepared with different APTES concentrations in chloroform (from 0.000010 to 1.0% w/v), water contact angles ranged from 51.7° to 63.7° depending on the amount of APTES deposited on the Si wafer. This increase is due to the presence of the carbon backbone of APTES, which is more hydrophobic than silanol groups. As previously reported in the literature, the typical contact angles for samples that have undergone the APTES process, giving a uniform silanization on the surface, is between 75° and 80° (Mittal 1993). In our case, the contact angle approached these values (75–80°) as the APTES
Table I. Measured static water contact (°) for films prepared after each immobilization step.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angle (°)</th>
<th>Sample</th>
<th>Contact angle (°)</th>
<th>Sample</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference (Si wafer)</td>
<td>35.9 ± 1.5</td>
<td>Up-to-APTES (0.00010% w/v)</td>
<td>51.7 ± 1.3</td>
<td>Up-to-GLA (10.0% v/v)</td>
<td>26.9 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-APTES (0.00050% w/v)</td>
<td>52.8 ± 1.0</td>
<td>Up-to-GLA (10.0% v/v)</td>
<td>33.3 ± 3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-APTES (0.0010% w/v)</td>
<td>55.9 ± 1.4</td>
<td>Up-to-GLA (10.0% v/v)</td>
<td>40.4 ± 3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-APTES (1.0% w/v)</td>
<td>63.7 ± 3.2</td>
<td>Up-to-GLA (10.0% v/v)</td>
<td>61.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-GLA (10.0% v/v)</td>
<td></td>
<td>Up-to-CaLB (2.0 mg mL⁻¹)</td>
<td>48.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-GLA (10.0% v/v)</td>
<td></td>
<td>Up-to-GLA (2.0% v/v)</td>
<td>49.5 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-GLA (10.0% v/v)</td>
<td></td>
<td>Up-to-GLA (2.0 mg mL⁻¹)</td>
<td>53.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-to-GLA (2.0% v/v)</td>
<td></td>
<td>Up-to-GLA (2.0 mg mL⁻¹)</td>
<td>66.9 ± 1.5</td>
</tr>
</tbody>
</table>

aStandard deviation values were calculated from six replicate experiments.

Concentration increased, meaning that increasing the APTES concentration in chloroform used for the silanization step resulted in a more uniform silanization. Using a lower silane concentration, the contact angle was close to the value of the clean Si wafer (35.9°), indicating inconsistent silanization.

Comparing the XPS spectrum of the up-to-APTES film produced with the lowest amount of APTES (Figure 4A) with the XPS spectrum of the up-to-APTES film produced with the highest amount of APTES (Fig. 4C) showed that the film produced with the highest amount of APTES was thicker. This trend had already been observed with FTIR spectroscopy and can also be seen by the ellipsometry results below.

Ellipsometry was used to determine the thickness of the APTES films. The resulting data are represented in Table II. The mean thickness of the APTES layer of 9 ± 1 Å corresponds to the thickness of an APTES monolayer, assuming an extended conformation perpendicular to the surface (Petri et al. 1999; Arranz et al. 2008). Ellipsometry results indicated...
Characterization of CaLB immobilized on Si surface

that a uniform APTES monolayer is formed only in the case of the highest APTES concentration used (1.0% w/v). On the other hand, high standard deviation values, especially for the lowest APTES concentration (0.000010% w/v), revealed ‘island’ monolayer formation, which means that silanization was not complete and full coverage was not accomplished. Such high standard deviation values are the consequence of inconsistent silanization and the APTES layer thickness depending on the position of the ellipsometry incident laser beam. These results are in excellent agreement with contact angle measurements.

Table II. The layer thickness (Å) of APTES films prepared in chloroform for 3 min.

<table>
<thead>
<tr>
<th>APTES concentration (% w/v)</th>
<th>APTES layer thickness (Å)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000010</td>
<td>6.2 ± 3.2</td>
</tr>
<tr>
<td>0.00050</td>
<td>7.5 ± 2.1</td>
</tr>
<tr>
<td>0.0010</td>
<td>9.1 ± 1.6</td>
</tr>
<tr>
<td>1.0</td>
<td>9.9 ± 0.9</td>
</tr>
</tbody>
</table>

*Standard deviation values were calculated from three replicate experiments.

The characterization of an enzyme-functionalized surface requires a careful investigation of surface topography. Therefore, AFM experiments were carried out on Si surfaces after each immobilization step. Morphological inspection gives a qualitative picture of how the CaLB molecules are assembled on the Si surface, whereas mean roughness values \( R_q \) provide a quantitative parameter of surface structure. Therefore, initially AFM images were obtained of Si surfaces that had been derivatized with different silane concentrations.

AFM images shown in Figures 5 and 6 indicate that the silanization process did not cause significant surface modification, at least not modification visible by AFM. The mean roughness values for the samples silanized with different APTES concentrations (from 0.000010 to 1.0% w/v) ranged from 0.16 nm to 0.21 nm and were not significantly higher than the roughness of the untreated Si wafer (0.10 nm).

The next step in the immobilization procedure was the deposition of GLA molecules. Therefore, transmission FTIR measurements of typical up-to-GLA films, previously silanized with APTES, were
carried out. All up-to-APTES films were treated with the same GLA solution in phosphate buffer, pH 8.0 (10.0% v/v). Figure 7 shows the FTIR spectrum of up-to-GLA films subtracted from typical FTIR spectra of up-to-APTES films, in order to obtain the signals that originate from deposited GLA molecules. The spectrum is dominated by the peak that originates from the aldehyde group (C = O stretching vibration).

Figure 6. Three-dimensional AFM images: (a) Si surface; (b) up-to-APTES film (1.0% w/v); (c) up-to-GLA film (10.0% v/v); (d) up-to-CaLB film (2.0 mg mL⁻¹). The vertical scale is 5 nm while the horizontal scales are both 250 nm.

Figure 7. Transmission FTIR spectra of up-to-GLA films subtracted from typical FTIR spectra of up-to-APTES films. The concentration of GLA in phosphate buffer, pH 8.0 was constant (10.0% v/v), while the concentration of APTES in chloroform was varied: (1) 1.0% w/v; (2) 0.0010% w/v; (3) 0.00050% w/v; (4) 0.000010% w/v.

Figure 8. Transmission FTIR spectra of CaLB immobilized on Si wafers with various concentrations of APTES in chloroform used for silanization (with no change in other immobilization steps): (1) 1.0% w/v; (2) 0.0010% w/v; (3) 0.00050% w/v; (4) 0.000010% w/v.
Characterization of CaLB immobilized on Si surface

vibrations at 1716–1660 cm\(^{-1}\)). Increasing the concentration of APTES solution in chloroform used for silanization of Si wafers resulted in an increase in the amount of APTES attached to the surface (Fig. 3), as well as an increase in the amount of GLA deposited on the already deposited APTES layer (Fig. 7).

Increasing the concentration of APTES solution used for silanization resulted in an increase of the contact angle of subsequently prepared up-to-GLA films (Table I). However, it has been reported that there is no significant difference between the contact angles before (up-to-APTES) and after activation with GLA (up-to-GLA) (Lee et al. 2003; Diao et al. 2005; Fernandez et al. 2008). We observed identical behavior only if the concentration of APTES solution in chloroform was high enough to create uniform silanization on the surface (1.0% w/v). If the concentration was too low for uniform silanization (from 0.000010 to 0.0010% w/v), GLA treatment of up-to-APTES films resulted in a large decrease in contact angle, approaching the contact angle of the clean Si wafer.

Glutaraldehyde-modified silicon surfaces

The activation of silanized wafers with GLA also did not change the surface topography significantly, although sporadic peaks up to 1.7 nm height and up to 21.8 nm in diameter appeared (Figures 5c and 6c). Moreover, the mean roughness value increased after GLA activation.

In order to prove the presence and to determine the amount of immobilized enzyme on the Si wafers, transmission FTIR measurements were performed. The area under the amide I band (centered at 1656 cm\(^{-1}\)), that originates from the C = O stretching vibration of the amide group coupled to the bending of the N–H bond and the stretching of the C–N bond, was followed. Figure 8 shows the FTIR spectrum of up-to-CaLB films subtracted from typical FTIR spectra of up-to-APTES and up-to-GLA films, in order to obtain the signals that originate from deposited enzyme molecules.

The areas under the amide I band (Figure 8) were quantified, performing the calibration with a known amount of CaLB in KBr pellets (Figure 9). Dispersing the CaLB homogeneously throughout the pellet is quite difficult. Nevertheless, three pellets were prepared for FTIR measurements and the relative standard error never exceeded 5%, providing a reliable calibration curve for quantification of the areas under the amide I band in Figure 8. Increasing the concentration of APTES used for silanization resulted in an increase in the amount of CaLB deposited on Si. The initial increase in APTES concentration used for silanization resulted in a significant increase in enzyme loading. Further increase in the APTES concentration resulted in an increase of enzyme loading, but in a more moderate manner. For instance, enzyme loading increased from 1.74 to 3.40, 5.05 and 13.2 μg cm\(^{-2}\) as the APTES concentration increased from 0.000010 to 0.00050, 0.0010 and 1.0% w/v. As previously mentioned, Subramanian et al. (1999) investigated enzyme immobilization on Si substrates by five different coupling procedures: (1) covalent coupling through a metal link reagent; (2) covalent coupling through APTES; (3) covalent coupling through an epoxide linker; (4) an entrapment technique using a thin layer of gelatin; and (5) an adsorption technique using poly-L-lysine. All of the covalent coupling procedures led to surface loadings approaching 1 pmol mm\(^{-2}\) (Subramanian et al. 1999). In our case a similar enzyme loading was obtained using 0.00050% w/v of APTES for silanization (1.02 pmol cm\(^{-2}\)), while four times greater loading was achieved if 1.0% w/v of APTES was used (3.97 pmol cm\(^{-2}\)).

Assuming that during immobilization the molecules of enzyme mainly keep their natural conformation (planar dimensions), 13.2 μg of CaLB is much higher than the theoretical maximum possible monomolecular load of enzyme molecules on this 1 cm\(^2\) surface of Si wafer, which is 0.46 μg cm\(^{-2}\) calculated on the basis of the average spherical diameter of CaLB (39.15 Å) (Dumitriu et al. 2003). Therefore, it is proposed that there must be multiple layers of the enzyme, with at least some part of the surface covered with enzymes having no catalytic activity (see Figures 5 and 6) (Saal et al. 2002).
Comparing the XPS spectrum of the up-to-APTES film with what was produced with the lowest amount of APTES (0.000010% w/v; Figure 4A) and the highest amount of APTES (1.0% w/v; Figure 4C) with the corresponding CaLB films (Figures 4B and 4D, respectively) also shows the dramatic increase in immobilized CaLB on the film that was produced with the highest amount of APTES in comparison to the film that was produced with the lowest amount of APTES.

As shown in Table 1, as the concentration of APTES used for silanization increased, the hydrophobicity of the up-to-CaLB films increased. As already published, lipase immobilization on the surface previously activated with GLA has no significant effect on the contact angle (Fernandez et al. 2008). In our case, as the concentration of APTES solution increased, the difference in contact angle between up-to-GLA and up-to-CaLB decreased, meaning that a higher APTES concentration resulted in more uniform silanization on the surface. Inconsistent silanization and hydrophobic groups on the enzyme, such as alanine-rich hydrophobic surface regions (Lutz 2004), explain the increased hydrophobicity.

**C. antarctica lipase B-modified silicon surfaces**

Immobilization of CaLB on the activated Si wafers revealed large irregularities on the surface. Irregular peaks were higher and wider, as the concentration of APTES solution used for silanization step decreased. For instance, using 0.000010% w/v APTES solution in chloroform resulted in an appearance of sporadic peaks of 4.2 nm height and 33.9 nm diameter (Figure 5), while 1.0% w/v solution resulted in sporadic peaks of 2.5 nm height and 30.0 nm diameter (Figure 6). This trend is more obvious in the profile images of the Si surfaces as a function of APTES concentration after enzyme immobilization, obtained by AFM (Figure 10).

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**Figure 10.** Profile images of the Si surface after enzyme immobilization (up-to-CaLB) as a function of APTES concentration: (a) 0.000010% w/v; (b) 0.00050% w/v; (c) 0.0010% w/v; (d) 1.0% w/v.
These results are in excellent agreement with contact angle measurements and support the conclusion that the surface is not uniformly silanized. Furthermore, inconsistent silanization is more obvious with lower APTES concentrations used for the silanization step.

The quantitative observations of the surface changes, expressed as mean roughness values, following the three steps of the immobilization protocol are given in Table III.

The AFM data are useful for the characterization of the morphology and roughness of each sample, but taken alone they present only a narrow picture of the actual character of the APTES films.

**Fluorescence enzyme activity**

The activity of CaLB immobilized on Si wafers with different concentrations of APTES (0.000010, 0.00050, 0.0010 and 1.0% w/v) used for silanization, with no change in other immobilization steps, was assessed by hydrolysis of DiFMU octanoate with 1-hexanol in dry acetonitrile at room temperature. Formation of DiFMU was followed with a fluorescence spectrometer, integrating the area between 400 and 530 nm. Enzyme fluorescence activity depended strongly on the APTES concentration used for silanization (Figure 11). An increase in APTES concentration caused an increase of enzyme activity (Figure 12).

Initially, increasing the silane concentration resulted in a significant increase of the enzyme fluorescence activity. For instance, the enzyme activity increased from 0.311 to 1.424 pmol DiFMU min$^{-1}$ cm$^{-2}$ as the silane concentration increased from 0.000010 to 0.0010% w/v. After such a significant

<table>
<thead>
<tr>
<th>APTES concentration (% w/v)</th>
<th>Mean $R_q$ (nm)</th>
<th>Si wafer</th>
<th>Up-to-APTES</th>
<th>Up-to-GLA</th>
<th>Up-to-CaLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000010</td>
<td>0.1</td>
<td>0.21</td>
<td>0.27</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
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<td>0.19</td>
<td>0.47</td>
<td>0.44</td>
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<td></td>
</tr>
<tr>
<td>0.0010</td>
<td>0.16</td>
<td>0.31</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.18</td>
<td>0.17</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 11. Determination of the activity of CaLB immobilized on Si surface with various concentrations of APTES in chloroform used for silanization (with no change in other immobilization steps): (1) 1.0% w/v; (2) 0.0010% w/v; (3) 0.00050% w/v; (4) 0.000010% w/v. Standard deviation values were calculated from three replicate experiments.

Figure 12. Fluorescence enzyme activity (expressed as pmol DiFMU min$^{-1}$ cm$^{-2}$) versus APTES concentration (semi-log diagram). Standard deviation values were calculated from three replicate experiments.

Figure 13. Fluorescence enzyme activity (expressed as pmol DiFMU min$^{-1}$ mg$^{-1}$) versus APTES concentration (semi-log diagram). Standard deviation values were calculated from three replicate experiments.
increase in enzyme activity, further increasing the silane concentration had almost no effect on enzyme activity. Saal et al. (2002) showed that glucose oxidase immobilized on mica carriers exhibited similar behavior.

Enzyme fluorescence activity expressed per mass of protein gave a different curve shape (Figure 13). Namely, as the APTES concentration used for Si wafer silanization increased, the fluorescence enzyme activity initially increased, showing a maximum for 0.001% w/v, and subsequently decreased. These results confirm that multilayer structures of the enzyme must be present, with some fractions of deposited enzyme molecules being inaccessible to the substrate and having no catalytic activity (Blasi et al. 2005). Substrate molecules typically reach only the enzyme available at the surface, while the buried enzyme is inaccessible.

**Conclusions**

CaLB was successfully immobilized on Si surfaces using the APTES–GTA modification approach.

It was shown that the initial silanization process is the most critical, since it subsequently determines the properties of the final enzyme preparations. If an excess of APTES is used, the subsequent immobilization step (activation with GTA) simply determines the quality of the immobilized enzyme and does not significantly change the immobilization protocol. Enzyme loading and enzyme activity are mostly influenced by the structure and architecture of the APTES films that can be controlled by choosing the reaction conditions (reaction time, solvent, temperature, etc.). Increasing the APTES concentration resulted in an increase in the amount of enzyme deposited on the silicon surface. On the other hand, an increase in the amount of enzyme immobilized did not always result in increased enzyme activity, since multiple enzyme layers were formed, and enzyme in the sub-layers could not contribute to catalytic activity.

Therefore, silanization reaction conditions that favor the formation of a uniform, full-coverage APTES monolayer have to be selected in order to obtain high enzyme activity (short reaction time, chloroform as solvent, certain APTES concentrations, etc.).

Both ‘island’ monolayer film formation and multilayer APTES formation (polymerization of APTES) should be avoided (long reaction time, toluene- or water-containing solutions as solvent, high APTES concentration, etc.), since these APTES formations give rise to the enzyme multilayer structure.

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**References**


