Journal of Colloid and Interface Science 345 (2010) 448–453

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Effect of oxidation level of n^+-type mesoporous silicon surface on the adsorption and the catalytic activity of *Candida rugosa* lipase

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ARTICLE INFO

Article history:
Received 2 November 2009
Accepted 22 January 2010
Available online 28 January 2010

Keywords:
Mesoporous silicon
Surface
Oxidation level
Lipase
Adsorption
Biosensor

ABSTRACT

In this work, we present the synthesis and characterization of n^+-type porous silicon (PSi) layers. Our final aim is the fabrication of a biosensor that exploits the semiconductive properties of this material. PSi wafers were used as a matrix for enzyme adsorption. These wafers, as a result of their porous nanostructure, had a high surface area (360 m^2/g) and pore size in the range 5–20 nm. The freshly prepared PSi was stabilized through controlled anodic oxidation. Two classes of samples differing for the level of oxidation were prepared. The first class was oxidized up to 2 V (LO-PSi), whereas the second class was oxidized up to 10 V (HO-PSi). Both samples were used for the adsorption of *Candida rugosa* lipase. A significantly higher loading was ascertained for LO-PSi (140 mg/g) compared to HO-PSi (47 mg/g). The different hydrophobic–hydrophilic balance of the PSi surfaces induced by the different oxidation voltage affects the physical interactions that address the adsorption process of the lipase. The higher loading achieved with the LO-PSi resulted in a higher activity of the immobilized biocatalyst but in a lower catalytic efficiency. The two biocatalysts showed an acceptable stability toward storage (pH 5 buffer solution at 5 °C) within 2 weeks.

1. Introduction

Porous silicon (PSi), discovered by Uhlir in 1956 [1], is currently finding interesting applications as transducers for biosensing devices [2]. PSi forms as a result of electrochemical etching of crystalline silicon in aqueous HF solution. In the field of biosensors, porous silicon shows great potentialities. The very large specific area and the possibility of choosing pores sizes from a few nm to more than 100 nm [3] represent peculiar characteristics. Consequently, the adsorption of a large amount of biomolecules onto small volumes can easily be performed, and the fabrication of small devices such as biosensors may become a valuable development. This becomes particularly interesting if PSi semiconductive properties can also be exploited [4].

The use of PSi as a matrix for biosensors allows for two different types of signal transductions [2]. Optical transduction through either Fabry–Perot fringes or microcavity resonators [4,5] has widely been investigated. More recently, also electrochemical transduction has been proposed [6,7]. In this context we recently reported a work where PSi was used for the realization of potentiometric biosensors for triglycerides quantification [8]. A PSi sample loaded with a lipase physically immobilized inside the mesopores constituted the anode of an electrochemical cell. The hydrolysis of an aqueous emulsion of tributyrin—a short chain triglyceride—was used to ascertain the activity of the immobilized lipase. The butyric acid formed as a result of the biocatalyst action produced a pH change that was quantified measuring the variation of the open circuit potential at the electrodes of the electrochemical cell. This type of biosensor is in principle reusable, thus allowing a significant cost reduction while maintaining high detection efficiency.

A recent review by Kilian et al. [9] pointed out the importance of the surface chemistry of PSi. Besides the already noted surface area and pore size, chemical modifications at the air–solid interfaces strongly affect the biosensing performance [10]. Indeed freshly prepared PSi is unstable due to the presence of highly reactive silicon hydride (Si–H_x, $x = 1$, 2, and 3) species that are very reactive both in air and in water [11]. Thus, in order to use PSi as a matrix for a biosensing device, a stable surface must be obtained. Several procedures addressed for this purpose have been reported. The most common is to grow an oxide layer on the PSi surface—to avoid spontaneous oxidation either in air or in water media—through a thermal [6] or a chemical (ozone) [10] treatment. Oxidation treatment can be followed by a silanization step to introduce suitable functional groups [9]. The biomolecule needed for biosensing can be adsorbed after either the oxidation (physical adsorption) or the silanization (chemical adsorption) steps. Whatever the kind of interaction between the PSi and the biomolecule, the

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doi:10.1016/j.jcis.2010.01.063

oxidation step is that on the basis of which the functioning of the biosensor depends. The formation of the SiO_2 layer during thermal or ozone oxidation cannot be controlled; therefore, it is not possible to obtain a reproducible surface in different PSi wafer sample preparations. Also, reproducibility, clearly, is a crucial step in the fabrication of a biosensor.

In the present work, PSi layers were oxidized through an electrochemical procedure referred to as "anodic oxidation" [12,13]. Different from other oxidation techniques, it is realized through a controlled procedure where a defined current intensity is made to flow into an electrochemical cell until an established oxidizing potential is reached. Through this procedure, PSi layers were oxidized at two different levels, i.e., "high oxidation level" (HO-PSi) and "low oxidation level" (LO-PSi) samples. *Candida rugosa* lipase (CRL) was then adsorbed on the oxidized PSi layers. The effect of the oxidation levels both on the loading and on the activity of the immobilized lipase was studied. The stability, in terms of reuse, of the immobilized lipase toward storage was finally investigated.

2. Experimental

2.1. Chemicals

Crystalline silicon wafers (n^+ doped with phosphorus) were purchased from Siltronix (Archamps, France). The chemicals used in the present work, their degree of purity, and the purchasing company are listed in Table 1.

2.2. Formation of PSi and anodic oxidation

Mesoporous silicon was fabricated by electrochemical etching in the dark of n^+-type crystalline (resistivity in the 3–$7 \times 10^{-3}\ \Omega$ cm range) bulk crystalline (1 0 0) Si wafers using a 15% HF hydro-alcoholic solution and a platinum cathode and a current density of 50 mA/cm^2 for 960 s. After the formation of the sample, the PSi was washed for 10 min under a continuous flow of distilled water. Anodic oxidation was carried out by flowing a current intensity of 9 mA in an electrochemical cell having a PSi sample as the anode and a platinum electrode as the cathode in contact with a 0.1 M KNO_3 solution. According to the required level of oxidation, the process was interrupted either at 2 V (low oxidation level PSi) or at 10 V (high oxidation level PSi). Finally the PSi samples were washed with distilled water and dried under an air flow. At least five different samples at each oxidation level were prepared and examined for biological tests (lipase adsorption and activity measurements).

Table 1
List of chemicals.

Materials and chemicals	Degree of purity (%)	Company
Fluoridric acid	15	Carlo Erba reagents
Potassium nitrate	99	Aldrich
Lipase from *Candida rugosa* (Amano AY)	–	Amano Enzymes
Bradford reagent	–	Sigma
Bovine serum albumin	98	Sigma
Gum arabic	–	Sigma
Glycerol	99	Sigma
Potassium dihydrogen phosphate	98	Sigma
Disodium hydrogen phosphate	98	Sigma
Sodium dihydrogen phosphate	99	Sigma
Sodium acetate	98	Sigma
Acetic acid	99–100	J.T. Baker
Ethanol	99.9	J.T. Baker
Tributyrin	⩾98	Fluka
Sodium hydroxide solution (10 mM)	–	Merck
Sodium chloride	99	Merck

2.3. Characterization of PSi

After the formation, the PSi layer was separated from the crystalline matrix by applying a high current intensity into the cell for few seconds. Textural analysis was carried out on a Thermoquest–Sorptomatic 1990, by determining the N_2 adsorption/desorption isotherms at 77 K. Before analysis, porous silicon was reduced in fragments and heated up to 250 °C at a rate of 1 °C min^{-1} under vacuum. The specific surface area and the pore size distribution were assessed by the BET [14] and DH [15] methods, respectively. Field effect gun scanning electron microscopy (SEM-FEG) studies were carried out by using a Zeiss S Ultra 55 microscope located at the CMTC-INP laboratory at the Institut National Polytechnique de Grenoble (France).

2.4. Adsorption of C. rugosa lipase on PSi

The n^+-type PSi layers were dipped into 5 mL of a *C. rugosa* lipase solution (0.5–30 mg/mL) in acetate buffer (20 mM; pH 5). The system was kept under a gentle continuous rotation to ensure an optimal diffusion of the solution into the layer's pores at a fixed temperature of 25 °C, for 24 h. All residual enzyme molecules that were not adsorbed into the PSi pores were removed by rinsing the sample with the phosphate buffer solution until no enzymatic activity was detected in the rinsing solution. A lipase solution of 3 mg/mL was used to study the effect of pH of immobilization on enzyme loading onto the PSi surface (20 mM acetate buffer for pH 4.0, 5.0, and 5.5 and 20 mM phosphate buffer for pH 6.0, 6.5, 7.0, 7.5, and 8.0). The PSi slab was then removed and washed with the same buffer used for the immobilization. The enzyme loading L ($mg_{protein}/g_{support}$) was calculated according the relation

$$L = \frac{E_i - E_r - E_w}{m_s}, \tag{1}$$

where E_i is the amount of enzyme in the initial solution; E_r is the residual amount of enzyme at the equilibrium in the solution; E_w is the amount of enzyme in the washing solution; m_s is the mass of support (g).

2.5. Determination of activity of lipase immobilized onto PSi

The activity of the lipase immobilized onto PSi was measured by the pH-stat method using 718 Stat Titrino equipment from Metrohm (Herisau, Switzerland). A sample of PSi carrying the adsorbed lipase was immersed into a gum Arabic-stabilized emulsion of tributyrin in distilled water at 25 °C. The value of pH 7.0 was continuously reestablished by titration with 10 mM sodium hydroxide solution. The substrate emulsion was prepared by homogenizing a mixture of tributyrin (3 mL), distilled water (47 mL), and an emulsifier prepared according to Ref. [16]. The catalytic activity of the immobilized lipases was expressed in terms of LU (lipase units) where 1 LU = 1 μmol of butyric acid per minute.

2.6. Statistical analysis

All data are presented as mean ± standard deviation (SD). Data were analyzed using GraphPad Prism software. Statistical significance of differences between data was evaluated by unpaired t test at a 95% confidence level ($P \leqslant 0.05$).

3. Results and discussion

3.1. Synthesis, characterization, and oxidation of PSi layers

Fig. 1a shows a real dimension picture of a PSi slab. After formation, PSi layers were characterized by means of SEM-FEG. Fig. 1b and c show the SEM images of a front view and of a cross section of the PSi surface, respectively. The SEM characterization reveals a columnar mesoporous layer with pores oriented perpendicularly to the surface, and having a thickness around 600 nm.

The lipase from *C. rugosa* (CRL) was immobilized onto PSi. This enzyme is a globular protein with a molecular weight of 64 kDa and a diameter of about 5 nm [17,18]. The pores size of the PSi matrix must be larger than the diameter of the enzyme molecules in order to use a high fraction of the surface area for the enzyme immobilization. Only if this condition is satisfied enzyme molecules can penetrate the PSi pores during the immobilization process. According to this fact, Tinsley-Bown et al. found that PSi layers having pores larger than 50 nm were needed to realize a α-rabbit IgG conjugated to horseradish peroxidise antibody-based biosensor [3]. For this reason, the material was also characterized by means of N_2 adsorption techniques. Fig. 1d reports the hysteresis cycle of N_2 adsorption/desorption onto PSi surface. The isotherm, classified as type IV by IUPAC, is typical of mesoporous materials (diameter range 2–50 nm). The application of the BET method [14] to adsorption/desorption data allows to calculate the surface area of the material (361 m^2/g). The application of the DH method [15] gives the pore diameter distribution. Fig. 1e shows the bimodal distribution given by a narrow peak at about 3.7 nm, and a broad one ranging from 5 to 20 nm, with a maximum at about 10 nm. As a consequence, enzyme molecules with their size around 5 nm are expected to penetrate PSi pores to a large extent.

As noted previously, freshly prepared PSi layers are chemically unstable because of the presence of a highly reactive Si–H surface groups [9,10]. Canham and co-workers found that freshly prepared PSi layers dissolve when immersed into solutions having a physiological pH [11]. A way to increase the stability of the PSi surface is to perform an oxidation through a thermal or chemical (ozone) treatment [9]. The oxidation process transforms the hydrophobic surface of Si into hydrophilic SiO_2, thus strengthening the PSi resistance to dissolution. Thermal oxidation can hardly be controlled,

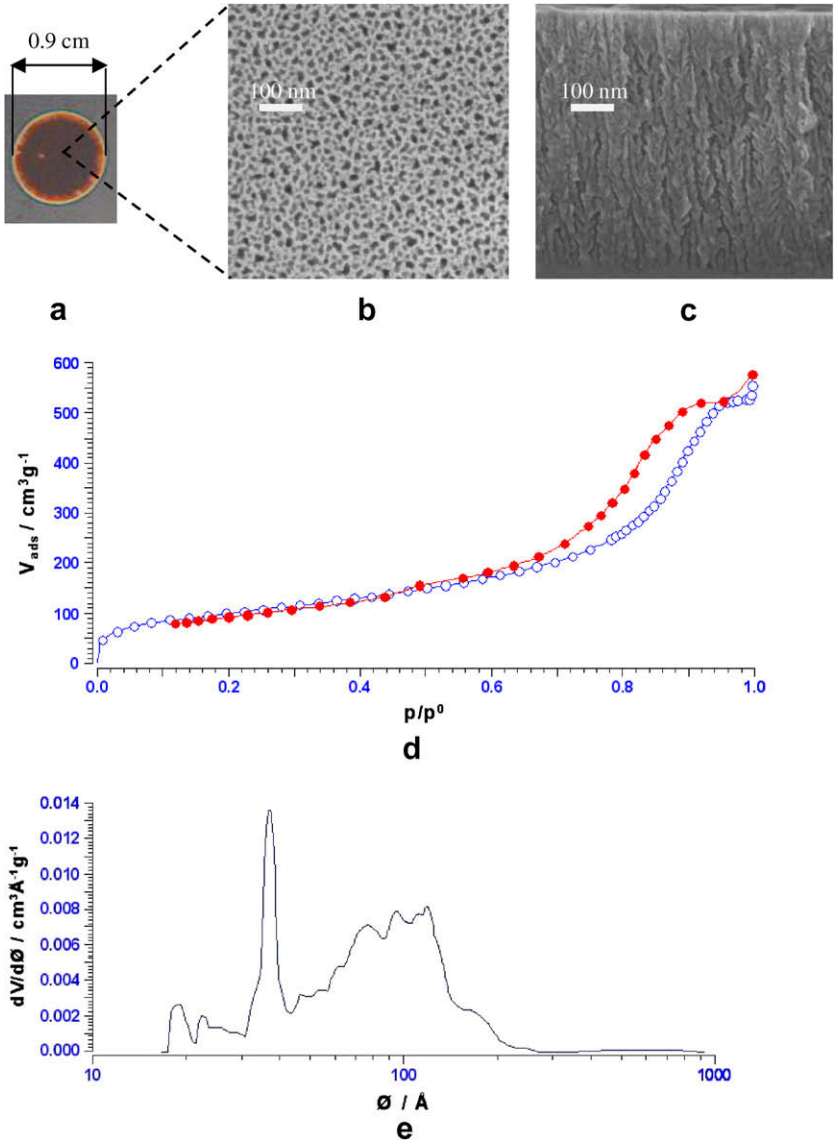

Fig. 1. Characterization of n^+-type PSi: real dimension picture (a). SEM image of a front view of the porous layers (b); SEM image of a cross section of the porous layers (c). N_2 adsorption (○) desorption (●) isotherms at 77 K (d). Pore size distribution (e).

whereas, as an alternative to thermal methods, anodic oxidation, using a KNO_3 0.1 M solution, allows control of the oxidation process, thus leading to highly reproducible samples [12,13]. This fact is important since, as reported below, the level of oxidation affects both the immobilization and the activity of the enzyme. The oxidation process is obtained by imposing a fixed current into the cell containing a 0.1 M KNO_3 solution and by following the potential increase with time. The threshold time is thickness dependent, but becomes a constant parameter for all samples with fixed thickness and fixed formation procedures.

This implies that the applied voltage is the main parameter in the choice of the level of oxidation. Fig. 2 shows the anodic oxidation process of the two series of samples that were ended at 2 V (about 13 min) and 10 V (about 35 min), respectively. This was done to obtain two types of differently oxidized surfaces in order to study the effect of the oxidation level on the adsorption of the lipase. The samples oxidized up to 10 V were named HO-PSi (high oxidation level porous silicon), whereas those oxidized up to 2 V were named LO-PSi (low oxidation level porous silicon).

3.2. Effect of pH on enzyme loading and activity

In our previous work the enzyme was immobilized from a buffer solution at a fixed pH [8]. In fact, the pH is an important parameter that affects both the enzyme loading and the activity. For this reason, the effect of pH on the immobilization process of CRL on HO-PSi was first studied. HO-PSi, as a result of its higher oxidation level and thus higher hydrophilic character, is expected to show a stronger dependence on pH also. Fig. 3a shows that the loading increases in the range of pH between 4 and 6, it remains constant between 6 and 7, and then it decreases at pH 7.5. The experimental trend depends on the surface properties of both the enzyme and the adsorbing material. Indeed the HO-PSi has a very hydrophilic surface due to the formation of a larger surface layer of SiO_2 during the anodic oxidation of the surface silicon atoms. Thus, the surface layer of HO-PSi should carry a different surface charge, depending on the pH of the immobilizing solution [19]. The sign of the surface charge will depend on the zero charge point of the material that is, unfortunately, unknown. Analogously, the surface charge of the protein will depend on the isoelectric point (p*I*), that for *C. rugosa* lipase is 4.3. At pH < p*I* the protein will be positively charged whereas at pH > p*I* it will be negatively charged. Assuming that the main driving force for the immobilizing process is the electrostatic interaction between the enzyme macromolecules and the PSi surface, we would expect that the maximal loading is reached when the interacting surfaces carry electric charges of different sign [19,20]. Thus, with a p*I* of the lipase 4.3 and the maximum of the loading reached in the range of pH 6–7 – when the protein carries a negative charge – a zero charge point of the material around 7 might be suggested. This is confirmed by the fact that at pH > 7 the loading decreases probably because of a repulsive electrostatic interaction due to the presence of negative charges both on the PSi surface and on the CRL enzyme.

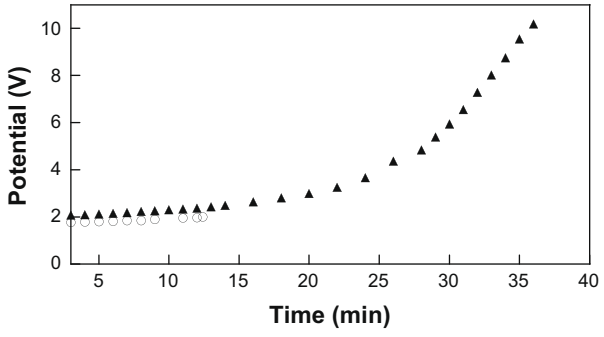

Fig. 2. Anodic oxidation curves for n^+-type PSi samples: sample HO-PSi (▲) and sample LO-PSi (○).

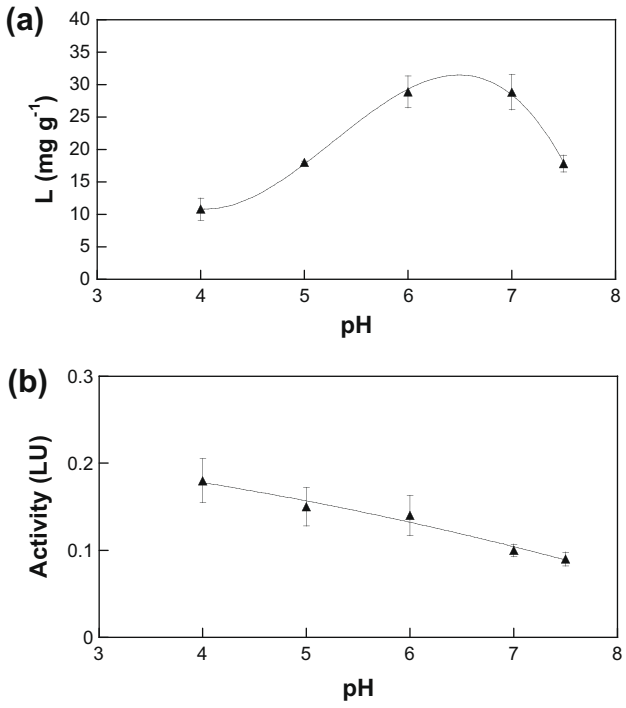

Fig. 3. (a) Effect of pH on the immobilization of *Candida rugosa* lipase onto HO-PSi. (b) Activity of the immobilized lipase as a function of pH of immobilization (HO-PSi sample).

The catalytic activity of the immobilized lipase at different pH values, measured through the hydrolysis of tributyrin assay, was then determined (Fig. 3b). In principle one should expect that the higher the loading the higher the activity of the immobilized preparation. In addition, it should be remarked that the pH at which the enzyme is immobilized has a strong influence on the catalytic activity. The balance between these two effects must be responsible for the experimental trend shown in Fig. 3b. The sample with the highest activity is that immobilized at pH 4. With increasing the immobilizing pH, the resulting biocatalysts show a linearly decreasing catalytic activity. Hence, pH affects both loading and activity giving a maximal loading at pH 6–7 and a maximal activity at pH 4. Thus, in order to obtain the best compromise between these two exigencies, the immobilization at an intermediate pH value (pH 5) was used.

3.3. Adsorption isotherms of C. rugosa lipase onto PSi layers

The adsorption isotherms (298 K, pH 5) of CRL on PSi layers having different levels of oxidation were then determined as shown in Fig. 4.

The curves reported in Fig. 4 were fitted through the Langmuir model [21]

$$L = \frac{L_{\max} C_E}{K_L + C_E}, \tag{2}$$

where L is the actual loading; $L_{\max}$ is the maximal loading; C_E is the enzyme concentration in the liquid phase at the adsorption equilibrium; and K_L (Langmuir constant) is the concentration of the free enzyme at half saturation of support. By fitting the experimental points, the maximal loading and the Langmuir constant for the

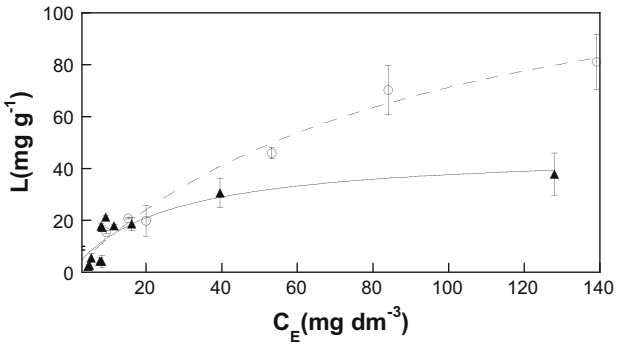

Fig. 4. Adsorption isotherms (loading versus residual protein concentration) for HO-PSi sample (▲) and LO-PSi sample (○). Adsorption experiments were carried out at 25 °C.

two PSi material were determined. LO-PSi achieved $L_{max} = 140$ $mg_{protein}/g_{support}$ and $K_L = 1920$ mg/dm^3 whereas HO-PSi achieved $L_{max} = 47$ $mg_{protein}/g_{support}$ and $K_L = 496$ mg/dm^3.

These results point out that LO-PSi is more efficient than HO-PSi toward CRL immobilization. This may be explained by hypothesizing different kinds of interactions between the enzyme and the two types of oxidized PSi surfaces. Indeed, the adsorption of CRL on the highly hydrophilic HO-PSi surface is likely due to ionic and dipolar interactions. Conversely, for LO-PSi a more hydrophobic surface is expected. Indeed, it is known from structural studies that *C. rugosa* lipase has a high hydrophobic surface in proximity of the active site that, in water solution, is buried by a lid made of an amphiphilic amino acid chain [17]. The presence of a hydrophobic interface, as that of LO-PSi, induces a substantial conformational change of the lipase architecture (lid opening) that leads to the adsorption of the lipase at the interface through its hydrophobic surface. On the other hand, in the case of HO-PSi electrostatic interactions, usually stronger than hydrophobic interactions, are always mediated by hydration molecules and can also be decreased by counterion binding in buffer solutions. The consequence is that a higher loading occurs onto the more hydrophobic surface of LO-PSi.

3.4. Catalytic performance of the lipase immobilized on PSi

The PSi samples with the immobilized lipase prepared for the study of the adsorption isotherm were tested for biological activity using the hydrolysis of tributyrin as biocatalytic assay. A comparison of the enzymatic activity of the lipase immobilized on the HO-PSi and LO-PSi slabs as a function of enzyme loading is reported in Fig. 5a. Both curves show a similar trend; i.e., enzymatic activity increases as the loading increases until a plateau is reached. The maximal activity of the lipase immobilized on the LO-PSi is higher than that immobilized on HO-PSi. This result agrees with the adsorption isotherms that showed a higher loading for the former matrix compared to the latter.

The optimal loading can be determined if the catalytic efficiency—defined as activity/loading (LU × g/mg)—versus loading is reported (Fig. 5b) [22]. For the lipase immobilized on HO-PSi the maximum of catalytic efficiency is reached at 2.57 mg/g, whereas for that immobilized on the LO-PSi the maximum is achieved at 15.8 mg/g. The maximal catalytic efficiency is obtained at a value of loading where all immobilized enzyme macromolecules are working at the best of their performance. Higher loadings lead to a decrease of catalytic efficiency since reagent and product diffusion becomes the limiting rate-determining step [16]. Fig. 5b shows that the maximal catalytic efficiency is higher for HO-PSi than for LO-PSi. Hence, although LO-PSi allows reaching high loadings of CRL, the resulting biocatalysts are less efficient than those obtained with HO-PSi.

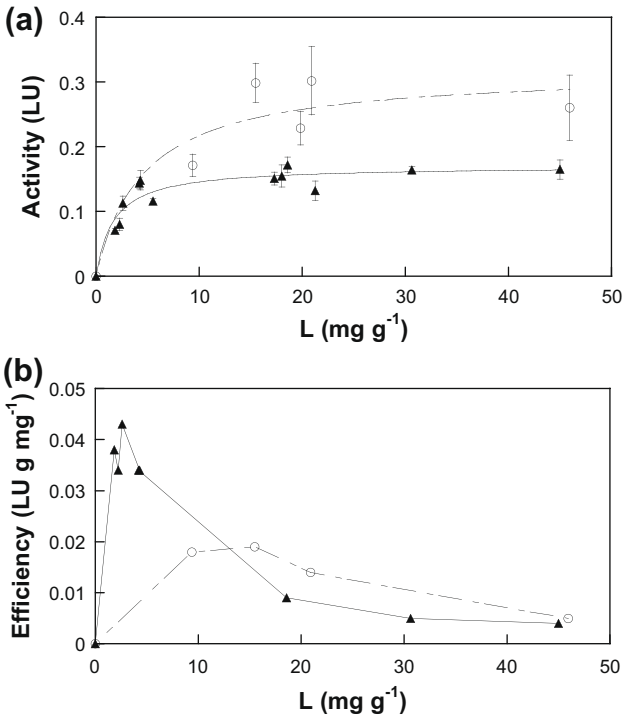

Fig. 5. Effect of loading (mg g^{-1}) on the activity (LU) (a) and the catalytic efficiency (LU g mg^{-1}) (b) of the immobilized lipases on PSi. HO-PSi samples (▲) and LO-PSi samples (○). 1 LU = 1 μmol of butyric acid per minute.

3.5. Stability toward storage of the immobilized lipase on PSi

In view of the use of the lipase immobilized on PSi for the realization of a reusable biosensor, the stability toward storage is a crucial factor. Tembe et al. found that their silicon-based conductive biosensor for catechol analysis was stable for almost 20 days afterwards a gradual loss of activity was observed [7]. Therefore, the activity of two series of samples of immobilized lipase on both LO-PSi and HO-PSi was checked once a week by the tributyrin assay. The samples were stored after the measurements in a buffer solution at pH 5, and at a temperature of 5 °C. Normalizing the first measurement as the 100%, the residual percentage activities of the immobilized lipases both on LO-PSi and on HO-PSi were determined. Fig. 6 shows the progressive decrease of the residual activity with time. After 5 weeks both immobilized biocatalysts had fully lost their activity. However, it should be noted that the trend of the residual activity decrease reflects the different nature of the surface in the two types of biocatalysts. The LO-PSi surface, compared to the HO-PSi one, loses its activity slowly in the short time

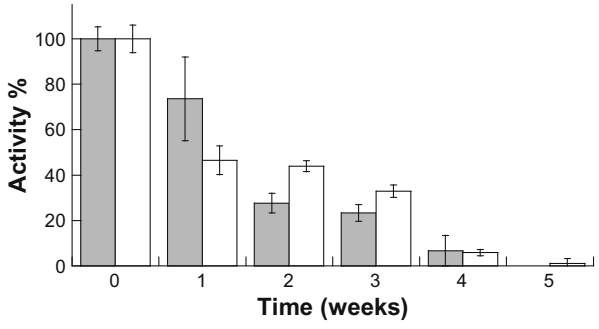

Fig. 6. Activity of the immobilized lipases as a function of storage time for HO-PSi (white) and LO-PSi (gray) samples.

of 2 weeks probably due to a more efficient loading of the biomolecules. After 2 weeks HO-PSi always displays higher activity than LO-PSi as a result of the higher stability induced by a thicker layer of SiO_2 groups.

4. Conclusions

In the present work n^+-type mesoporous silicon was prepared through electrochemical etching. The material had a high surface area (360 m^2/g) and pore size in the range 5–20 nm. These two features are very important for the immobilization of macromolecules of biological origin as enzymes. The stabilization of a porous silicon surface is a fundamental problem that must be solved before using it as a transducer element for both optical and electrochemical biosensors. In the present work we used anodic oxidation for the surface stabilization of n^+-type mesoporous silicon. This method can easily be controlled, and is highly reproducible. Anodic oxidation confers a higher hydrophilic character to the internal surface of PSi due to the formation of a layer of SiO_2. It has been shown that a different extent of oxidation produces significant effects on loading and catalytic activity of the immobilized enzyme. A low oxidation level results in a mainly hydrophobic PSi surface, whereas a high level of oxidation produces a rather hydrophilic PSi surface. Therefore different types of interactions address the physical adsorption of the lipase. It is remarkable that, although LO-PSi allows for a higher lipase loading and a higher catalytic activity, HO-PSi results in a more efficient biocatalyst. Finally the two biocatalysts showed an acceptable stability toward storage (pH 5 buffer solution at 5 °C) within 2 weeks.

Acknowledgments

Dr. V. De Montis of CMTC-INP laboratory at the Institut National Polytechnique de Grenoble (France) is thanked for SEM-FEG images. FUSINT project and MIUR (Rome) are acknowledged for financial support. F.C. thanks the "Master & Back" project financed by RAS.

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