Chemical stabilization of porous silicon for enhanced biofunctionalization with immunoglobulin

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Abstract

Porous silicon (PSi) is widely used in biological experiments, owing to its biocompatibility and well-established fabrication methods that allow tailoring its surface. Nevertheless, there are some unresolved issues such as deciding whether the stabilization of PSi is necessary for its biological applications and evaluating the effects of PSi stabilization on the surface biofunctionalization with proteins. In this work we demonstrate that non-stabilized PSi is prone to detachment owing to the stress induced upon biomolecular adsorption. Biofunctionalized non-stabilized PSi loses the interference properties characteristic of a thin film, and groove-like structures resulting from a final layer collapse were observed by scanning electron microscopy. Likewise, direct PSi derivatization with 3-aminopropyl-triethoxysilane (APTS) does not stabilize PSi against immunoglobulin biofunctionalization. To overcome this problem, we developed a simple chemical process of stabilizing PSi (CoxPSi) for biological applications, which has several advantages over thermal stabilization (ToxPSi). The process consists of chemical oxidation in H₂O₂, surface derivatization with APTS and a curing step at 120 °C. This process offers integral homogeneous PSi morphology, hydrophilic surface termination (contact angle θ = 26°) and highly efficient derivatized and biofunctionalized PSi surfaces (six times more efficient than ToxPSi). All these features are highly desirable for biological applications, such as biosensing, where our results can be used for the design and optimization of the biomolecular immobilization cascade on PSi surfaces.

Keywords: biofunctionalization, porous silicon, nanostructured materials, material stability, protein adsorption, biomaterial

1. Introduction

Porous silicon (PSi), which can be described as a porous assembly of silicon nanocrystals, was first considered for optoelectronic applications owing to its light emission properties and relatively simple fabrication methods [1]. More recently, PSi has been considered for biological and biomedical applications, including biosensing [2], therapeutic delivery [3, 4] and as a molecular probe in cellular tracking [5]. PSi has a higher specific surface area (up to 500 m² cm⁻³) than that obtainable with planar silicon technology [6], providing a platform for various biomolecular interactions. By exploiting this property, several research groups have used PSi as a biomolecular sensing...
platform, creating new devices such as biosensors that can detect proteins, DNA, cells, glucose and other small molecules [7–10].

The as-prepared PSi surface is predominantly SH terminated and is highly reactive [6]. It is usually stabilized and/or derivatized using various chemical reactions, yielding PSi surfaces with diverse properties. Previous works have proposed the derivatization of PSi through covalent binding and physical adsorption [11–14].

In antibody–antigen biosensing applications (immunosensing), it is known that protein–surface interactions are crucial for the design and performance optimization of a biosensor. Moreover, from the bioengineering viewpoint, the optimum condition for biofunctionalization is given by the density of antibodies that are properly (actively) biofunctionalized onto the transducer surface. Thus, biological applications such as biosensing require a process yielding densely biofunctionalized PSi surfaces.

The importance of pre-stabilization of PSi surfaces for the subsequent biofunctionalization is poorly documented. Currently, thermal oxidation is the most popular stabilization process for PSi. Whereas such stabilization is crucial for optoelectronic technologies, its necessity for biological applications is yet uncertain [15–19]. In particular, it is unclear whether the direct derivatization of a fresh PSi surface is adequate for subsequent biological experiments.

Among the wide range of PSi types, mesoporous PSi (with pores in the range of 2–50 nm) is undoubtedly the most interesting form for biosensing applications. In this work, stabilized and non-stabilized mesoporous PSi surfaces were derivatized with 3-aminopropyl-triethoxysilane (APTS) and biofunctionalized with mouse immunoglobulin aiming to investigate the relevance of the stabilization process. We introduce a simple chemical oxidation process for stabilizing PSi (CoxPSi), which is compared with thermal oxidation (ToxPSi) in terms of convenience for biological immobilization. We specifically focus on the hydrophilic character, the mechanical stability of the mesoporous film and the efficiency of different immobilization cascades.

2. Experimental approach

2.1. Fabrication

2.1.1. Porous silicon (PSi) fabrication and stabilization. PSi layers were fabricated galvanostatically by the electrochemical etching of single-crystalline p-type Si wafers (boron-doped, orientation (100), resistivity 0.05–0.1 Ω cm) in a HF:ethanol (1:2) electrolyte solution. A current density of 80 mA cm–2 was applied for 30 s under illumination with a 150 W halogen lamp.

After etching, the PSi surface is predominantly SH terminated [6]. To investigate the role of the stabilization process, we used both stabilized PSi and non-stabilized PSi. Stabilized PSi samples were prepared by two methods: (a) chemical oxidation (Cox), embedding PSi in H2O2 (30% v/v) for 5 or 90 min, or (b) thermal oxidation (Tox) for 30 min at 500 °C in a furnace in ambient air [20]. Then, the samples were rinsed in absolute ethanol and derivatized and biofunctionalized with immunoglobulins.

2.1.2. Derivatization and biofunctionalization of PSi with immunoglobulins. Surface derivatization of both non-stabilized and stabilized PSi surfaces was performed using APTS (Sigma Aldrich) dissolved in dry methanol (2% v/v). PSi chips were incubated in an APTS-based solution for 15 min, followed by cleaning in absolute ethanol. After drying of PSi, they were cured at 120 °C for 20 min in a furnace.

PSi surfaces were biofunctionalized with mouse immunoglobulins diluted (1:200 v/v) with a mixture of phosphate-buffered saline and Tween 20 (PBS-T). After incubation for 60 min, the surfaces were cleaned three times with PBS-T and dried in a dry nitrogen stream.

2.2. Characterization

2.2.1. Microscopy techniques. Morphological characterization of the PSi layer was carried out using a field-emission scanning electron microscope (FESEM; Philips XL–40FEG) and a conventional SEM (Philips XL30), both operated at 20 keV. Derivatization of PSi with APTS was evaluated using fluorescein isothiocyanate (FITC, Sigma). FITC is a fluorescent molecular probe that reacts covalently with the NH2 group of APTS. Immunoglobulin immobilization on PSi was monitored using a fluorescently labeled (1:500) anti-mouse secondary antibody and an inverted fluorescence microscope (Olympus IX81) coupled to a CCD color camera.

2.2.2. Physical and chemical techniques. After each step of surface modification, the samples were characterized by the following techniques. Fourier transform infrared (FTIR) spectroscopy was used for the identification of molecular species. The spectra were acquired with a Bruker IFS66v spectrometer, in vacuum, in the 4000–550 cm–1 range, with 2 cm–1 resolution, in the diffuse reflectance configuration. Reflectance UV–visible spectra were recorded using a Jasco V-560 double-beam spectrophotometer, and water contact angles were measured with a KSV CAM-101 system.

2.3. Statistical analysis

The data were analyzed with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). The statistical difference was established using t-test, and the significance level was set at p < 0.05.

3. Results and discussion

Surface biofunctionalization is crucial for biological applications, and different structural configurations of PSi have been described [9, 14, 21, 22]. Here, we used a single layer of PSi obtained by electrochemical anodization of crystalline silicon. Figure 1 shows its top view and cross section (inset; SEM images). This PSi layer is characterized by column-like pores of 1.8 µm height and an average diameter of 20 nm. These properties are highly desirable for
biological applications because they ensure a large surface area (where the biomolecular recognition takes place) and appropriate layer thickness and pore diameter for biomolecule infiltration. For immunosensing applications, it should be kept in mind that the diameter of the immunoglobulin molecule is about 5.6 nm [23].

It is unclear whether a stabilization process is required for biological applications of PSi and whether derivatization of as-prepared PSi can stabilize its surface. Therefore, we conducted surface derivatization of non-stabilized PSi with APTS to allow further biofunctionalization with immunoglobulin. Figure 2 shows top and cross-sectional views of the morphology of the non-stabilized PSi surface after derivatization and biofunctionalization. Derivatization with APTS did not change the morphology of the non-stabilized PSi surface, as seen in the inset of figure 2(a). However, after biofunctionalization with immunoglobulin the structure of the non-stabilized PSi collapsed. This is evident from the groove-like dendritic features in figure 2(a) and the layer detachment in figure 2(b). Presumably, this mechanical instability and strong capillary stress on the surface originate from protein adsorption. Likewise, the high surface area due to the nanometric pore size and high reactivity of the non-stabilized PSi surface [6] are responsible for the detachment. It was reported that DNA hybridization can lead to PSi erosion mediated by the electrostatic effect of DNA molecules [24]. Furthermore, in physiological fluids, part of the silicon matrix can be dissolved as orthosilicic acid [25]. However, in our experiments, the porous structure has not been completely lost, and derivatization of non-stabilized PSi with APTS could not stabilize PSi for protein biofunctionalization.

UV–visible reflectance spectra were recorded for both as-prepared PSi and biofunctionalized non-stabilized PSi (figure 3(a)). As-prepared PSi layers show the interference
Figure 4. SEM images obtained after the immunoglobulin biofunctionalization of (a) non-stabilized PSi, (b) CoxPSi for 5 min and (c) CoxPSi for 90 min.

Figure 5. (a) FTIR spectra and (b) water contact angles of as-prepared PSi, CoxPSi, derivatized CoxPSi and biofunctionalized CoxPSi.

The chemical composition of CoxPSi at each step of the process was evaluated by FTIR spectroscopy. Figure 5(a) shows FTIR spectra of the as-prepared PSi, CoxPSi and derivatized and biofunctionalized CoxPSi. The as-prepared PSi has a characteristic band at 615 cm\(^{-1}\) referred to as SiH. After Cox, two bands appear at every step of surface modification: a strong band at 1050–1100 cm\(^{-1}\), assigned to asymmetric stretching in SiO\(_2\), and a weak band at 835–795 cm–1 from the Si–OH bond. It is clear that Cox stabilization leads to mostly oxidized PSi. However, a small SiH band remains at 615 cm\(^{-1}\). After surface derivatization with APTS, a Si–O–Si band is observed at 1180 cm–1, proving that a siloxane bond was formed between APTS and CoxPSi. After biofunctionalization with
immunoglobulins, two bands appear at 1535 and 1653 cm\(^{-1}\) related to the N–H and C=O bonds, respectively [28, 29]. These chemical groups are characteristic of proteins, and their presence indicates that the surface is biofunctionalized.

The wettability of PSi surfaces was evaluated by measuring the water contact angle (figure 5(b)). As expected, as-prepared PSi shows a hydrophobic behavior with a contact angle of 108.41°. This value stems from both the presence of a surface nanotopography and the abundant terminal Si–H bonds. After Cox, the contact angle decreased to 21.47°, revealing a hydrophobic to hydrophilic transition due to the surface Si–OH and Si–O groups [30]. Likewise, hydrophilic behavior is maintained when the surface of CoxPSi is derivatized with APTS and biofunctionalized with immunoglobulins, resulting in contact angles of 28.71° and 26.16°, respectively.

Representative visible reflectance spectra of different PSi samples are shown in figure 6. Compared with CoxPSi surfaces, there are redshifts of 25 and 52 nm after surface derivatization with APTS and biofunctionalization with immunoglobulins, respectively. Interference emerges from the thin film effect of PSi, which is composed of silicon, silica and air. The shift of the interference spectrum is caused by changes in the effective refractive index, demonstrating that biomolecules are infiltrating into the pores. Note that the thin-film behavior of as-prepared PSi is not lost after oxidation, derivatization and biofunctionalization. This result indicates that the Cox preserves the porosity of the PSi layer, keeping its internal surface available for the biomolecule attachment. By exploiting this optical shift, PSi devices can be used as label-free optical biosensing systems [16, 31, 32].

Cox does not involve high temperatures and is simpler than Tox, which is the most common approach to achieve partial or complete oxidation of PSi structures for stabilization purposes. Tox has been widely applied for optoelectronic applications to improve the photostability and light-emitting properties of PSi [30]. However, the potential effects of this process on biofunctionalization with proteins have not been investigated. In figure 7, the derivatization and biofunctionalization of CoxPSi and
ToxPSi are evaluated by fluorescence microscopy. Results indicate that Cox leads to a highly homogeneous surface derivatization and immunoglobulin biofunctionalization. Also, the negative control images show a negligible response. More interestingly, the CoxPSi surface has a higher APTS coverage and a higher density of biofunctionalized immunoglobulins than the ToxPSi surface (figure 7(a)). Likewise, quantification of the fluorescence intensity for derivatization and biofunctionalization treatments reveals that CoxPSi has a higher efficiency than ToxPSi ($p < 0.05$, figure 7(b)). These results can be explained by the presence of surface SiOH groups in CoxPSi, which can easily react with APTS molecules and induce derivatization according to a partial or total condensation of ethoxy groups [15]. Such a mechanism is not obvious for the terminal SiO$_2$ present in ToxPSi [20]. Thus, since the APTS molecule functions as a cross-linker between the PSi matrix and biomolecules, biofunctionalization with immunoglobulin is expected to be higher on CoxPSi than on ToxPSi. This improvement in biofunctionalization is an interesting feature of Cox. It may be crucial for biosensing applications because immunoglobulin is the key biomolecular recognition agent in immunosensor devices. Taken together, these results suggest that ToxPSi may not be the most appropriate choice for biological applications requiring a high degree of surface biofunctionalization, because it results in a low biomolecular coverage of the surface. The absence of this drawback in CoxPSi makes it an interesting alternative for biological applications, such as biosensing.

When CoxPSi and ToxPSi are compared in terms of chemical composition, interesting differences are revealed after each fabrication step (figure 8). Figure 8(a) shows the differences in the FTIR spectra as chemical or thermal oxidation treatment is used for stabilizing PSi. The most intense band at 1050–1100 cm$^{-1}$ corresponding to asymmetric stretching in SiO [27] is present both in CoxPSi and ToxPSi; however, the intensity of this band is weaker in CoxPSi. This means that ToxPSi is a harsher oxidation process than CoxPSi, as also evident from the disappearance of the SiH peak at 615 cm$^{-1}$ in ToxPSi. Another important difference is the absence of the Si–OH mode at 835–795 cm$^{-1}$ [28] in ToxPSi, which is important for the following derivatization step. In the FTIR spectra of derivatized surfaces (figure 8(b)), the Si–O–Si band at 1180 cm$^{-1}$, which proves the formation of the siloxane bond [19], is weaker in ToxPSi than in CoxPSi. Consistent with this result, there is a pronounced decrease in the Si–OH and SiO peak areas after derivatization of CoxPSi, which is not observed for ToxPSi. The presence of these terminal SiOH groups in CoxPSi, can partly explain the results shown in figure 7, as Si–OH can easily react with APTS molecules according to a partial or total condensation of ethoxy groups [15]. Such a mechanism is less obvious for the terminal SiO$_2$ present in ToxPSi [20]. These results on surface chemical composition are directly relevant to the further biofunctionalization (figure 8(c)): the two characteristic protein bands at 1535 and 1653 cm$^{-1}$, which are related to the N–H and C=O bonds, respectively [28, 29], are stronger in CoxPSi than in ToxPSi.

4. Conclusions

This work demonstrates that non-stabilized mesoporous PSi is prone to detachment upon biomolecular immobilization,
resulting in the formation of groove-like dendritic structures on the surface after its biofunctionalization with a protein. Therefore, a stabilization process must be considered before biofunctionalizing mesoporous PSI. A simple chemical process for the stabilization of PSI for biological applications has been presented, which involves immersion in H$_2$O$_2$, derivatization with APTS and curing at 120 °C. This process results in an integral PSI morphology, and both homogeneous and highly efficient derivatization and biofunctionalization with immunoglobulins. Moreover, the process is simple and does not require the high temperatures used in thermal oxidation. The latter technique is unfavorable for biofunctionalization with immunoglobulins because it results in a low density of biomolecules on the PSI surface. In contrast, the chemical oxidation results in a highly biofunctionalized, hydrophilic and mechanically stable PSI surface. All these features are highly desirable for biological applications such as biosensing, where the reported results can be used for the design and optimization of the biomolecular immobilization cascade on PSI.

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