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Fabrication and characterization of a chemically oxidized-nanostructured porous silicon based biosensor implementing orienting protein A

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ABSTRACT

Nanostructured porous silicon (PSi) elicits as a very attractive material for future biosensing systems due to its high surface area, biocompatibility and well-established fabrication methods. In order to engineer its performance as a biosensor transducer platform, the density of immunoglobulins properly immobilized and oriented onto the surface needs to be optimized. In this work we fabricated and characterized a novel biosensing system focusing on the improvement of the biofunctionalization cascade. The system consists on a chemically oxidized PSi platform derivatized with 3-aminopropyltriethoxysilane (APTS) that is coupled to *Staphylococcus protein A* (SpA). The chemical oxidation has previously demonstrated to enhance the biofunctionalization process and here "by implementing SpA" a molecularly oriented immunosensor is achieved. The biosensor system is characterized in terms of its chemical composition, wettability and optical reflectance. Finally, this system is successfully exploited to develop a biosensor for detecting asymmetric dimethylarginine (ADMA), an endogenous molecule involved in cardiovascular diseases. Therefore, this work is relevant from the point of view of design and optimization of the biomolecular immobilization cascade on PSi surfaces with the added value of contribution to the development of new assays for detecting ADMA with a view on prevention of cardiovascular diseases.

1. Introduction

Biosensing technology is a rapidly advancing field that benefits from the possibility to use the properties of biological systems combined to functional advanced materials. This synergy offers transduction platforms possessing extreme control on the material properties at the nanoscale level to specific biomolecular recognition elements, such as immune systems, genomic pairs or enzyme catalyzed reactions [1]. Among advanced materials, nanostructured porous silicon (PSi) elicits as a very attractive one for future biosensing systems [2,3]. PSi has interesting intrinsic properties, such as large specific surface area (200–500 m^2cm^{-3}), biocompatibility and simple fabrication methods, besides its potential integration with standard microelectronic techniques [4,5]. Moreover, by using diverse chemical or physical reactions, the nature of PSi surfaces can be easily adapted and modified for allowing further biofunctionalization with different biomolecules. All these features make PSi very attractive for biotechnology and bioengineering applications such as biosensing.

From the optical point of view, PSi is considered to be composed of silicon, silica and air. Thus, as molecules infiltrate into the pores, a shift can be observed in the interference spectrum of a PSi layer due to the change in the effective refractive index. This effect enables PSi devices to be applied as a label-free optical biosensing system [6]. However, most of these devices are fabricated using randomly immobilized antibodies onto the surface, with the consequent loss of sensing efficiency due to the presence of inoperative antibodies immobilized on the transducer surface. Regarding the immunoglobulins density, we have recently reported a chemical process that enhances the density of immunoglobulins immobilized onto the PSi surface [7]. A strategy is however required to molecularly orient the antibodies on this system. One interesting possibility to achieve this orientation is by using *Staphylococcus aureus* protein A (SpA), a bacterial protein that binds specifically to the Fc portion of antibodies. Indeed, protein A is widely used in chromatography for the purification of antibodies in both laboratory and industrial procedures [8] and has already been integrated in biosensor immobilization cascades [9,10].

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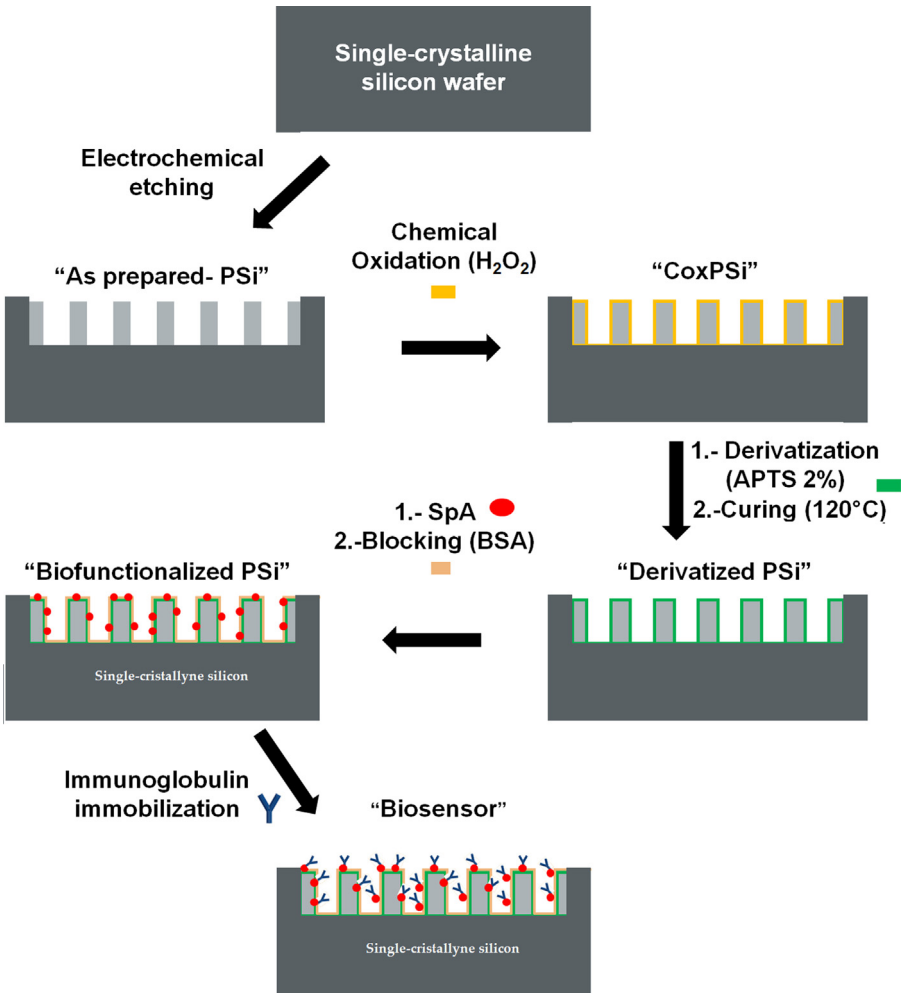

Fig. 1. Experimental flowchart followed for the fabrication of the PSi based biosensor featuring molecularly oriented immunoglobulin.

Herein, chemically oxidized PSi surfaces (CoxPSi) are derivatized with 3-aminopropyltriethoxysilane (APTS) and biofunctionalization with SpA is addressed with the aim to immobilize molecularly oriented antibodies. Fig. 1 shows the schematic representation of the bioassembly for constructing the biosensor. Firstly, PSi is fabricated by electrochemical etching of single-crystalline p-type Si wafers. This as-prepared PSi layer is chemically oxidized in order to stabilize the structure and allow an enhanced derivatization process with APTS, which is known to expose NH_2 groups onto the surface [6]. Then, as a first step of the immobilization cascade, the surfaces are biofunctionalized with SpA, in order to bind immunoglobulins specifically through interaction with the antibodies Fc domains. After this step, the surface is blocked using BSA in order to prevent any unspecific interaction in the final step, which consists in a second biofunctionalization carried out to potentially immobilize molecularly oriented immunoglobulins onto the surface. Using this immobilization cascade it is possible to fabricate a molecularly oriented biosensing system.

We characterize the immobilization cascade in terms of chemical composition, surface wettability, biofunctionality and optical reflectance. Moreover, we apply this molecularly oriented prototype biosensor system for the biosensing of asymmetric dimethylarginine (ADMA) in PBS solution. ADMA is an endogenous biomolecule involved in the inhibition of the nitric oxide synthesis, one of the main vasodilator in the cardiovascular system. Herein lies its importance since it is involved in endothelial dysfunction and vasoconstriction becoming an important biomarker of adverse cardiovascular events [11].

2. Materials and methods

2.1. Formation, stabilization and derivatization of PSi

Fabrication of PSi layers was carried out 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:EtOH (1:2) electrolyte solution. The current density applied was 80 mA cm^{-2} during 30 s under a 150 W lamp illumination. In order to stabilize as-prepared PSi we realized a chemical process as previously reported [7]. Briefly, chemical oxidation of PSi was performed immersing PSi in H_2O_2 (30%, v/v) for 90 min. PSi samples were cleaned in absolute ethanol prior to surface derivatization performed using 3-aminopropyltriethoxysilane (APTS; Sigma–Aldrich) dissolved in dry methanol (2%, v/v) for 15 min followed by cleaning step in absolute ethanol. After drying of PSi, the samples were cured in a furnace at 120 °C for 20 min.

2.2. Immobilization cascade onto PSi

In order to bioassemble the molecularly oriented immunoglobulin biosensor the following immobilization cascade was conducted onto the PSi surface. Firstly, biofunctionalization of surfaces with

diluted SpA (50 μg/ml; Sigma–Aldrich) in phosphate buffer-Tween 20 (PBS-T) for 60 min was realized. After incubation with SpA, PSi surface was cleaned for three times using PBS-T. Surface blocking was realized using Bovine Serum Albumin (1%, w/v BSA; Sigma–Aldrich) in order to prevent any further unspecific interaction between the surface and biomolecules. Finally, surface was biofunctionalized with immunoglobulins diluted in PBS-T (1:200, v/v) for 60 min, cleaned for three times using PBS-T, milli-Q water and dried under nitrogen flow stream.

2.3. Morphological characterization

Morphological characterization of PSi layer has been studied by field-emission scanning electron microscopy (FESEM; Philips XL-40FEG), operating with an acceleration potential of 20 keV. The biofunctionalization events onto PSi were detected using fluorescently labeled anti-SpA or anti-mouse immunoglobulin secondary antibodies (1:500) labeled with Alexa 568 or Alexa 488, respectively. Samples were observed in an inverted fluorescence microscope (Olympus IX81) coupled to a CCD color camera.

2.4. Physical and chemical characterization

The samples were characterized after each step of fabrication. Fourier transformed infra-red (FT-IR) and X-ray photoelectron (XPS) spectroscopies were used for identifying the relevant molecular species at each step. The FT-IR spectra were obtained using a Bruker IFS60v FT-IR spectrometer in the 4000–550 cm^{-1} range with 2 cm^{-1} resolution. XPS spectra were acquired in a spectrometer (SPECS SAGE HR 100) equipped with a 100 mm mean radius PHOIBOS analyzer, using a Mg-anode X-ray source. All XPS binding energies were referenced to the C 1s peak at a binding energy of 284.6 eV to compensate the surface charging effects. XPS peaks were deconvoluted using the XPSPEAK 4.1 software.

For optical characterization, reflectance UV–visible spectra were recorded using a Jasco V-560 double-beam spectrophotometer equipped with an integrating sphere. To evaluate the wettability of samples, water contact angles were measured by using an optical contact angle measuring system (KSV CAM-101).

2.5. Biosensing experiments for label free-optical ADMA detection

ADMA solutions were prepared and incubated with the acylation reagent for 20 min. The resultant solutions were added into the CoxPSi biofunctionalized with rabbit anti-Acyl-ADMA antibody (Immunodiagnostik AG) and incubated for 1 h. The chips were cleaned twice with PBS and sequentially rinsed twice in milli-Q water (18 Ω cm) in order to prevent any PBS crystallization. Finally, samples were dried under a dry nitrogen stream.

3. Results and discussion

3.1. Morphological characterization of the PSi single layer

In a biosensing system the biomolecular recognition events take place in the transducer element. For that reason, the transducer material should provide high surface area to increase the sensitivity of the system. This argument is in favor of porous transducer materials if designed with appropriate thickness and pore diameter providing the required biomolecular accessibility through infiltration. In this sense, PSi offers these desirable features making it an excellent aspirant for future biosensing technology. In this study, single layer PSi obtained by electrochemical anodization of silicon wafers was chosen as transducer. Fig. 2 presents its surface and cross-section view (inset; FESEM images). As seen, this PSi layer is characterized by column-like pores of about 2 μm depth and diameter of 30 nm, satisfying the above mentioned requirements.

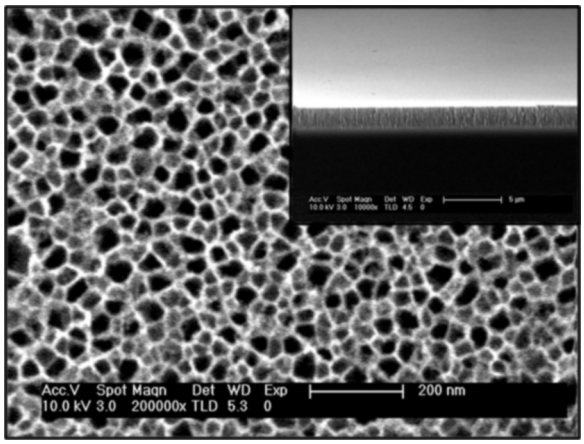

Fig. 2. Surface (main view) and cross-sectional (inset view) FESEM images of the typical PSi layer structure prepared in this study (p-type Si, resistivity 0.05–0.1 Ω cm; current density applied of 80 mA cm^{-2} during 30 s).

3.2. FT-IR characterization

With the aim of characterizing the chemical composition of this biosensor model we carried out an FTIR study. Fig. 3 shows the FT-IR spectra of CoxPSi, derivatized CoxPSi, CoxPSi biofunctionalized with SpA and after immunoglobulin immobilization. CoxPSi shows two bands corresponding to the characteristic asymmetric stretching mode of SiO at 1050–1100 cm^{-1} [12] and the Si—OH bond at 825 cm^{-1} [13]. Derivatization with APTS resulted in a band related to Si—O—Si at 1180 cm^{-1}, which confirms that the siloxane bonding between APTS and CoxPSi has taken place [14]. After biofunctionalization with SpA two specific bands appear at 1535 cm^{-1} and 1653 cm^{-1} related to N—H and C=O bonds, respectively [13,15]. The intensities of these bands increase after biofunctionalization with immunoglobulins.

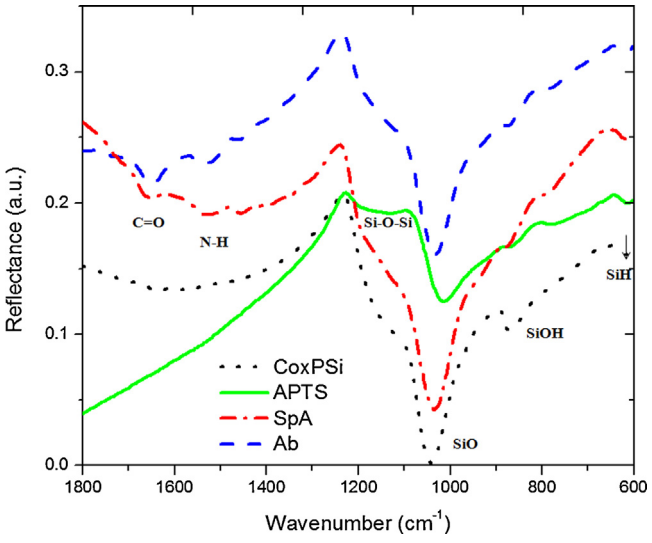

Fig. 3. FT-IR spectra showing the functional groups of chemically oxidized PSi (CoxPSi), CoxPSi derivatized with aminopropyltriethoxysilane (APTS) and after biofunctionalization with *Staphylococcus aureus protein A* (SpA) and immunoglobulin (Ab).

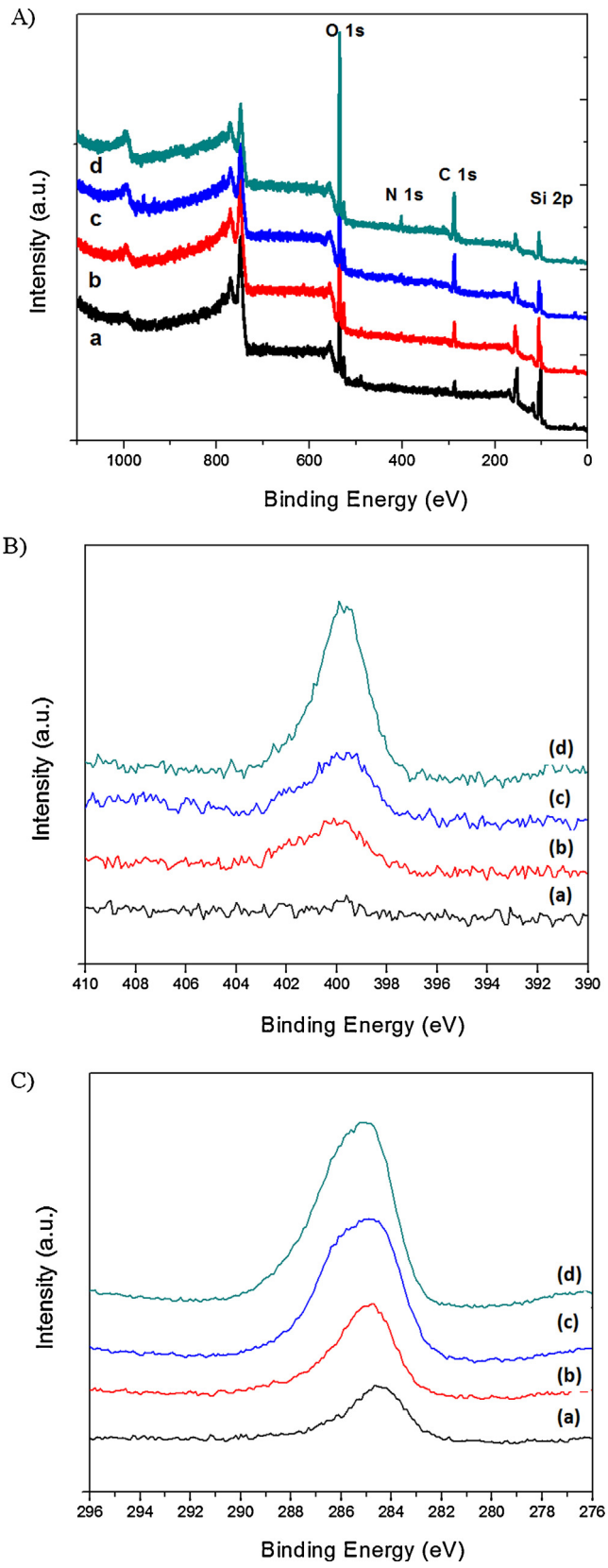

Fig. 4. XPS survey spectra (A), high resolution spectra of core level N 1s (B) and C 1s (C) from the different surfaces studied: CoxPSi (a), APTS derivatized CoxPSi (b), CoxPSi biofunctionalized with SpA (c) and after biofunctionalization with immunoglobulin.

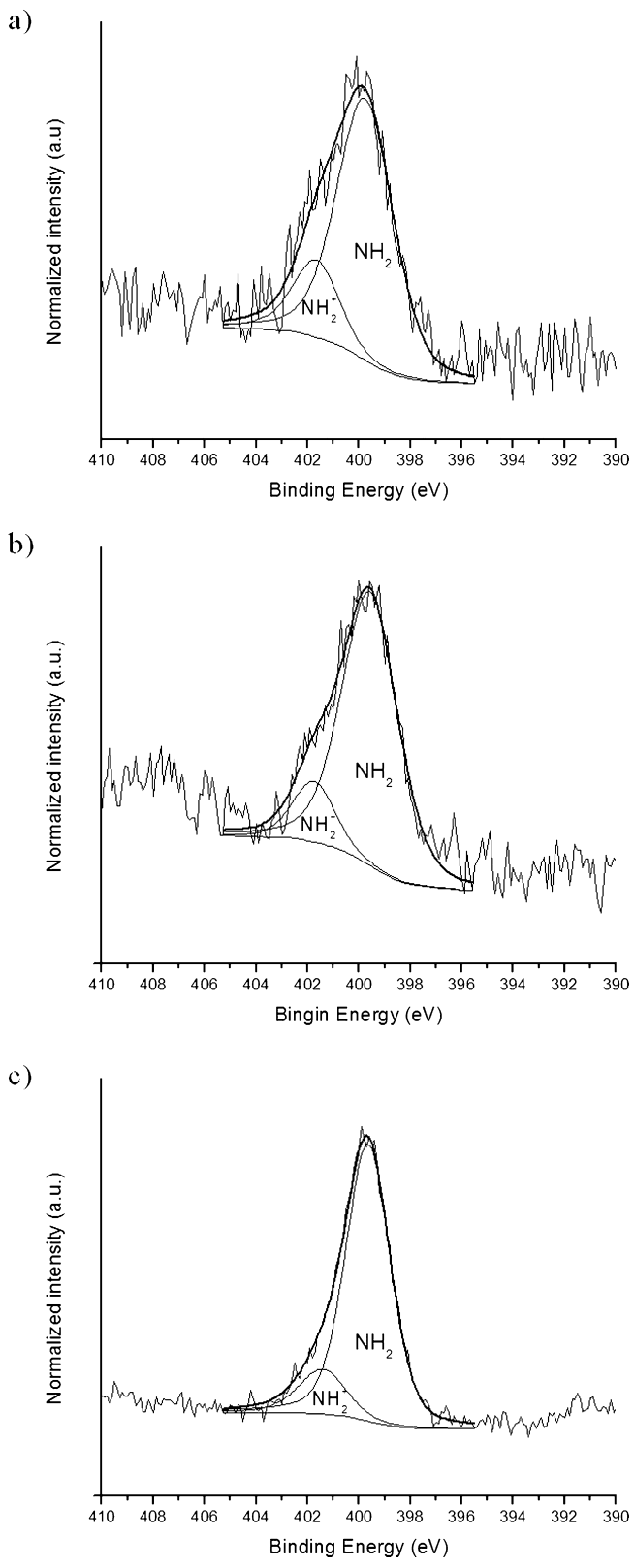

Fig. 5. Deconvoluted N 1s core level XPS spectra of derivatized CoxPSi (a), CoxPSi biofunctionalized with SpA (b) and CoxPSi biofunctionalized with SpA/immunoglobulin (c).

3.3. XPS analysis

Fig. 4a shows the XPS survey spectra of CoxPSi, derivatized CoxPSi, CoxPSi biofunctionalized with SpA and after biofunctionalization with immunoglobulins. The predominant signals were C 1s, O 1s, N 1s and Si 2p peaks that are elements related to the building blocks of the bioassembly. The atomic composition in the samples surfaces shows that, on the one hand, immobilization of SpA results in a small increase of N 1s peak (1.7%) and C 1s (29%), and on the other hand, after immunoglobulin biofunctionalization higher peaks of N 1s (4.2%) and C 1s (35.8%) are found. The increase of the signal of both nitrogen and carbon indicates that immobilization of biomolecules onto the PS surface has taken place. The spectra of all samples exhibit two Si peaks centered at about 154 eV (Si 2s) and 102.7 eV (Si 2p), C 1s peak centered at 284.6 eV and the O 1s signal centered at 532.2 eV. In correlation with the previously described features, the silicon peaks decreased after derivatization and biofunctionalization due to the APTS and protein layer deposition. Core level spectra of N 1s and C 1s are shown in Fig. 4b and c for the whole series of samples respecting original intensity in each sample. In fact, the N 1s peak at about 400 eV appears only after derivatization of CoxPSi with APTS (Fig. 4b). The analysis of biofunctionalized samples confirmed the increase of N and C content during the successive biofunctionalization steps (Fig. 4a and b).

In order to visualize the possible functional groups present on the surface of our biosensor model, deconvolution of high resolution spectra in the region of C 1s and N 1s were carried out. Fig. 5 shows the deconvolution of N 1s core level spectra. It reveals two contributions, located at about 399 eV and 401 eV, which are related to the amine (NH_2) and protonated amino group (NH_3^+), respectively [16]. The subsequent biofunctionalization with SpA and immunoglobulin are reflected mainly by the increase in the amine group (NH_2) contribution in the N 1s XPS spectra [16,17]. Fig. 6 shows the deconvolution of C 1s at each step of fabrication. CoxPSi presents two characteristic contributions at about 284.6 eV (C1) corresponding to C—C and C—H components and about 286.7 eV (C2) related to C—C—O [18]. Derivatization results in a new contribution at about 285.7 eV (C3) that is related to C—N bond belonging to the amine group in the structure of APTS [19]. Interestingly, after the biofunctionalization process a shoulder appears by the contribution of a new type of bond. This additional peak centered at around 288.1 eV (C4) is assigned to O═CO—NH— peptide bond, which is the most characteristic bond in proteins [17,19].

All these results concerning C 1s and N 1s core level XPS are in line with FTIR results, where a new peak due to amide I bond has appeared clearly in the spectra of biofunctionalized CoxPSi.

3.4. Fluorescence microscopy, water contact angle and optical reflectance

In Fig. 7a the biofunctionalization processes are evaluated using fluorescence microscopy. As seen, the fabrication procedure leads to a highly homogeneous biofunctionalization with SpA and immunoglobulins onto the PSi surface.

Water contact angle was used to evaluate the wettability of the PSi biosensor as seen in Fig. 7b. As previously observed, as-prepared

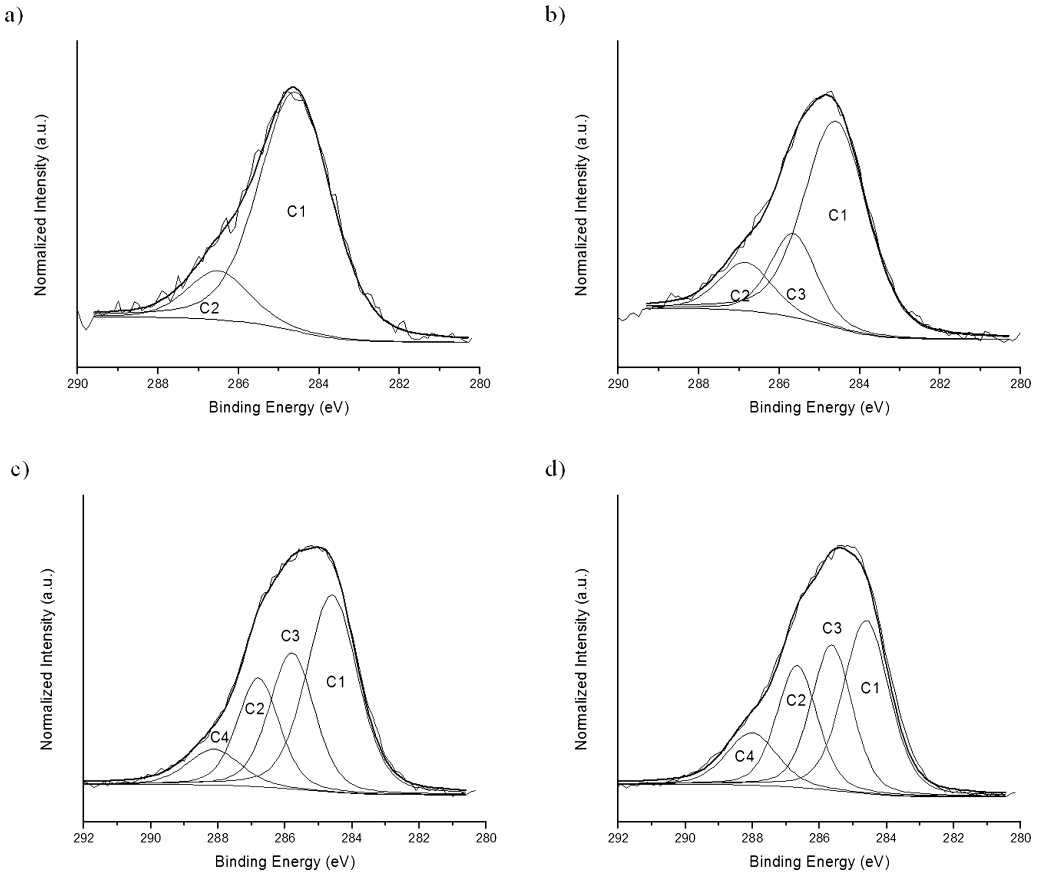

Fig. 6. Deconvoluted C 1s high resolution XPS spectra of CoxPSi (a), derivatized CoxPSi (b), CoxPSi biofunctionalized with SpA (c) and CoxPSi biofunctionalized with SpA/immunoglobulin (d).

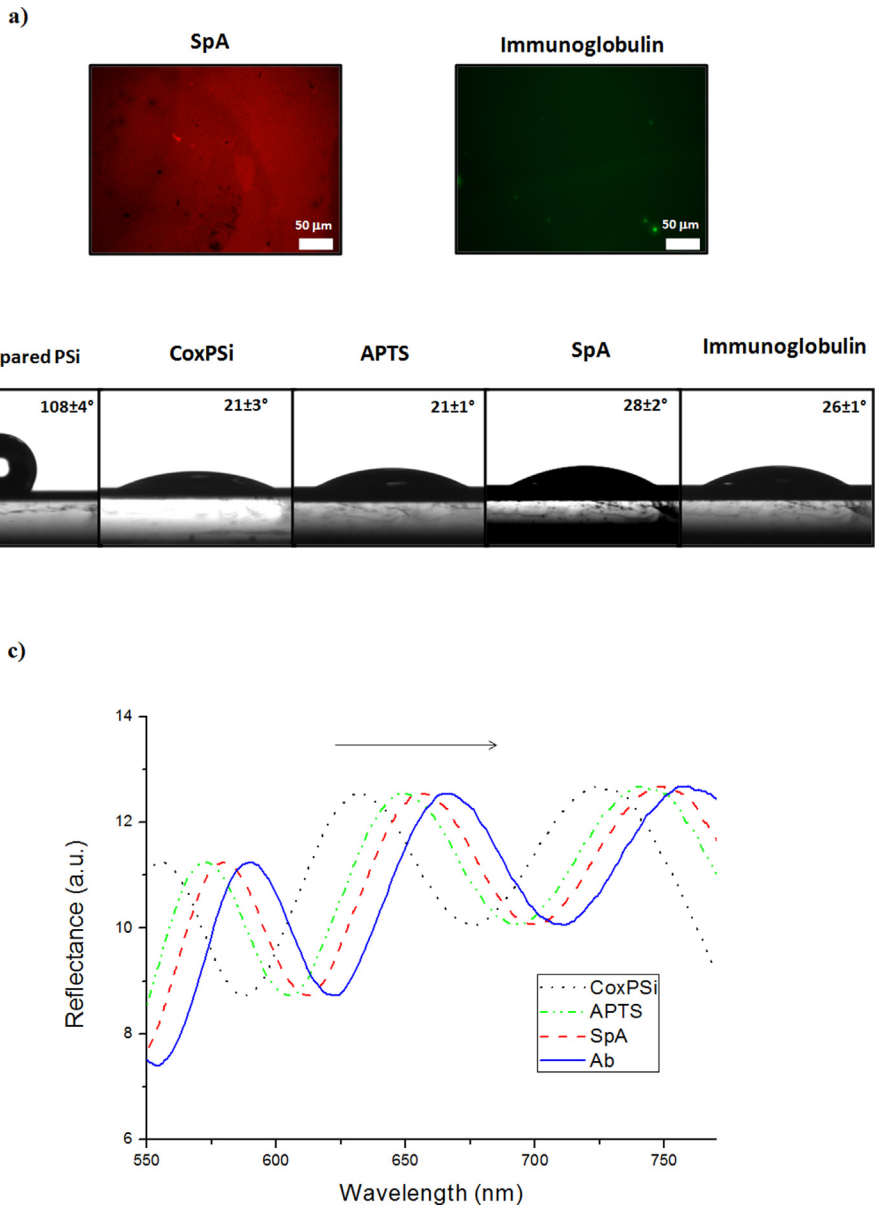

Fig. 7. (a) Fluorescence microscopy image of the surface biofunctionalization by SpA and immunoglobulins, (b) surface wettability studied by water contact angle after each step of fabrication and (c) UV–visible spectra of CoxPSi, derivatized CoxPSi and CoxPSi after each biofunctionalization step.

PSi results in a contact angle of $108 \pm 4^\circ$ showing its hydrophobic behavior [20]. The contact angle of CoxPSi is $21 \pm 3^\circ$, mainly explained by the terminal Si—OH and Si—O groups onto the surface [21]. Likewise, hydrophilic behavior is maintained as the surface of CoxPSi is derivatized with APTS ($21 \pm 1^\circ$). Biofunctionalization with SpA and immunoglobulins, results in contact angles of $28 \pm 2^\circ$ and $26 \pm 1^\circ$, respectively.

The representative visible reflectance spectra of different PSi samples are shown in Fig. 7c. It is shown that, with respect to CoxPSi surfaces, there is a redshift of 16 nm and 34 nm after surface derivatization with APTS and biofunctionalization (SpA and immunoglobulins), respectively. The interference spectra emerge from the thin film effect of PSi considered as an effective medium composed of silicon, silica and air. This shift of the interference spectrum is caused by changing the effective refractive index and suggests that biomolecules are infiltrating into the pores.

3.5. Biosensing experiment

By exploiting the shift in the visible spectrum, PSi devices can be used as label-free optical biosensing systems [6,22,23]. In fact, to apply this biosensing platform, experiments were conducted to detect ADMA. Five concentrations of ADMA in PBS solution were tested in order to evaluate if this biosensing system is able to detect acyl-ADMA. Fig. 8 shows the calibration curve of the biosensing experiments for detecting ADMA. There was a certain range of µM concentrations where we could observe a linear dependence between wavelength shift and the ADMA concentration. After circa 2.5 µM concentration the shifts tend to saturation. This indicates that, though in a limited range, our biosensing system is able to detect ADMA and could be used for the ADMA quantification. However, further studies are necessary in order to optimize the performance and extend the detection range.

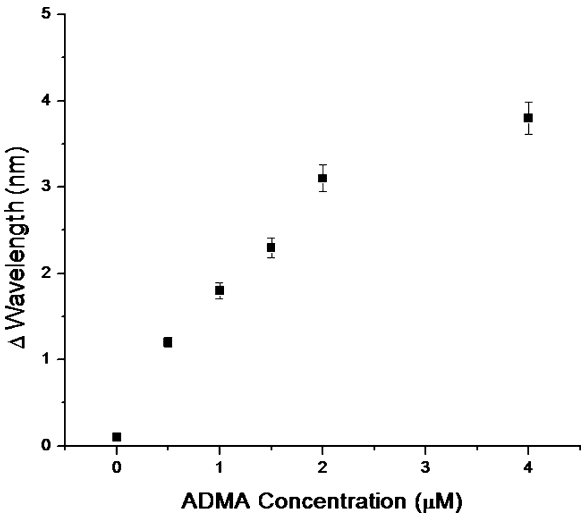

Fig. 8. Biosensing of ADMA biomarker after immersion of the prototype CoxPSi/SpA biosensor system of this study in different PBS solutions with graded ADMA concentrations.

4. Conclusion

In the present study, we have fabricated and characterized a biosensing system based on the immobilization of SpA onto chemically oxidized PSi. The use of chemically oxidized PSi enabled an enhanced derivatization process with APTS and a further enhanced biofunctionalization process. SpA immobilization onto CoxPSi results in a biofunctional surface detected by FTIR and XPS. Moreover, a homogeneous biofunctionalization surface was obtained. This surface containing SpA enabled the subsequent immobilization of molecularly orientated immunoglobulins, which were evidenced by FTIR, XPS and fluorescence microscopy. The biofunctionalization pattern after immunoglobulin immobilization was highly homogeneous. All these features are desirable for biosensing applications. As a proof of concept of this sensing platform, we successfully exploited this biosensing system for detecting ADMA (an endogenous molecule involved in cardiovascular diseases) in PBS solutions. Therefore, this work is relevant for the design and optimization of the biomolecular immobilization cascade on PSi surfaces as well as to the new development of assays for detecting ADMA. Further progress is required to considerably increase the detection range of ADMA using PSi platforms.

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