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Dual detection biosensor based on porous silicon substrate

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ABSTRACT

Due to the high surface-to-volume ratio (hundreds of m^2/cm^3) porous silicon became during the last years a good candidate material as substrate for biosensor application. Moreover, the versatility of surface chemistry allows different functionalization approaches and large number of molecules to be captured on well-defined areas. This paper reports a dual detection method for protein recognition processes developed on different nanostructured porous silicon (PS) substrates, based on using two complementary spectroscopic techniques: fluorescence and electrochemical impedance. The structures were tested for biomolecular recognition – biotin–strepavidin couples – in order to achieve an optimum surface for protein's immobilizations. Comparative analyses of the attachment degree and preservation of the biomolecules activity on the porous silicon surfaces and silicon slides are also described.

1. Introduction

Even if it is a non-covalent interaction, the avidin–biotin complex has a strong affinity coupling, with $K > 10^{-12}$, which recommends it to be used for a wide range of specific targeting applications such as immunoassays, high-throughput screening, detection and immobilization of DNA, biofunctionalization, or even in vitro cytotoxicity, to mention only a few [1].

The bond formation between biotin and avidin is a very rapid process, and once formed, it is unaffected by extremes of pH, temperature, organic solvents and other denaturing agents. Besides avidin, the streptavidin is also a tetrameric protein, with four identical binding sites for biotin, and additionally it has a mildly acidic isoelectric point ($pI = 5$) being therefore preferably used to avoid the nonspecific interactions (the avidin pI is 10.5). Thus, the small size and stability of biotin, as well as its high binding capacity of the biomolecules, without causing interference and modifying their functions, were exploited for development of tests with high sensitivity and also with a high degree of reproducibility [2,3]. In this view, the authors took into consideration the biotin–streptavidin system for preliminary tests of new nanostructured sensing surfaces and the corresponding detection system.

For a dual detection, and consequently a double confirmation of the results, the proposed system is dedicated to fluorescencet and electrochemical impedance detection spectroscopies. Moreover, a controlled deposition of biomolecules has been achieved using the microarray technology to allow further tests of the detection limit and monitorization of more complex biointeractions towards diagnostic applications.

One of the important steps for a successful microarray experiment is immobilization of the biomolecules onto the solid surface, in order: (i) to obtain maximum binding efficiency; (ii) to avoid the unspecific binding; and (iii) not to affect the biomolecules functionalities and conformations [4]. Both covalent and non-covalent attachment of biomolecules (protein and DNA) have been reported for immobilization of the capture agents onto various types of substrates and surfaces [5,6], and more close to our approach, Ressine et al. [7–9] have proposed porous silicon as a suitable and promising alternative to commonly encountered glass slides for microarray analysis.

Since the discovery of porous silicon (PS) by Arthur Uhlir Jr. and Ingeborg Uhlir in 1956, this material has been extensively studied so far, on one hand for a better understanding of the mechanisms behind its surprising properties, and on the other hand for finding the applications that would facilitate new advances. Thus, different optoelectronic devices, like LEDs or waveguides, have been developed on the basis of its photoluminescent property [10], and also supports for cell growth and even tissue engineering have been proposed since its biocompatibility was demonstrated [11]. In this context, Ressine group has reported the use of both macroporous and nanoporous silicon as candidate substrates for forward and reverse phase microarrays. Furthermore, the IMT group published, also, the results obtained in the detection of the C-reactive protein (CRP) levels based on the antigen–antibody reaction using different protein microarray platforms, including porous silicon [12]. It was demonstrated that, due to its 3D porous structure and consequently high surface area-to-volume ratio, the PS substrates can be used as antibody array support to detect even pM range analytes. However, in spite of these remarkable achievements, the difficulty

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to control the chemistry of such a large internal area represents an important issue, and a parallel test method should be required to confirm the data. A dual detection system on PS was reported by Chen et al., where the hydrogenated PS surface was used as substrate for gel pad formation, and both fluorescence spectroscopy and the molecular mass analysis have been used [13].

The electrochemical impedance spectroscopy (EIS) based detection transduces changes in interfacial properties between the electrode and the electrolyte induced by different molecules' attachment (immobilization) on surface. This technique offers information not only about the surface modification, but it has been used to monitor the DNA hybridization, conformational changes, or damages [14]. If the other detection schemes require the labelling of the target DNA with a fluorophore, the EIS detection is a label-free tool and, thus, possesses advantages of low cost, simplicity, and ease of miniaturization [15]. These advantages represent the main reasons for employing the electrochemical impedance and consequently for developing the capacitance biosensors to study also cell growth [16], protein binding at surfaces [17], or antibody–antigen binding [18]. Finding a substrate with high sensitivity and selectivity are important for gaining all the advantages of this electrochemical simple tool, and the use of nanomaterials represents an alternative [19], the first choice being the increase of the sensor surface area, electrical conductivity and connectivity.

Herein, we report the fabrication of different nanostructured porous silicon surfaces and their ability to efficiently immobilize biotin. These structures have been tested by both fluorescence and impedance spectroscopies for bio molecular recognition – biotin–strepavidin couples – in order to achieve an optimum surface for protein's immobilizations.

2. Experimental procedure

2.1. Materials and protein labelling

The porous silicon substrates were prepared using p-type silicon wafers from SiMAT (Germany), and HF-50% and ethanol absolute from Sigma Arldrich (Germany), respectively.

Biotin, streptavidin and HEPES were purchased from Sigma Arldrich (Germany).

The strepavidin fluorescence labelling was performed using commercial Cy3-NHS ester (Amersham, GE Healthcare, UK) using the manufacturer protocol: 1 mg/ml in phosphate buffered saline (PBS) (pH 7.4) protein solution was mixed with 1 mg Cy3-NHS ester in DMSO and stirred in the dark for 45 min at room temperature. The reaction was quenched by addition of one-tenth volume of PBS. The labelled protein was purified on NAP columns (Amersham, GE Healthcare, UK) and only those aliquots with fluorophore-to-protein ratio higher than two were further used.

The PBS used as printing buffer in half dilution was purchased from ArrayIt Corporation (Sunnyvale, US).

2.2. Porous silicon preparation

Two types of porous silicon (PS) layers were fabricated by electrochemical etching of (100) crystallographic orientation, p-type Si substrates, with resistivities of 5–10 Ωcm (***Si 1***) and 19.5–22.5 Ωcm (***Si 2***), respectively, using a single-tank wet etching system (AMMT GBH) for 4 in. diameter silicon wafers with programmable power supply and dedicated software for time-based current profiles. To provide an ohmic contact for homogeneous anodization, a thin aluminium layer has been sputtered on the wafer backsides.

Before anodization, the silicon wafers were cleaned in Piranha solution (H_2SO_4:H_2O_2, 3:1) at 80 °C for 30 min in order to remove organic impurities, rinsed for 20 min in deionised water (18 MΩ), and finally dried under stream of nitrogen. The process parameters were adjusted in order to obtain PS layers with high porosity, few nanometers' pore diameters, highly homogeneous over its surface, as well as being of optimal porosity to avoid stress in the porous structure causing subsequent crack pattern after drying [20–22]. Thus, we have conducted a series of experiments to develop a PS layer that meets all these requirements for each of the Si substrates used, and the most appropriated conditions are: 1:1 (v/v) electrolyte solution of 50% HF and 95% ethanol, 30 mA/cm^2 current density, and 300 s process time.

During the porosification process, the current and also the voltage have been monitored, and the recordings obtained for each of the PS test layers – ***PS 1*** and ***PS 2*** – are presented in Fig. 1. The plots contain many features considering that the Si etching is performed with a constant current. While the current fluctuates generally around 4 mA (which represents ∼2‰ from the applied value), the recorded voltage has a different profile. There is a transient region in the beginning where necessary charges are built up at surface before etching starts, followed by an irregular pattern which may be due to the local oxide build-up and etching or due to hydrogen bubbles interfering with electrolyte flows. Also, modification of Si wafer resistivity during porosification, and more important, at silicon-electrolyte interface may be also responsible for this apparent noise; the potential variation is more accentuated for the substrate with an initial higher resistivity (***PS 2***).

After the etching process, the porosified Si wafers were washed in ethanol and deionised water to remove residual HF, and then dried under stream of pure nitrogen. The test structures where obtained by dicing the wafers into individual 2 × 2 cm^2 chips.

Since immobilization of a biomolecule on a substrate requires the presence of chemical active groups, the following protocol has been used for surface modification:

- silanization in 1% APTES (3-aminopropyltriethoxysilane) solution in ethanol at room temperature during 6 h.
- successive washing steps in deionized water and ethanol for 10 min to remove the unbounded silane and then the slides were placed in oven for 30 min at 110 °C.
- the silanized samples were stored in ethanol at 4 °C until required for the study.

To assess the performances of the newly developed nanostructurated substrates as biointeraction dual mode transducers, we compared their responses with those of standard flat silicon substrates.

2.3. Biotin–strepavidin reaction

The test slides, both flat (***Si 1/Si 2***) and the porosified (***PS 1/PS 2***) silicon, were printed with one dilution of biotin in 8 × 8 spots per sub-array configuration, using a contact printer OmniGrid Micro printer (Genomic Solutions-UK). The stamp time for all the samples was 500 ms and pin size 200 μm; the humidity in the printer chamber was 80% and temperature 20 °C. In order to control the pin contact force and duration, the following motion parameters of the instrument set-up were established: acceleration 2000 mm/s^2, velocity between 10 and 170 mm/s.

The biotin dilution for printing was obtained by dissolving 11.1 mg biotin in DMSO and PBS. The samples' printing was followed by an overnight incubation at 4 °C in the dark, being then washed twice in HEPES for 5 min. To prevent the nonspecific binding of the interest molecules, a high protein, BSA (bovine serum albumin) dissolved in HEPES – 1 M BSA in 0.01 M HEPES solution (2 ml HEPES in 198 ml DI) – has been used for surface blocking (20 h reaction time). After these steps, the samples were washed in HEPES and then rinsed with DI.