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Immobilization strategies and electrochemical evaluation of porous silicon based cytochrome c electrode

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ABSTRACT

Quasi-reversible and direct electrochemistry has been observed at a new electrochemical interface constructed by functionalization of porous silicon microcavities (PSiMc) with either amino (APTES) or mercapto (MPTS) propyltriethoxysilane and the redox protein cytochrome c (cyt c). The high specific surface area, tunable pore size (observed by scanning electron microscopy) and functional surface of PSiMc permitted the infiltration of molecular binders and cyt c into the porous structure. The optical response of PSiMc was evaluated during all modification steps. In all the cases a red shift in the reflectance spectra after substrate modification was observed, indicating the infiltration and immobilization of organic binders and the cyt c protein. Dose-response curves demonstrated that biofunctionalization with APTES followed by glutaraldehyde (GTA) amine activation provides higher amounts of immobilized protein with 4.73 nmol protein/cm^2 of etching area compared with 1 nmol protein/cm^2 when using MPTS as a molecular binder. Infrared spectroscopy (FTIR) also demonstrated the adsorption of the protein within the surface nanostructure. The PSiMc/cyt c hybrid devices showed high electrochemical stability due to the retaining of the protein's redox activity even after several months of its immobilization

1. Introduction

The electron transfer reactions of proteins and enzymes at electrodes can be studied and controlled under a variety of conditions that mimic some of their native reaction properties. Understanding of these reactions fundamentally can provide insight into physiological electron transfer processes as well as impetus for the further development of biosensors and bioelectrocatalytic systems [1–6]. One of the most promising enzymes for electrochemical studies, for its fast response and outstanding redox activity, is cytochrome *c* (*cyt c*), a metalloprotein that is primarily known as an electron-carrying protein between ubiquinone-cytochrome *c* oxidoreductase (Complex III) and cytochrome *c* oxidase (Complex IV) in the inner mitochondrial membrane [7]. It consists of a single polypeptide chain of 104 amino acids (MW: 12400 Da) with a redox-active heme group covalently attached to two cysteine residues. Some of the notable characteristics of *cyt c* are its activity over a wide range of pH (from 2 to 11), and its ability to perform biocatalytic reactions even at relatively high temperatures. Exploiting its redox activity, *cyt c* has been immobilized onto electrodes to be used for the detection of the short-lived superoxide radical $O_2^{\bullet -}$, which is implicated in the pathogenesis of several diseases, and in the determination of the extracellular H_2O_2 released by living cells of human hepatoma [8–10].

Numerous studies on cytochrome's electrochemistry when adsorbed on Pt, Hg, Au, Ag, and other electrodes have shown that direct enzyme adsorption can produce large conformational changes often to the extent of denaturation [11,12]. Methods to limit denaturing after adsorption are available through the modification of the electrode surfaces. The adsorption of the enzyme on chemically modified macroscopic electrodes facilitates direct electron transfer and stabilizes the enzyme against denaturation, retaining its biological activity [13]. Porous electrodes are considered some of the most attractive platforms for the immobilization of biomolecules. Therefore, porous materials have been tested as supports for immobilizing *cyt* c, evaluating the electrochemical activity and stability of physically adsorbed *cyt c*, for instance on macroporous active carbon [14] and multilayer nanozeolite modified indium-tin-oxide (ITO) [15]. The covalent attachment of *cyt c*

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has been evaluated e.g. on amino-functionalized mesoporous silica thin films [4]. The results of these works demonstrated that the redox activity of the protein is retained after the physical or covalent immobilization procedure; moreover, the hybrid substrates in all the cases presented a quasi-reversible faraday behavior. Other studies have been conducted in the same direction by using macroporous gold films [16], mesoporous films of antimony doped tin oxide (ATO) [17], nanotextured diamond surface [18], mesoporous molecular sieves [19], NaY zeolite matrix [20], or mesoporous tin-rich ITO [21]. The main challenge in the development of these hybrid substrates was to obtain effective protein immobilization and the maintenance of the bioactivity to get a stable and sensitive response signal. Here we report porous silicon (PSi) substrates in an effort to assess the behavior of the redox-active *cyt c* attached to a semiconductor electrode surface. This promising material has many superior properties compared with those mentioned above, such as tunable porosity, high thermal stability, chemical inertness, easily modifiable surface, low cost, large surface area (up to 800 m^2/g), biodegradability (for in vivo measurements), photoluminescence and photonic properties [22]. PSi has already been used in a number of cases as substrate to immobilize cells, such as human neuronal [23], rat pheochromocytoma, or human lens epithelial cells [24], or biomacromolecules, e.g. the photosynthetic reaction center from the purple bacterium Rhodobacter sphaeroides [25], glucose oxidase [26], bovine serum albumin [27], as well as horseradish peroxidase as a biosensor for phenol monitoring [28]. These pieces of work have proved that the enzymatic activity of the immobilized proteins is preserved and demonstrated the excellent biocompatibility of PSi. The physical properties of a PSi electrode covered by a redox active monolayer have been studied showing the capability of the PSi material to support electron transfer [29]. However, to our knowledge this work presents the first attempt in evaluating the electrochemical properties of a PSi/*cyt c* based electrode. In this work we used porous silicon microcavities (PSiMc) which are 1D photonic bandgap structures that include a spacer layer positioned between two Bragg reflectors. The microcavity structure produces a narrow photonic resonance that is shown as a dip in the reflectance spectrum. The resonance peak is highly sensitive to changes in the effective refractive index such as those arising from binding of biomolecules onto the surface [30,31]. This provides an easy method to evaluate infiltration of organic and biological molecules inside the pores. The immobilization of *cyt c* on the functionalized PSiMc substrate was performed via covalent binding through either its lysine or cysteine amino acid residues to APTES/GTA or MPTS binders, respectively. The redox activity of the immobilized protein has been demonstrated by protein film voltammetry.

2. Experimental

2.1. Fabrication of porous silicon devices

PSi samples were prepared by the wet electrochemical etching process using highly doped, p-type (boron doped) silicon wafers (thickness 500-550 μm) with a 0.001-0.005 ohm-cm resistivity, and with the crystallographic orientation of (100), purchased from WRS materials. The electrolyte consisted of hydrofluoric acid (48 wt%, Golden Bell) and ethanol (100%, Sigma-Aldrich) in the volumetric ratio of 3:7. The anodization time and current density were controlled by a computer interfaced electronic circuit. Samples were fabricated at room temperature and freshly etched samples were washed with ethanol and dried with pentane. To perform this work we have selected the PSi microcavity (PSiMc) configuration, mainly due to its optical features in the reflectance spectra that easily allow the detection of the infiltrated material into the porous structure. PSiMc configuration consists of an active porous layer embedded between two multilayered mirrors (Bragg reflectors). The PSiMc were produced by alternating layers of high porosity (H; refractive index, n = 1.14395) and low porosity (L; n = 1.25865), with current densities of 70 and 30 mA/cm^2. Anodization times of 6.35 s and 10.67 s for H and for L, respectively, were used for the fabrication of the corresponding dielectric Bragg mirrors. The PSiMc structures were fabricated with the configuration of (HL)x5 HH (LH)x5, where (HL)x5 corresponds to the first Bragg reflector, HH to the cavity and (LH)x5 to the second inverted Brag reflector. In order to assure a good molecular penetration, the first layer of the structure was prepared as a high porosity one [26] (it also acts as a sacrificial layer). The PSiMc samples were thermally oxidized at 600 °C for 30 min in O_2 atmosphere to stabilize and protect them against environmental contaminants and/or natural aging.

2.2. Functionalization of PSiMc and covalent protein immobilization

Two *cyt c* binding strategies on PSiMc devices were tested with one and two-step procedures using mercapto (MPTS, 95%, Sigma-Aldrich) and amino-propyltriethoxysilane (APTES, 99%, Sigma-Aldrich), the latter together with glutaraldehyde as a crosslinker reagent (GTA, 25%, Sigma-Aldrich).

Method 1. The PSiMc devices were dipped in 3-aminopropyltriethoxysilane (5% in toluene) for 1.5 h, after this reaction time the non-specific silane binding was eliminated by rinsing several times the modified PSiMc samples with toluene, the samples were then dried with N_2 and baked in an oven at 110 °C for 15 min [31]. The activation of the silane amine group was performed by 20 min incubation of silanized PSiMc samples in freshly prepared glutaraldehyde (GTA) solution (2.5% in 3.8 mM PBS, pH 7.2 buffer, from Sigma). The non-bound GTA was removed from the surface by rinsing a number of times with PBS buffer; the derivatized PSiMc samples were dried under a stream of N_2. The biofunctionalization of PSiMc was performed by exposing the PSiMc/APTES/GTA hybrid devices to *cyt c* (from Saccharomyces cerevisiae, ≥85% based on Mol. Wt. 12,588 basisfrom Sigma, Inc.) solutions for 2 h resulting in lysine amino acid binding. PSiMc/APTES/GTA/*cyt c* devices were prepared at various protein concentrations (10, 25, 50, 100, 200 and 560 μM) in 3.8 mM PBS, pH 7.2 buffer. Non-specific protein binding was avoided by strongly rinsing the *cyt c* modified structures with PBS buffer. For buffer solutions pure water was used throughout, which was obtained using a Millipore Q water purification equipment. Its resistivity was 18.1 MΩ cm.

Method 2. This protocol used 3-mercaptopropyltrimethoxysilane (5% in isopropanol) as functional reagent, the experimental procedure was similar to the above described protocol for APTES, with the exception that GTA is not necessary here. This method provided thiol groups (S-H) on the PSiMc surface that promote the protein molecular binding through the formation of a disulphide bridge (S-S) with the cysteine amino acid residue. Fig. 1 shows the chemical adsorption mechanisms. The PSiMc biologically interfaced samples were stored at 4 °C until their characterization. During the electrochemical studies the PSiMc, PSiMc/APTES/GTA, PSiMc/APTES/GTA/*cyt c*, PSiMc/MPTS and PSiMc/MPTS/*cyt c* samples were the working electrodes.

2.3. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed with a UHR Dual Beam FEI Helios Nanolab 600 field emission scanning electron microscope. The samples were mounted on a conductive carbon tape as prepared. Images were captured at magnifications of 20 000, 200 000, 400 000x. To characterize the element distribution