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Bioelectrochemical studies of azurin and laccase confined in three-dimensional chips based on gold-modified nano-/microstructured silicon

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ABSTRACT

Double-sided three-dimensional porous silicon chips, 6 mm × 6 mm, covered with a 40 nm gold (nano)layer, were fabricated from a porous silicon wafer. Scanning electron microscopy along with electrochemical characterisation showed sample conductivity, mechanical stability, and high surface area of the thus fabricated devices, *viz.* 10 times higher electrochemically active surface area compared to the geometric area. The three-dimensional gold coated silicon chips were further modified with thiol layers, followed by immobilisation of a simple copper-containing redox protein, azurin, or a complex multi-copper redox enzyme, laccase. The bioelectrochemical studies showed very high surface concentrations of azurin and laccase, *i.e.* close to the theoretical monolayer coverage. However, direct electron transfer reactions between the biomolecules and gold surfaces were observed only for a small percentage of the immobilised redox protein and enzyme, respectively. Thus, highly efficient oxygen-bioelectroreduction on laccase-modified 3D thiol-gold-porous silicon chips (as compared to planar laccase-modified gold electrodes, 42 μA/cm^2 *vs.* 7 μA/cm^2, respectively) was obtained only in the presence of an efficient soluble redox mediator.

1. Introduction

Following the first reports by Uhlir and Turner (Turner, 1958; Ulhir, 1956), porous silicon (PSi), an interesting material for a diversity of applications, including chemical (King et al., 2007; Schmedake et al., 2002) and biochemical sensing (Cunin et al., 2002; Drott et al., 1997; Laurell et al., 1996; Orosco et al., 2006; Setzu et al., 2007), became readily available. The versatility of PSi can be explained by several unique features: intrinsically high surface-to-volume ratio (S/V); a range of three-dimensional (3D) pore morphologies that can be easily realised by adjusting the multi-parameter fabrication process; tuneable optical and electrical properties; biocompatibility; and finally relatively simple technologies for large volume PSi fabrication that also are compatible with standard microelectronic techniques and microelectromechanical systems.

PSi can be fabricated by anodic etching, a multi-parameter electrochemical process affecting the morphology and geometry of PSi elements obtained (Christophersen et al., 2003; Föll et al., 2002). Still, applications of PSi are limited, especially regarding bioelectronic devices, *e.g.* amperometric and potentiometric biosensors, biofuel cells, and biocomputing devices, where an electric signal is translated into the bio-signal and *vice versa*, mainly because of the intrinsic low conductivity of PSi. Macro-, micro-, and nanoscale electronic elements of different types based on organic and inorganic precursors have been exploited as building-blocks of bioelectronic devices, such as metal electrodes, field-effect transistors (FETs), and piezoelectric crystals (Katz, 2006); conductive polymers (Berggren and Richter-Dahlfors, 2007); as well as silicon-based materials (Yakimova et al., 2007) including PSi (Buckberry and Bayliss, 2001; Schoning et al., 1997; Thust et al., 1996). However, there are a very limited number of publications describing possible applications of PSi-based amperometric bioelectronic devices (Alves et al., 2008; Jin et al., 2006; Lopez-Garcia et al., 2007; Song et al., 2006). Moreover, to our best knowledge there is still no study providing a detailed bioelectrochemical investigation of redox proteins and enzymes loaded into PSi matrixes.

For successful applications of PSi in amperometric bioelectronics it is essential to increase the PSi conductivity while maintaining

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its high S/V ratio. A number of methods were reported to improve the conductivity of PSi, such as coating with a conducting polymer (Abalyaeva and Efimov, 2000), doping procedures (Zhao et al., 2006), and metal deposition into the PSi matrix using sputtering or wet depositions techniques (Garcia Salgado et al., 2008).

Below we describe bioelectrochemical investigations of nano-/microstructured PSi chips (NMPSi), which were covered with a thin layer of gold and modified with a small copper containing redox protein, azurin, and a multicopper redox enzyme, a fungal laccase. The *Pseudomonas* sp. azurin and *Trametes* sp. laccase, used in our studies, are bioelements widely used in different types of amperometric bioelectronic devices, *viz*. biosensors (Scheller et al., 2005; Shleev et al., 2006a,c), biofuel cells (Coman et al., 2008; Kamitaka et al., 2007; Service, 2002; Soukharev et al., 2004), biodiodes (Alessandrini et al., 2005; Bonanni et al., 2005; Rinaldi and Cingolani, 2004), and biotransistors (Shleev and Ruzgas, 2008).

2. Materials and methods

2.1. Chemicals and buffers

Unless otherwise specified, all chemicals were purchased from Sigma–Aldrich GmbH (Schnelldorf, Germany). Dimethylformamide (DMF), 99.8%, and hydrofluoric acid (HF), 40% (w/v), was from Merck KGaA (Darmstadt, Germany). Silicon wafers of p-type, <1 0 0> orientation and resistivity of 6–8 Ω cm, were obtained from Addison Engineering Inc. (San Jose, USA). All solutions were prepared using water purified with a PURELAB UHQ II system from ELGA Labwater (High Wycombe, UK).

2.2. Proteins

Pseudomonas aeruginosa azurin (MW 14.0 kDa) was from Sigma–Aldrich GmbH and was used without further purification. *Trametes hirsuta* laccase (MW 70.0 kDa) was obtained from the basidiomycete *T. hirsuta* (*Wulfen*) *Pilát* (*Coriolus hirsutus* (*Wulfex Fr.*) *Quél.*), strain *T. hirsuta* 56, provided by the laboratory collection of the Moscow State University of Engineering Ecology (Russia). The basidiomycete was grown by submerged cultivation (Gorshina et al., 2006) and laccases were isolated from a culture medium as described previously (Shleev et al., 2004). The homogeneous preparation of the enzyme (10 mg ml^{-1}), as judged from SDS-PAGE, was stored in 100 mM phosphate buffer, pH 6.5, at 253 K.

2.3. Electrochemical measurements

Cyclic voltammetry (CV), linear scan voltammetry, and chronoamperometry of planar and 3D electrodes were performed in a 20 ml electrochemical cell, carrying a SCE (240 mV *vs*. NHE) reference electrode and a platinum mesh counter electrode, using a potentiostat/galvanostat (model # 2059, combined with a 7800 function generator) from Amel Instruments (Milano, Italy). All potentials in the manuscript are reported *vs*. NHE.

2.4. Fabrication of PSi electrodes

The setup for the highly reproducible fabrication, the chemical mechanism of the reaction, and the influence of the fabrication parameters on the 3D nanostructured layers were detailed previously (Ressine et al., 2006, 2007). Briefly, 3-in. double-sided polished silicon wafers (<1 0 0> orientation, 6–8 Ω cm resistivity, *p*-type), were anodised on both sides in a two-step process. The anodisation was performed in a mixture (1:10, v/v) of HF and DMF for 1 h with the backside illuminated (to avoid backside doping) at a current density of 2 mA cm^{-2} in a two-compartment electrochemical cell, where the wafer served as the cell divider. During the procedure, the positive charge carriers in the silicon wafer migrated towards the anodic side of the wafer, solubilising the silicon at the anodic side, and thus forming the nano-/microstructured layer. Sequentially, the procedure was repeated for each side of the wafer by switching the polarity of the power supply. The etched Si wafers were subsequently diced into 6 mm × 6 mm square chips, providing 90 electrodes from a single wafer, with high morphological reproducibility (Ressine et al., 2005). Electrical contacts were applied by gluing wires to both sides of the fabricated 3D structured chips with a conductive adhesive (Kemtron Ltd., UK). Finally, chip surfaces were coated with 10 nm or 40 nm gold films by argon plasma facilitated sputtering on both sides using a S150B Sputter Coater from Edwards (West Sussex, UK). The thickness of deposited gold layers was controlled by an Edwards FTM Film Thickness Monitor. Nonporous chips for reference purposes were sputtered with gold at the same processing cycle to provide precisely the same thickness of the deposited layers as on porous chips.

2.5. Characterisation of PSi chips

Imaging and structural characterisation of Si chips were performed using a FIB/SEM – FEI NanoLab 600. Prior to measurements, Au/Si and Au/NMPSi chips were electrochemically cleaned by cycling 30 times between 0 mV and 1900 mV *vs*. NHE in 0.5 M H_2SO_4. The diffusional electrode area (A_{diff}) was calculated from the reversible voltammograms of the clean gold electrodes immersed in 0.1 M phosphate buffer pH 7.0 containing 5 mM $K_3[Fe(CN)_6]$ using the Randles–Sevcik equation (Supplementary information) (Bard and Faulkner, 1980), assuming the diffusion coefficient of ferricyanide to be equal to 7.5×10^{-6} cm^2 s^{-1} (Adams, 1969). Microscopic (real) electrode areas (A_{real}) were calculated using the theoretical charge density (σ_t) associated with the reduction of gold oxide, *i.e.* 390 μC cm^{-2} (Supplementary information) (Trasatti and Petrii, 1991). For comparison, in addition to nonporous Si biochips, a gold disk electrode from Bioanalytical Systems (model MF-2014, West Lafayette, IN, USA) with a geometrical area of 0.02 cm^2 was also used as a well-characterised planar electrode control. To obtain a very smooth surface the gold electrode was polished with a DP-suspension, *i.e.*, a high performance diamond-based abrasive (1.0 μm, Struers, Copenhagen, Denmark), and a de-agglomerated alumina polishing suspension (0.1 μm, Struers), rinsed with Millipore H_2O, and sonicated in Millipore H_2O for 10 min after each polishing step.

2.6. Bio-modification of the electrodes with bioelements

P. aeruginosa azurin was immobilised using the procedure described in Gaigalas and Niaura (1997). Briefly, chips were immersed in 2 mM hexanethiol (ethanol solution) and left to react for 17 h at room temperature. After washing 10 min with 10% aqueous ethanol, the SAM-modified electrode was immersed in 100 μl solution of 1 mg ml^{-1} azurin in 10 mM phosphate buffer pH 7.4 for 2 h.

For laccase immobilisation, the electrode was immersed in 2 mM 4-aminothiophenol (ethanol solution) and left to react for 16 h at room temperature. After washing for 10 min with 10% ethanol, the SAM-modified chips were immersed in 100 μl solution of 2 mg ml^{-1} laccase in 10 mM phosphate pH 6.0 and incubated for 1.5 h to let the enzyme diffuse through the electrode. 100 μl of 10 mM phosphate pH 6.0 containing 35 mM N-hydroxysuccinimide (NHS) and 52 mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were added for covalent binding of the enzyme to the chips surfaces and left to react for 3 h.