Sensors and Actuators B 244 (2017) 142–150

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Reversible sensing of heavy metal ions using lysine modified oligopeptides on porous silicon and gold

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ARTICLE INFO

Article history:
Received 20 August 2016
Received in revised form
20 December 2016
Accepted 27 December 2016
Available online 29 December 2016

Keywords:
Heavy metal ion detection
Phytochelatins
Porous silicon
QCM
Corrosion

ABSTRACT

Phytochelatins (PCs), oligomers of glutathione, naturally chelate heavy metals (HMs) in aqueous solution; small peptides such as these cannot be used as covalently bound bioprobes on transducer surfaces due to their propensity to induce corrosion of standard sensor supports such as gold and porous silicon (PSi). In this work, we chemically modify a commercial PC oligopeptide with a six poly-lysine (Lys) chain, thereby changing its isoelectric point from 4.2 to 6.9. PC-Lys bioprobes were successfully immobilized on both PSi multilayers and flat gold surfaces. The interaction of PC-Lys and HM ions, namely Lead (II), Cadmium (II) and Arsenic (III) in aqueous solution was quantified by optical spectroscopic reflectometry and quartz crystal microgravimetry. As a result, it was proven that the biomolecular interactions are reversible and the affinities between PC-Lys complex and HM ions are in the range of 10^{-12} M.

1. Introduction

Water and soil pollution caused by the prevalence of heavy metal (HM) ions is a worldwide environmental problem predominantly caused by industrial activities but also attributed to natural phenomena such as concentrated sediments of particular minerals [1]. Beyond remedial actions, there is a need for the monitoring of HM ion levels in primary resources such as soil and drinking water [2,3]. Although commercially available instruments for HM detection do exist, there is strong interest in developing innovative instruments such as biosensors that can accurately measure polluting levels of HM ions in water and soil.

Biosensors are generally composed of a biological recognition element, the so-called bioprobe (of either a DNA single strand, an enzyme, a protein, and so on) which is properly conjugated with a transducer component that converts biomolecular interactions into signals (optical, electrical, electrochemical, gravimetrical), thereby providing a final readout to end-users. Over the last twenty years, a silicon-derived material, namely porous silicon (PSi), has been widely studied due to its particular properties [4–7]. PSi is fabricated by an electrochemical etching of doped crystalline silicon in hydrofluoridric acid (HF) water solution [8], exhibiting a sponge-like morphology characterized by a specific surface area up to 200–500 $m^2\,cm^{-3}$ and is therefore very sensitive to the presence of biological or chemical species which penetrate inside its pores [9]. Moreover, since silicon dissolution is a charge-mediated, self-stopping process, tuning the etching parameters (i.e. etch time, HF concentration, doping level, and so on) it allows for the modulation of PSi porosity in each layer, which in turn permits the fabrication of multilayered structures. Due to high air content (up to 80–85%), PSi is an almost perfect electric insulator while, from an optical point of view, its low porosity and high porosity layers are very smooth and therefore high quality optical spectra in both a transmission and reflection mode can be obtained from a visible to near-infrared wavelength region (500–1600 nm). Several photonic multilayer devices were demonstrated, such as optical microcavity, Bragg mirrors, rugate filters, and the Thue-Morse (T-M) sequences, all providing high quality factors and sharp optical resonances. Since, upon exposure to biochemical substances, the average refractive index changes drastically, PSi can be used as a smart optical transducer material [10].

As a result of our experiments we already demonstrated that a T-M PSi optical structure, due to the characteristic alternation of its porosity layers, is more sensitive than a symmetric multilayer [11]. The main drawback, however, of this fascinating material is its chemical instability: as-etched PSi ages quickly upon exposure to the atmosphere since Si-H bonds tend to be substituted by Si-O-Si ones, and oxidized PSi is also easily corroded in aqueous environments [12–14].

Chemical and biological passivation procedures allowing functionalization and stability of PSi supports have previously been

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http://dx.doi.org/10.1016/j.snb.2016.12.132
0925-4005/

published [15,16]. Another quantitative measuring technique commonly used in the development of biosensors is the Quartz Crystal Microbalance (QCM) technology [17–20]. QCM is currently used to measure extremely small mass changes with high sensitivity down to nanograms thereby becoming a cost-effective tool in biosensing. Standard QCM exploits the properties of piezoelectric quartz resonator (QRs) in quantifying the resonance frequency shift Δf when a mass m is absorbed into or desorbed from the QRs' surface, according to Sauebrey's equation (1):

$$\Delta f/f0 = -(\Delta m/A\rho l) \quad (1)$$

where $f0$ is the fundamental frequency of QR, A is the area of the layer of gold partially covering the QR, Δm is the mass variation corresponding to frequency shift Δf and ρ and l are the quartz density and thickness, respectively [21].

The PSi and QR surfaces can both be functionalized by bioprobes in order to realize optical and nanogravimetric biosensors, respectively. The key point is bioconjugation, which is the procedure that should be used for the immobilization of biological sensing elements onto the transducer surface. One of the most useful biomolecular probes for heavy metal ion detection is a family of oligopeptides with the ability to bind them, known as Phytochelatins (PCs). PCs are small, heavy metal-binding proteins with the general structure of $(\gamma\text{-Glu-Cys})_n\text{Gly}$ (n = 2–11) that complex with toxic metal ions: a well known mechanism developed in the vegetal world to protect fungi and plants in nature [22,23]. However PCs cannot be easily covalently grafted to solid surfaces due to that fact that, in the presence of ligands, molecular binding triggers charge-mediated corrosion of bioconjugated supports.

In this work we describe the synthesis of Phytochelatin 6 (PC_6) as modified by a Lysine chain (six amino-acid tail, Lys_6) and its immobilization on PSi T-M and QR surfaces by proper functionalization strategies. The novel PC_6-Lys_6 synthetic oligopeptides fix support corrosion and effectively detect HM ions in solutions. In our experimentation, spectroscopic reflectometry, gravimetric analysis and scanning electron microscopy were used as characterization techniques.

2. Materials and methods

2.1. Porous silicon fabrication

PSi structures were fabricated by electrochemical etching of crystalline Si (0.001 Ω cm resistivity, ⟨100⟩ oriented, 500 μm thick) in HF (50% in volume), water and ethanol solution (1:1:1) in the dark and at room temperature (RT). PSi-based T-M realization and characterization have been previously studied [24]. Briefly, starting from a bilayer characterized by a low porosity (high refractive index, H)-high porosity (low refractive index, L) couple, the 2n layers T-M sequence was generated following the substitution rules H → HL and L → LH into the 2n-1 layers' structure, so that the sequence of the 64 layers could be expressly written as LHHLHLLHHLLHLHHLHLLHLHHLLHHLHLLHHLLHLHHLLHHLHLLHLHHLHLLHHLLHLHHLHLLHHLHLLHLHHLHLLHHLHLLHLHHL. A current density equal to 100 mA/cm^2 was applied for 1.6s, in order to obtain the high refractive index (n_H = 1.79 at λ = 753 nm) layers, with a thickness d_H = 120 nm, and a current density of 200 mA/cm^2 for 1.2 s for the low refractive index (n_L = 1.53 at λ = 753 nm) layers with a thickness of d_L = 156 nm. Refractive indexes and thicknesses were determined by spectroscopic ellipsometry on single layer samples (data not shown here). For each layer, time intervals of 5 s were used during the etching process in order to recover HF concentration at dissolution edge and start the next layer formation with zero current density, so that variations in the current were always the same for each layer. The etching area was 0.98 cm^2. After the electrochemical process, the dimension of the pores was increased by rinsing the "as-etched" porous silicon structures in KOH-ethanol solution (1.5 mM) for 15 min [25]. Even if the PSi stacks were made of several tenths of porous layers, the reflectivity spectra were of a very high quality within a wide range of wavelengths, as shown in Fig. S1(A) of Supporting information, together with a scheme of T-M sequence (Fig. S1(B)). After this the devices were thermally oxidized in order to prevent uncontrolled environmental aging and limit corrosion in alkaline solutions [26]. Oxidation was performed in an oven on exposure to pure O_2, applying a two-step process: 30 min at 400 °C followed by 15 min at 900 °C.

2.2. Modified peptides synthesis and characterization

PC_6-Lys_6 (4 mg) was produced by Primm s.r.l. (Italy) following our indications on the position of the Lysine chain with respect to PC_6 orientation by a standard solid-state synthesis procedure. A solid, sugar-like powder with a solubility of 1 mg/mL in H_2O was obtained and released. Molecular weight and purity were characterized by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Spectrometry and High Performance Liquid Chromatography, respectively. Mass spectrometry revealed 2235.23 Da for the oligopeptides, while a chromatogram revealed a purity higher than 95%.

2.3. Chemical modification

2.3.1. Porous silicon modification

PSi surfaces were modified by following a well-established method [27]. Fig. S2(A) shows the functionalization procedure needed to obtain primary amine on PSi surfaces. The first passivation step was the oxidation of PSi in order to stabilize the surface against spontaneous aging due to the Si-H groups on its surface just after fabrication. Oxidized PSi was treated in piranha solution (H_2SO_4:H_2O, 4:2) at RT for 30 min in order to activate Si—O—Si in Si—OH groups. After several rinses in distilled water (DI-H_2O) and drying under N_2, samples were treated by a solution of 5% 3-(aminopropyl)triethoxysilane (APTES) (Sigma Aldrich) in anhydrous toluene for 30 min at RT, washed with anhydrous toluene three times, cured on a heater at 100 °C for 10 min and washed again twice with anhydrous toluene. The silanized PSi samples were then treated with the cross-linker Bis [sulfosuccinimidyl] suberate (BS^3 by Thermo scientific, USA) 1.7 mM in PBS 1X at 4 °C for 4 h, washed three times with PBS 1X and once with DI-H_2O. BS^3 brings a sulfo-N-hydroxysulfosuccinimide (sulfo-NHS) group that reacts with primary amines at pH 7–9 to form stable, covalent amide bonds [28]. In this way the BS^3 binds from one side to the primary amines available on PSi silanized surface, and on the other side, to the primary amine exposed by PC_6. The PC_6 (AnaSpec IGT group) and PC_6-Lys_6 (Primm s.r.l.) were diluted in PBS 1X in order to obtain a 2 mM concentration. The samples were incubated at 4 °C for 2 h.

2.3.2. Modification of quartz resonators

Fig. S2(B) reports the modification of QR using thiol-PEG-amine to obtain primary amines on QR gold surfaces. Thiol PEG Amine, (JenKem Technology USA) was flowed in the QCM cell at a 2 mM concentration in PBS 1X and left in incubation for 2 h at RT. After extensive rinsing of PEGylated-QR (5 min at 5 rpm) in PBS solution, functionalization was completed, the same as in the case of PSi, using BS^3 and PC_6 and PC_6-Lys_6 (see Schemes in Fig. S2(C) and (D)).

2.4. Monitoring of HMs ion interaction

Interaction between the modified transducer (i.e., PSi and gold) surfaces and Lead (II), Arsenic (III) and Cadmium (II) ion solutions was stationary, characterized by spectroscopic reflectometry using