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Protein conformational changes revealed by optical spectroscopic reflectometry in porous silicon multilayers

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Abstract

The protein–ligand molecular interactions imply strong geometrical and structural rearrangements of the biological complex which are normally detected by high sensitivity optical techniques such as time-resolved fluorescence microscopy. In this work, we have measured, by optical spectroscopic reflectometry in the visible–near-infrared region, the interaction between a sugar binding protein (SBP), covalently bound on the surface of a porous silicon (PSi) microcavity, and glucose, at different concentrations and temperatures. Variable-angle spectroscopic ellipsometric (VASE) characterization of protein-functionalized PSi layers confirms that the protein–ligand system has an overall volume smaller than the SBP alone.

1. Introduction

Porous silicon (PSi) structures are quite ideal transducers for chemical sensing and monitoring of biological interactions, due to their high specific area, up to 600 $m^2\ cm^{-3}$, low cost and compatibility with standard integrated circuit processes. PSi is fabricated by the electrochemical etching of a doped silicon wafer in a hydrofluoridic aqueous solution. This simple, but non-trivial, process allows the production of several photonic structures, characterized by different geometries: from single layers, which optically act like Fabry–Perot interferometers, to multilayers, such as Bragg mirrors, rugate filters and microcavities [1]. The optical response of these devices is strongly sensitive to any chemical or biological matter infiltrated into the sponge-like matrix of the PSi, since it obviously changes the average refractive index of the structure and, consequently, causes a redshift of its reflectivity spectrum or a variation of the photoluminescence emission intensity [2–4]. Unfortunately, this mechanism is not specific, so that PSi-based optical devices cannot be used as sensors for recognizing different substances in a complex mixture. On the other hand, the surface of the PSi microstructures can be chemically modified in order to bind bioprobes, such as enzymes, DNA single strands and proteins, which naturally interact with their biological counterparts, the so-called ligands, with high specificity. By interfacing the bioprobes with the PSi optical structures, it is possible to realize sensitive, specific and label-free devices which can be used as biosensors [5, 6] or in the monitoring of molecular events, such as DNA hybridization [7].

The proteins purified from thermophilic organisms are characterized by high stability in a harsh environment such as high temperature, up to 100 °C, high ionic strength, extreme pH values, even in the presence of elevated concentrations of detergents and chaotropic agents so that they can be successfully used as very effective bioprobes in sensor design [8]. In particular, it has been recently demonstrated that the D-trehalose/D-maltose-binding protein (SBP), which is part of the sugar uptake system in the hyperthermophilic organism archaeon *T. litoralis*, is in its native state up to 80 °C and is also able to bind glucose molecules. This result suggests the possibility of using SBP as a stable probe within a biological recognition system for glucose monitoring [8]. SBP is a monomeric 48 kDa macromolecule constituted by two domains and containing twelve tryptophan residues [9]. The presence of these fluorescent residues has been used to study the interaction between SBP and glucose at different temperatures by means of steady state

fluorescence spectroscopy by Herman *et al* [8]: they found that the highest affinity between the protein and this ligand was at a temperature around 60 °C, with a dissociation constant (K_d) of about 40 μM. At room temperature SBP still binds substrates while the activity of the SBP-based transport system becomes negligible [10, 11] and this behavior can be ascribed to an increased rigidity of the SBP structure at room temperature [12]. Like other sugar binding proteins, SBP consists of two globular lobes linked by a hinge region made of a few polypeptide chains. The deep cleft formed between the two lobes, which present a similarly tertiary structure, contains the ligand binding site [11, 13]. The binding of glucose to SBP is strongly related to a movement of the protein lobes as well as to profound conformational changes in the hinge region. The lobes envelope onto the binding site and cause a net reduction of the protein–ligand complex volume, as confirmed by fluorescence measurements [8].

In a recent published paper [6], we have found that, at room temperature, the reflectivity spectra of a PSi-distributed Bragg reflector (DBR), properly functionalized with SBP, undergo a redshift on exposure to glucose solutions. This effect means that the PSi–SBP–glucose system gains, after the molecular event, a greater optical density, i.e. the product between the refractive index and the physical thickness of the sample. In particular, we observed a redshift of about 1.2 nm after the interaction with a 150 μM glucose solution. The estimated sensitivity of the monitoring method was 0.03 nm μM^{-1}. On the other hand, some preliminary experiments conducted at 60 °C [14] showed that the reflectivity spectra of different PSi-based structures, functionalized with SBP, undergo blueshifts, which means a decrease in the optical path, as a consequence of the interaction with glucose. Since the layer thickness is fixed by the physical dimension of the PSi matrix, the variation is clearly due to changes in the average refractive index. The decrease in the average refractive index of the PSi–SBP–glucose system is related to the increase of the total voids present in the layer due to the reduction of the protein–ligand complex volume. In this work, we explain these apparently conflicting results by focusing our investigation on two PSi devices, both SBP-functionalized: an optical microcavity (PSMC) we have characterized by optical spectroscopic reflectometry, and a thin PSi monolayer as a simplified material system for the ellipsometric characterization.

2. Materials and methods

D-glucose and all the other chemicals used in the present study were from Sigma. All commercial samples were of the best available quality. The SBP was purified and supplied by Dr S D'Auria of the Institute for Protein Biochemistry, National Council of Research, Napoli, Italy. Proteins were expressed, purified and quantified by his laboratory as described in [10] and references therein.

In this study we have designed and fabricated two PSi-based structures: a monolayer, which is a porous layer with fixed porosity, and a microcavity, which is constituted by a low porosity (high refractive index) layer between two Bragg

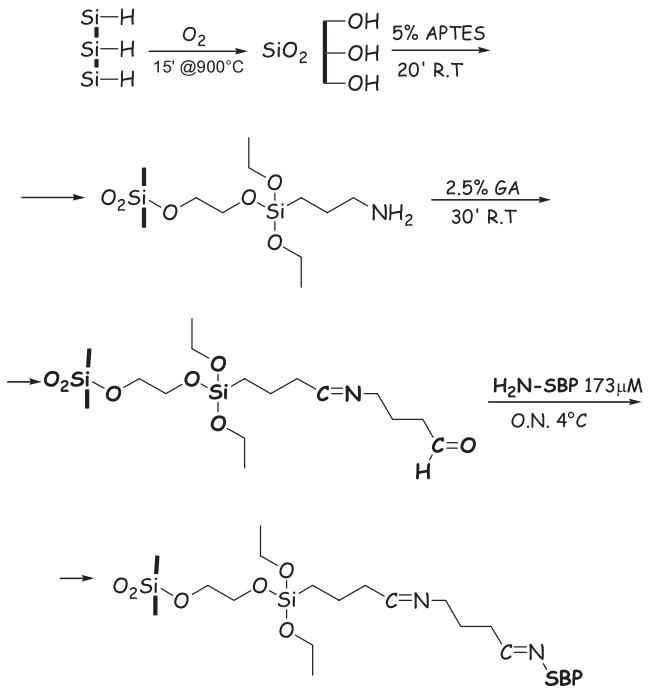

Figure 1. Porous silicon functionalization scheme: from the as-etched material up to the organic–inorganic chip.

reflectors, each one obtained by alternating low and high porosity layers seven times. Both the samples were produced by electrochemical etching of a very highly doped n^{++}-silicon wafer (Siltronix Inc., USA), ⟨100⟩ oriented, 0.001 Ω cm resistivity, 400 μm thick, in an HF-based solution. The silicon was etched using a 50 wt% HF/ethanol solution with halogen lamp illumination and at room temperature. Before anodization the substrate was placed in the HF solution to remove the native oxide. The monolayer, characterized by variable-angle spectroscopic ellipsometry (Horiba–Jobin–Yvon, model UV-VISEL) in the wavelength range between 400 and 1700 nm, was 503 ± 1 nm thick with a porosity of 77.5 ± 0.3%. The porous silicon microcavity (PSMC) was electrochemically etched by applying a current density of 400 mA cm^{-2} for 0.3 s to obtain the low refractive index layers, with a porosity of 59 ± 1%, while one of 50 mA cm^{-2} was applied for 0.5 s for the high index layers, with a porosity of 50 ± 1%. This optical structure has a characteristic resonance peak at 1017 nm, centered in a 105 nm wide stop band. Due to the electrochemical etching, the porous silicon surface is almost completely hydrogenated and thus very reactive and thermodynamically unstable: it is therefore mandatory to passivate, by proper chemical treatment, the PSi surface. By substituting the Si–H bonds with Si–C or Si–O–C it is not only possible to stabilize the surface but also to covalently link the biological probes on it.

The covalent binding of the SBP on the porous silicon surface is based on a three-step functionalization process as reported in figure 1: the first step is the PSi structures' thermal oxidation in O_2 atmosphere, at 900 °C for 15 min; then, we have treated the surfaces with a proper chemical linker, aminopropyltriethoxysilane (APTES), and used a

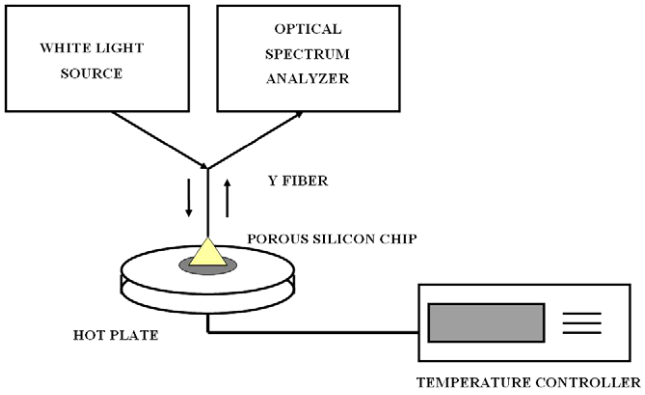

Figure 2. Experimental set-up used to measure the optical reflectivity spectra of porous silicon microcavity.
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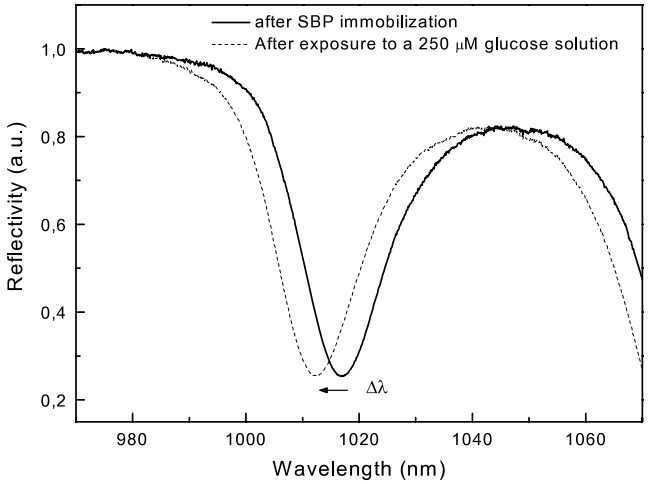

Figure 3. Resonance blueshift of a functionalized PSi microcavity on exposure to a 250 μM glucose solution.

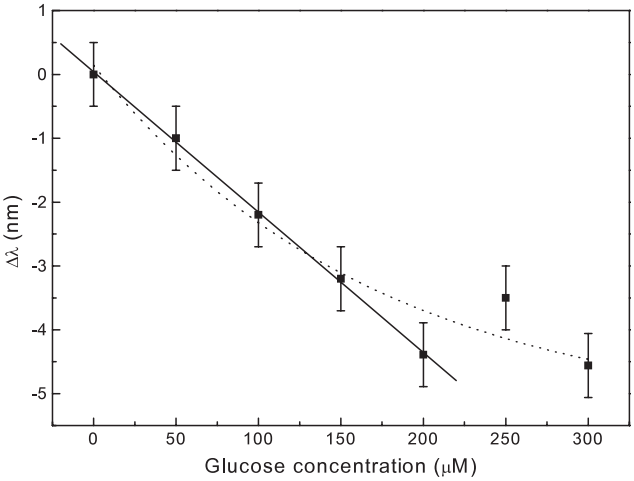

Figure 4. Dose–response curve for functionalized PSMC exposed to different concentrations of glucose. The first five experimental points have been fitted by a straight line (continuous line), while the entire dataset is fitted by an exponential monotonic decay curve (dotted line).

bi-functional linker, glutaraldehyde (GA), which exposes an aldehyde (O=CH–) able to react with the amino (NH_2) groups present on the protein surface. To this aim, samples have been rinsed by dipping in a 5% solution of APTES and a hydroalcoholic mixture of water and methanol (1:1) for 20 min at room temperature. We have then washed the chips with deionized water, and methanol, and dried in N_2 stream. The silanized devices were then baked at 100 °C for 10 min. We have immersed the chips in a 2.5% glutaraldehyde (GA) solution in 20 mM HEPES buffer (pH 7.4) for 30 min, and then rinsed them in deionized water and finally dried in N_2 stream.

The experimental set-up (figure 2) we have used to measure the reflectivity spectra is constituted by a white light, as source, directed onto the porous silicon chip through a Y fiber. The same fiber was used to guide the output signal to an optical spectrum analyzer (OSA, Ando, Japan). The spectrum was measured over the range 600–1400 nm with a resolution of 0.2 nm. A hot plate, driven by a temperature controller, has been used to perform measurements at 60 ± 1 °C, verified by a thermocouple placed directly on the chip.

The functionalized PSi samples work as active substrates for the SBP protein: we have spotted on each PSi optical structures 20 μl of a 173 μM SBP solution in 2 mM phosphate buffer solution (pH 7.3) and incubated the system at 4 °C overnight. After incubation and three washing steps of 5 min each, used to remove the excess biological matter non-covalently linked to the PSi surface, we have optically verified the presence of the protein in the spongy structure as a redshift of about 60 nm in the reflectivity spectrum in the case of PSMC.

3. Experimental results and discussion

When recorded at the temperature of 25 °C, the PSMC characteristic resonance peak undergoes only very small redshifts, of the order of 1 nm, on exposure to glucose solutions with increasing concentrations, up to 250 μM, according to the low affinity of SBP with glucose at room temperature [6]: at this temperature a few proteins can rearrange their conformational structure and efficiently bind the ligand. The results of this biomolecular interaction change dramatically if the same measurements are performed at 60 °C: in figure 3 we reported the peak shift of the functionalized PSi microcavity on exposure to a 250 μM glucose concentration. The optical resonance undergoes a 4.5 nm shift toward lower wavelengths. The dose–response curve, describing how the device responds to the measurand variations, is shown in figure 4: the curve is linear in the range of glucose concentrations between 0 and 200 μM. In this linear regime we can estimate the sensitivity to solute concentration variations as the slope of the straight line equal to 0.022 (4) nm μM^{-1}. For higher glucose concentrations, the blueshift did not increase any more, which means that all the active SBP proteins have bound the glucose molecules.

The large blueshift recorded corresponds to a decrease of the optical path that the light exploits when reflected by the porous structure. Since the optical path is defined

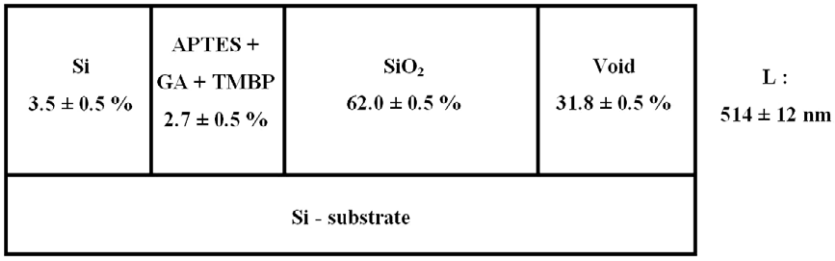

Figure 5. Ellipsometric scheme used to fit the experimental data, together with the estimated results of the fit, in the case of the functionalized monolayer.

Table 1. Porosity decrease of the PSi monolayer due to the functionalization steps.

	Porosity (%)
As-etched	77.5 ± 0.3
After oxidation	49.1 ± 0.3
After APTES treatment	41.7 ± 0.5
After GA treatment	35.5 ± 0.4
After SBP incubation	31.8 ± 0.5

as the thickness times the average refractive index, and there is no variation of the multilayer thickness, we can attribute the blueshift to an increase of the porosity due to the protein's volume reduction after the interaction with the glucose. Moreover, the chemically passivated surface of the PSi is highly stable with respect to the biological chemical buffer solutions, so that there cannot be any oxidation nor any corrosion of the silicon matrix. In order to confirm these results, we have used a very sensitive optical technique, variable-angle spectroscopic ellipsometry (VASE), to quantify the porosity changes of a thin PSi layer after SBP functionalization and on exposure to increasing concentrations of glucose solutions. From the optical point of view, the thin porous silicon layer can be considered, in the frame of an effective medium approximation such as the Bruggeman theory [15], like a multi-component material with a percentage of voids, i.e. the porosity. In figure 5 we show, as an example, the material scheme used to fit the VASE experimental data after the complete functionalization of the PSi microcavity. In table 1 the porosities estimated by ellipsometry after each functionalization step of the monolayer are reported for a comparison: the chemical and biological treatment steps considerably reduce the average porosity of the PSi layer.

Ellipsometric spectra have also been acquired after each addition of glucose solutions: the results, i.e. the changes in porosity of the PSi layer, ΔP, as estimated from the ellipsometric data, are shown as a function of glucose concentration in figure 6. The VASE characterization gives a direct measurement of the variation of each layer component: using these experimental data, we have estimated, after the addition of 250 μM of glucose, an increase of more than 3.5% in the PSi monolayer porosity.

The porosity increase saturates with the glucose concentration and the experimental data can be fitted by an exponential monotonic growth curve (continuous line in figure 6).

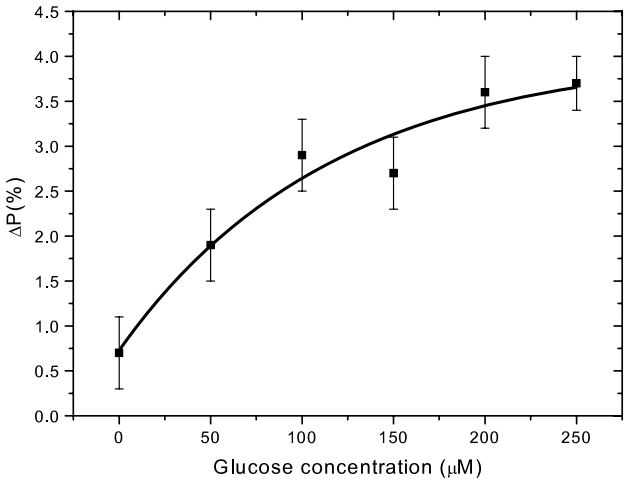

Figure 6. The porosity increase of a functionalized PSi monolayer as a function of glucose concentration. On exposure to a concentration of 250 μM of glucose, an enhancement of more than 3.5% in porosity is obtained. The experimental points have been fitted by an exponential monotonic growth curve (continuous line).

On the other hand, we can also numerically calculate which is the effect of such a porosity increment on the reflectivity spectrum of the PSi microcavity. We simulated the reflectivity spectrum of a functionalized PSi microcavity, with the same porosities and thicknesses as the one we have realized, before and after the protein–ligand interaction. A realistic picture of the PSi–SBP–glucose system requires a vertical distribution of the biological matter in the PSi matrix [16]. The organic phase of our system has been modeled by supposing a Gaussian distribution for both the SBP molecules' presence throughout the upper layers of the microcavity and also for the corresponding increment of porosity, ΔP, due to the glucose interaction. In particular, the ΔP distribution was peaked at the top of the structure (with a maximum value of 3.5%) and a full width at half-maximum of 3 μm, which equals the half-width of the first Bragg reflector in the microcavity structure. The reflectivity spectra have been simulated by the standard transfer matrix method [17] and results are reported in figure 7.

The result of this simulation is a blueshift of the resonant peak of 6.0 ± 1.0 nm, which is consistent with the experimental one of 4.6 ± 0.5 nm (see figure 3), observed after the addition of a glucose solution, 250 μM.

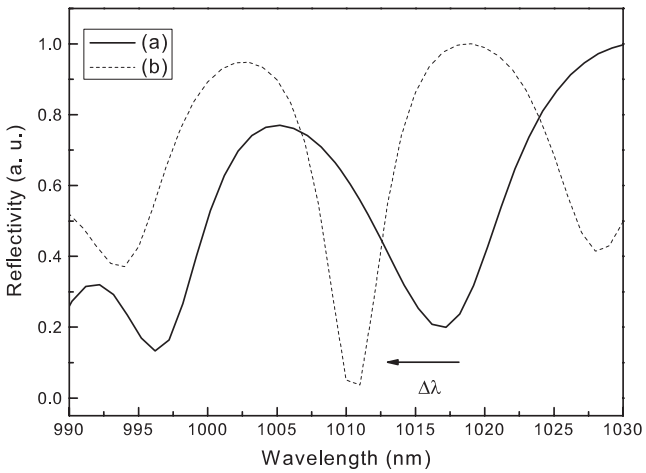

Figure 7. (a) Simulated reflectivity spectrum of the PSi microcavity used in the experiment. (b) Simulated reflectivity spectrum of the same structure after a non-homogeneous increase of porosity.

Even if highly specific, the protein–ligand interaction is in general reversible, so that proteins release completely the target molecules, especially when in buffer solutions. But when bound on the surface of a nanostructured material, proteins could be limited in their activity: after an overnight dialysis in deionized water, we have found a 2 nm redshift of the resonant peak, which means that the ligand has been released only by some proteins. Anyway, when we have repeated the measurements, we have obtained always about a 5 nm blueshift with a 250 μM concentration of glucose, so that we have concluded that the effect was reproducible, at least for five replicas we have made under the same experimental conditions, but was not perfectly reversible.

4. Conclusions

We have studied the optical response of a PSMC, functionalized by means of SBP, on exposure to different concentrations of glucose. Measurements were performed at 25 and 60 °C. At 60 °C, i.e. the temperature corresponding to the maximum affinity of SBP for glucose, we have observed blueshifts in the characteristic resonance peak of the PSi structure when the concentration of the added glucose solution increased. We have demonstrated, by direct measurement but also with numerical simulation, that the observed decrease in the effective optical thickness of the PSi–SBP system is due to a porosity increase, due to a reduction of the SBP volume after the interaction of the protein with its ligand, glucose, as was already demonstrated by time-resolved fluorescence measurements [8, 12]. We would like to underline that optical spectroscopic reflectometry in photonic porous silicon resonant devices could be a very useful and sensitive technique in biomolecular interaction monitoring.

Acknowledgments

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