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Biochips at work: porous silicon microbiosensor for proteomic diagnostic

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Abstract

The molecular interactions between the glutamine binding-protein (GlnBP) from *Escherichia coli* and its main ligands the L-glutamine (Gln) and the gliadin, a toxic peptide containing three Gln sequences, are detected by means of an optical biosensor based on porous silicon (PSi) technology. The binding events are optically transduced in the wavelength shifts of the porous silicon reflectivity spectrum. In the first case, the hydrophobic interaction links the GlnBP, which acts as a molecular probe for Gln, to the hydrogenated porous silicon surface area. A more stable coupling between the protein and the chip surface can be obtained by a proper functionalization process. Even if the GlnBPs are covalently bonded to the PSi, they are able to selectively recognize the gliadin in micromolar concentration.

1. Introduction

Porous silicon (PSi)-based optical biosensors are becoming increasingly used in the label-free detection and identification of organic molecules due to the noteworthy properties of this material [1, 2]. PSi has a spongy structure with a large specific surface area of the order of 100–500 m^2 cm^{-3} [3], so a very effective interaction with several adsorbates is assured. When a biological solution penetrates into the PSi pores, it substitutes the air, so the average dielectric properties of the heterogeneous silicon–air–liquid system change. The effect depends on the refractive index value of the solution but also on how it penetrates into the pores. Due to this sensing mechanism, PSi optical devices cannot identify the single components of a complex mixture. In order to enhance the sensor selectivity, several biological probes, which exploit

very specific interactions only with selected biochemical molecules, can be linked to the PSi surface: most common examples are DNA strands, proteins, enzymes, antibodies, and so on. The ligand-binding proteins are very effective biological probes in highly specific biosensors for small analytes [4]. In particular, the GlnBP from *E. coli* is a monomeric protein composed of 224 amino acid residues (26 kDa) responsible for the first step in the active transport of Gln across the cytoplasmic membrane. Of the naturally occurring amino acids, only the Gln is bound by the GlnBP with a dissociation constant K_d of 5×10^{-9} M [5]. We have recently published some results about the optical detection of the molecular interactions between the GlnBP and Gln or peptides containing this amino acid [6, 7].

In this work, we report further experimental data on these experiments, useful for the development of a reagentless microsensor for the optical detection of Gln and gliadin, a peptide which is toxic for coeliac patients. The results obtained show that, quite independently of the binding between the protein and the PSi surface, the GlnBP can sufficiently strongly interact with a biological solution and work as an efficient molecular probe for the detection of the ligands.

2. Material and method

Proof of PSi in biosensing has been experimentally demonstrated in a variety of sensor devices, but it is crucial, in order to exploit the advantages of this material, that the porous morphology enables a homogeneous diffusion of the biological matter everywhere in the matrix.

For this study, we fabricated a Fabry–Perot interferometer, with 10 μm thickness and 70% porosity, by electrochemical etching of p^+-type (100) crystalline silicon (resistivity 3–5 mΩ cm) in HF/EtOH (30:70) solution. The electrochemical dissolution process leaves nanosized residues which partially obstruct the pores in the PSi layer. To remove the nanostructures and improve the pore infiltration of the biomolecular probe–target system, a KOH post-etch process can be used [8]. PSi monolayers have been treated in a diluted KOH solution (1.5 mM) for 15 min: this exposure is sufficient to remove the nanostructures in the pores and also slightly increase the pore diameters (by about 15%). Unfortunately, the KOH post-etch treatment removes the silicon–hydrogen bonds and partially oxidizes the porous silicon surface. The presence of the Si–H bonds on the porous silicon surface, monitored by infrared spectroscopy with a Fourier transform infrared (FT-IR) spectrometer (Nicolet Nexus), is required either by the hydrophobic interaction with the outer amino acids of GlnBP, which are also highly hydrophobic, or by the chemical functionalization process we used to passivate the PSi surface. The Si–H bonds have been restored by immersing the PSi chip in a dilute HF solution for 30 s.

The PSi surface was chemically modified by a photo-activated treatment based on the ultraviolet (UV) exposure of a solution of alkenes functionalized with an ester group: the PSi chip was covered with 10% N-hydroxysuccinimide ester (UANHS) solution in CH_2Cl_2. The chip was exposed to a UV lamp (500 W, 40 μW cm^{-2} measured) for 4.5 min. The UANHS was synthesized in house, as described in the literature [9]. After the photoreaction, the chip was washed by dichloromethane in an ultrasonic bath for 5 min three times and rinsed with acetone to remove any adsorbed alkene from the surface. The carboxyl-terminated surface obtained acts as a substrate for the chemistry of the subsequent attachment of the protein. In figure 1, the two possible schemes of interaction between the PSi surface, as etched or chemically modified, and the GlnBP are reported.

The protein–ligand binding has been monitored by following the changes in the reflectivity spectrum of the optical biosensor in the wavelength range between 700 and 1200 nm with a resolution of 0.1 nm.

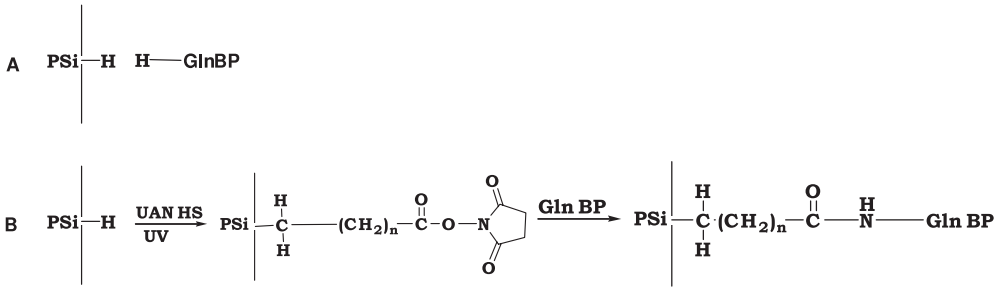

Figure 1. Schemes of interaction between the PSi surface and the GlnBP: (A) hydrophobic link; (B) covalent photo-induced link.

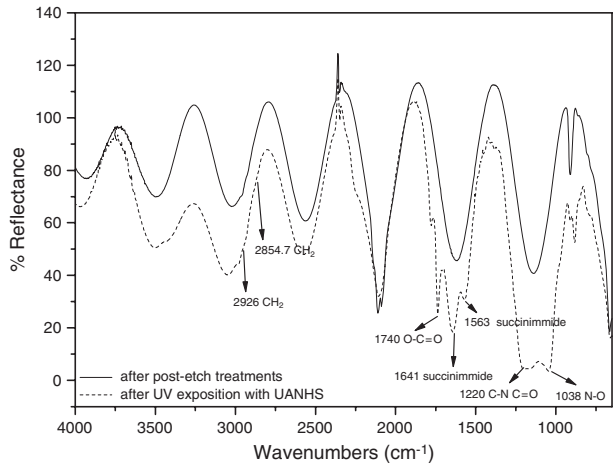

Figure 2. FT-IR spectra of the porous silicon monolayer before and after the photo-induced functionalization process based on UV exposure.

3. Experimental results

Most of the outer amino acid residues of the GlnBP present an NH terminal group, so they can interact with the Si–H-terminated PSi surface with a bond strength that is weaker than the covalent link, but stronger than the ionic one. We have verified that the proteins spotted on the chip surface are still there after several rinses, up to 15, with each one being for 5 s, in water or in buffer solution [6]. A step towards a completely reusable biosensor, since the binding of Gln–GlnBP is completely reversible [5], is to obtain a stable passivation of the PSi surface by covalently linking the protein. The chemical grafting process can be controlled by infrared spectroscopy: in figure 2 the FT-IR spectra of the porous silicon monolayer before and after the photo-induced functionalization process are reported. It is well evident that almost all the Si–H groups have reacted and have been substituted by the Si–C bonds: the characteristic peak of Si–H bonds at 2100 cm^{-1} has almost completely disappeared after the passivation treatment, while the C–H_x, C–O and C–N peaks are well evident. Since all the protein–ligand interaction experiments are made in aqueous solution, we have verified that the FT-IR spectra are unchanged, in particular that the characteristic peaks of the Si–O–Si bonds (at 1050 cm^{-1}) are absent.

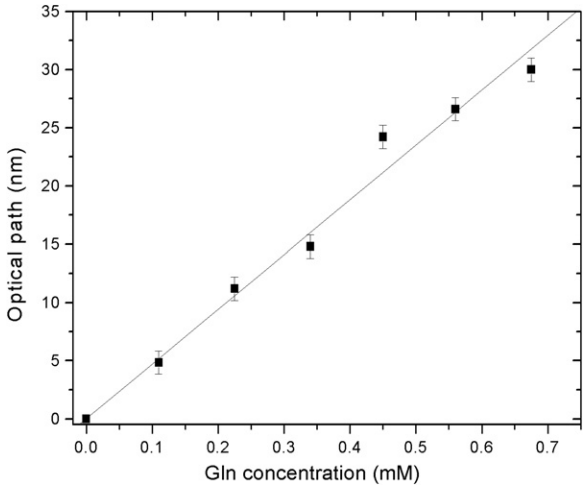

Figure 3. Dose–response curve: determination of biosensor sensitivity to ligand (Gln) concentration.

The experimental measurement to detect the binding of the Gln, or the gliadin, to the GlnBP is a two-step procedure: first, we have registered the optical spectrum of the porous silicon layer after the GlnBP absorption on the chip surface and after the Gln (gliadin) solution has been spotted on it. The organic material excess has been removed by a further rinse in the buffer solution. When the biological matter (refractive index $n \cong 1.45$) penetrates into the porous silicon matrix and substitutes the air ($n = 1$) in the pores, the average refractive index of the structure increases, resulting in a fringe shift in wavelength, which corresponds to a change in the optical path nd.

In figure 3 we have shown the dose–response curve of the PSi-based biosensor due to the presence of Gln, i.e. the changes in the optical path registered by the Fabry–Perot interferometer as a function of the amino acid concentration. A sensitivity of 47 ± 1 nm μM^{-1} (which corresponds approximately to 0.9 nm μg^{-1} l) can be estimated by the curve slope, which is in good agreement with that recently published [6].

Gliadin is a peptide, i.e. a protein constituted by few amino acids (<15), which contains a sequence of three Gln residues. Due to the high social interest in coeliac disease, it is of extreme importance to find new analytical methods for detecting toxic biomolecules in foods. We have recently estimated the sensitivity of the optical interferometer to the concentration of gliadin as $S_{Gli} = 448$ (57) nm μM^{-1} [7]. In figure 4 we report the dose–response curves as a function of the gliadin concentration in the case of two sets of measurements performed on different days, with the same PSi chip after an overnight dialysis in buffer solution. Due to the reversibility of the ligand–protein binding process, the dialysis treatment removes all the ligands that have interacted with the proteins. The sensor responses are very similar: both the curves have been fitted by a monomolecular growth model, $y = A - Be^{-kx}$, where A is the offset, B the amplitude, k the rate and $y' = Bk$ the sensitivity. In the first experiment we have obtained a sensitivity of 392 (12) nm μM^{-1}, and in the second a sensitivity of 156 (11) nm μM^{-1}. The estimate of this not dramatic sensitivity decrease after multiple reuse is very useful for experimental procedure design. The lowering of the sensor response can be mainly attributed to protein denaturation during the chip regeneration process. In both experiments, we have checked the selectivity of GlnBP in detecting Gln, pure or as sequence

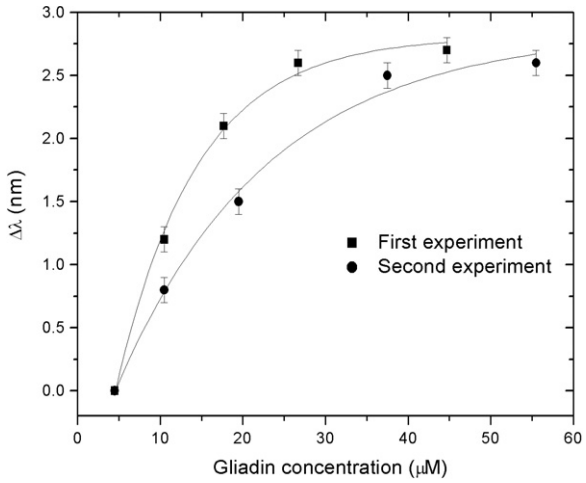

Figure 4. Dose–response curves. Optical variations of the immobilized GlnBP as a function of the gliadin concentration.

in gliadin, by control measurements: we have used biological solutions containing different molecules, such as glucose and a non-toxic peptide, the rice prolamine, as Gln and gliadin interference substances respectively, without observing any change in the optical path.

4. Conclusion

In conclusion, in this work we have presented additional experimental results about an optical porous silicon microsensor for protein–ligand and protein–protein interaction detection. We used both a fresh PSi surface and a functionalized one to immobilize in the porous silicon the L-glutamine binding protein of *E. coli*, which selectively recognizes the target analytes. The binding of Gln–GlnBP and gliadin–GlnBP is completely reversible, and we have shown that the sensor can be reusable. The sensitivities estimated are of the same order, or even better, as that of other optical biosensors recently proposed [10].

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