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ORIGINAL PAPER

Enzymes and proteins from extremophiles as hyperstable probes in nanotechnology: the use of D-trehalose/D-maltose-binding protein from the hyperthermophilic archaeon *Thermococcus litoralis* for sugars monitoring

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Abstract The D-trehalose/D-maltose-binding protein (TMBP), a monomeric protein of 48 kDa, is one component of the trehalose and maltose (Mal) uptake system. In the hyperthermophilic archaeon *Thermococcus litoralis*, this is mediated by a protein-dependent ATP-binding cassette system transporter. The gene coding for a thermostable TMBP from the archaeon *T. litoralis* has been cloned, and the recombinant protein has been expressed in *E. coli*. The recombinant TMBP has been purified to homogeneity and characterized. It exhibits the same functional and structural properties as the native one. In fact, it is highly thermostable and binds sugars, such as maltose, trehalose and glucose, with high affinity. In this work, we have immobilized TMBP on a porous silicon wafer. The immobilization of TMBP to the chip was monitored by reflectivity and Fourier Transformed Infrared spectroscopy. Furthermore, we have tested the optical response of the protein-Chip complex to glucose binding. The obtained data suggest the use of this protein for the design of advanced optical non-consuming analyte biosensors for glucose detection.

Keywords Trehalose/maltose-binding protein · Biosensors · Porous silicon · Glucose · Diabetes · Archaeon

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Introduction

The general interest in biomolecules, isolated from thermophilic organisms was originally due to the biotechnological advantages offered by the utilization of these highly stable molecules in industrial processes (Brock 1985). In fact, enzymes and proteins isolated from thermophilic microorganisms exhibit a high stability in conditions usually used to denature proteins: high temperature, ionic strength, extreme pH-values, elevated concentration of detergents and chaotropic agents (Jaenicke 1994). In addition to the potential industrial applications, it is important to highlight that proteins and enzymes that are stable and active over 100°C represent good models to shed light on the molecular adaptation of life at high temperature (D'Auria et al. 1998).

The D-trehalose/D-maltose-binding protein (TMBP) is one component of the trehalose (Tre) and maltose (Mal) uptake system, which, in the hyperthermophilic archaeon *Thermococcus litoralis*, is mediated by a protein-dependent ATP-binding cassette (ABC) system transporter (Xavier 1996). TMBP from *T. litoralis* is a monomeric 48 kDa two-domain macromolecule containing 12 tryptophan residues (Diez et al. 2001). TMBP shares common structural motifs

with a number of other sugar-binding proteins. This class of biomolecules is composed of proteins, whose structure consists of two globular domains connected by a hinge region made of two or three short peptide segments. The two domains are formed by non-contiguous polypeptide stretches and exhibit similar tertiary structure. The ligand-binding site is located in the deep cleft between the two domains and the binding is accompanied by a movement of the two lobes as well as by conformational changes in the hinge region (Sun et al. 1998).

The gene coding for the thermostable TMBP from the archaeon *T. litoralis* has been cloned, and the recombinant protein has been expressed in *E. coli* (Horlacher et al. 1998). The recombinant TMBP has been purified to homogeneity and characterized. It exhibits the same functional and structural properties as the native one (Horlacher et al. 1998).

In a recent work (Herman et al. 2006), we have investigated the binding of several sugars to TMBP by time-resolved fluorescence spectroscopy, and molecular dynamics methods. We found that TMBP is also able to bind glucose molecules. Since human blood does not contain Tre and Mal, it is not outrageous to envisage the use of the TMBP as a probe for the design of a minimally invasive biochip for glucose detection.

In this work, we have extended our investigation on the utilization of TMBP for sensing glucose by immobilizing the protein on a porous silicon chip. The TMBP-based chip has been characterized and tested as regards the response to glucose. The obtained results are discussed.

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.

Purification of TMBP and protein concentration determination

An mount of 20 g wet weight of the *E. coli* cell pellet containing the expressed TMBP were resuspended in 50 mM Tris–HCl, pH 7.5, 500 mM NaCl (100 ml), ruptured by a French pressure cell at 16,000 psi, and centrifuged for 15 min at 19,000 *g*. The supernatant was heated to 90°C for 20 min and centrifuged for 15 min at 19,000 *g*. The supernatant was collected and dialyzed for 12 h against 10 mM Tris–HCl, pH 7.5 at 4°C. The solution was loaded onto a DEAE column previously equilibrated in 10 mM Tris–HCl, pH 7.5. After washing the column with 10 mM Tris–HCl, pH 7.5, TMBP was eluted by a NaCl gradient (0.0–1.0 M NaCl). Centricon 30 concentrator (Amicon, MA, USA) was used to concentrate and to dialyze TMBP against 10 mM Tris–HCl, pH 7.5. The purified protein was passed through a Blue A affinity column (Amersham, NJ, USA) in order to obtain DNA-free TMBP. The purity of TMBP was verified by SDS-PAGE and absorption spectra.

The protein concentration was determined by the method of Bradford (Bradford 1976) with bovine serum albumin as standard on a double beam Cary 1E spectrophotometer (Varian, Mulgrade, Victoria, Australia).

Chip preparation and spectroscopic characterization

In this study, we used as sensor an apodized Bragg reflector obtained by alternating high (*A*) refractive index layers (low-porosity) and low (*B*) refractive index layers (high-porosity) whose thicknesses satisfy the following relationship: $n_A d_A + n_B d_B = m\ \lambda/2$, where m is an integer and λ is the Bragg resonant wavelength. The distributed Bragg reflector (DBR) was produced by electrochemical etch of a highly doped p^+-silicon, ⟨100⟩oriented, 0.04 Ω cm resistivity, 400 μm thick, in a HF-based solution. The silicon was etched using a 30 wt.%HF/ethanol solution in dark and at room temperature. Before anodization the substrate was placed in HF solution to remove the native oxide. A current density of 148 mA/cm^2 for 2.31 s was applied to obtain the low refractive index layer (effective refractive index $n_L \cong 1.505$, thickness $d_L \cong 598$ nm) with a porosity of 72%, while one of 110 mA/cm^2 was applied for 2.34 s for the high index layer ($n_H \cong 1.585$, thickness $d_H \cong 568$ nm) with a porosity of 69%. This structure is characterized by a first order ($m = 1$) resonance at 3,600 nm. In Fig.1, the experimental

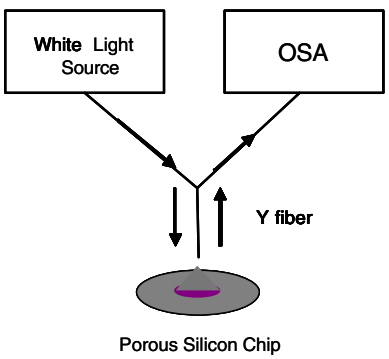

Fig. 1 Experimental setup used to measure the optical reflectivity spectrum of porous silicon chip

setup, used to measure the reflectivity spectrum, is reported. We have used as source a white light directed on the porous silicon chip through a Y-fiber. The same fiber was used to guide the output signal to the optical spectrum analyzer. The spectrum was measured over the range 800–1,600 nm with a resolution of 0.2 nm. The porous silicon surface after electrochemical etching is hydrogenated and for this reason, very reactive and thermodynamically instable; in order to realize a sensor more stable and able to link the biological probes it is necessary to substitute the Si–H bonds with the Si–C or Si–O–C ones.

To promote a covalent link between the porous silicon and the TMBP, we have exploited a three-step functionalization process, based on the chemical passivation of the PSi surface after oxidation. We have first thermally treated the PSi DBR, in O_2 atmosphere, at 1,000°C for 30 min, to remove all the Si–H bonds and create an oxide layer on the pores' surface to assure the covalent attachment with a proper chemical linker, the minopropyltriethoxysilane (APTES). To this aim, we have rinsed by immersion the DBR in a 5% solution of APTES and a hydroalcoholic mixture of water and methanol (1:1), for 20 min at room temperature. After the reaction time, we have washed the chip with DI-water and methanol and dried in N_2 stream. The silanized device was then baked at 100°C for 10 min. The next step consists in creating a surface able to link the carboxylic group of the proteins: we have thus immersed the DBR in a 2.5% glutaraldehyde solution in 20 mM HEPES buffer (pH 7.4) for 30 min, and then rinsed it in DI-water and finally dried in N_2 stream. The glutaraldehyde reacts with the amino groups on the silanized surface and coats the internal surface of the pores with another thin layer of molecules.

We have monitored all the reaction steps by Fourier Transformed Infrared (FT-IR) Spectroscopy and the consequent modification of the optical response of the PSi device by reflectivity spectroscopy.

The modified surface obtained works as an active substrate for the chemistry of the following attachment of the protein: we have spotted on the PSi chip 20 μl of 7.5 μM sodium bicarbonate buffer (pH 7.35) containing a rhodamine labeled TMBP and incubated the system at –4°C overnight. Even if our aim is the realization of a label-free optical biosensor based on the PSi nanotechnology, we have used a fluorescent protein to control the distribution of the biological matter on the chip surface and to test the chemical stability of the covalent link between the TMBP and the PSi surface.

After this assessment phase, we have also optically detected the ligand-binding interaction by following the wavelength shift of the reflectivity spectrum. The experimental measurement of the TMBP–glucose binding is a two step procedure: first, we have registered the optical spectrum of the porous silicon layer after the TMBP immobilization on the DBR surface and after the Glucose solution has been spotted on it.

Results

In Fig. 2, we have reported the optical spectra of our device as etched and after each step of the chemical

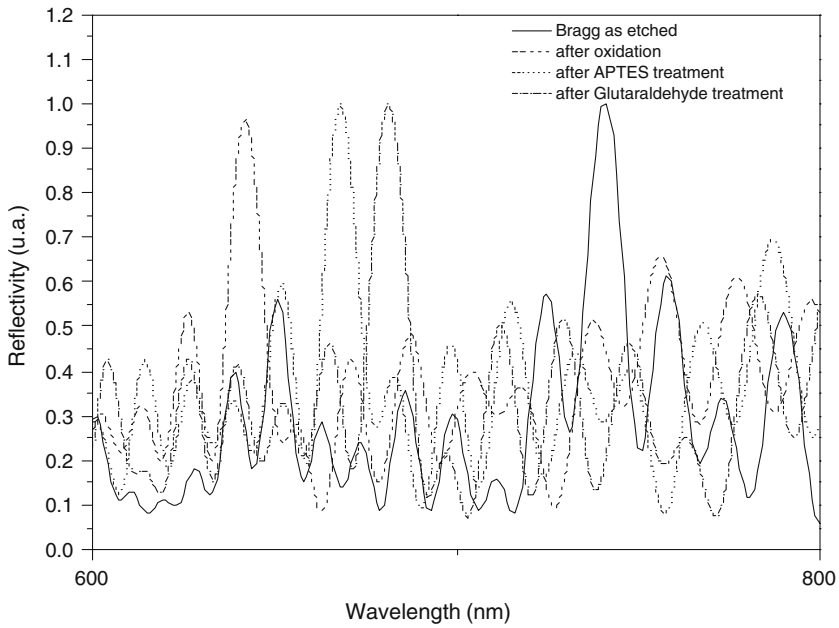

Fig. 2 Optical reflectivity spectrum of the Bragg reflector as-etched (*continues curve*), after oxidation (*dashed curve*), after APTES treatment (*dot curve*) and after glutaraldehyde treatment (*dot-dashed curve*)

treatment in the range 600–800 nm, where the $m = 5$ Bragg resonance peak at about 720 nm is present. The oxidation process causes a blue shift of the reflectivity spectrum of 99.5 nm due to the lower value of the SiO_2 refractive index ($n_{oxi} \cong 1.5$)with respect to the Si refractive index ($n_{Si} \cong 3.2$). On the contrary, the silanisation steps by APTES and glutaraldehyde produce red shifts of the reflectivity spectrum of 28 and 17 nm, respectively, corresponding to an increasing of the average refractive index of the layers due to a filling of the pores by the organic layers.

Since the protein distributes uniformly on the pores surface, the TMBP attachment causes a new detectable red shift of only 9 nm in the reflectivity spectrum.

The FT-IR spectra of the oxide PSi sample and after the silanization process are reported in Fig. 3: the main characteristic peaks of silicon dioxide (at 1,124 cm^{-1}), of the APTES amino groups (at 3,300 and 3,352 cm^{-1}) and of gluteraldehyde cyano group (at 1,404 cm^{-1}) are easily recognized.

In Fig. 4a, is shown the DBR observed by a Leica Z16 APO fluorescence macroscopy system after incubation. By illuminating the chip spotted with the labeled protein by a 100 W high-pressure mercury source, we found that the fluorescence is very high and homogeneous on the whole surface. We have also qualitatively tested the strength of covalent bond between the protein and the porous silicon surface by washing the device in a dialysis membrane overnight in DI-water. Since the fluorescent intensities differ of only few counts, we can conclude that the PSi-TMBP double layer is very stable.

We have also measured the signal response to the glucose concentration after the interaction with the protein in a range between 10 and 150 μM. The maximum shift of the Bragg wavelength is 1.2 nm. Figure 5 shows the dose–response curve to glucose additions. The estimated sensitivity of the TMBP-Chip is 0.03 nm/μM. Interestingly, the concentration of glucose that induces a optical response of the protein is very close to the amount of the sugar present in the human interstitial fluids. This result suggests the use of this protein in designing of a non-consuming and minimally invasive biosensor for the

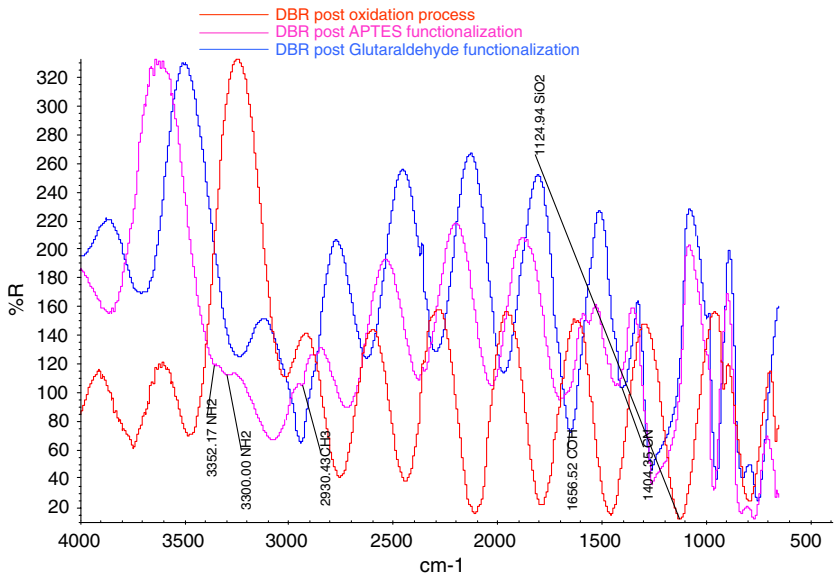

Fig. 3 FT-IR spectra of the Bragg reflector after oxidation and APTES/ glutaraldehyde treatment

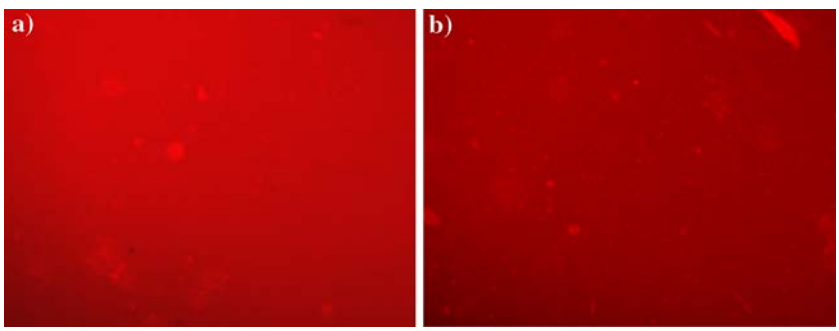

Fig. 4 **a** Porous silicon chip after incubation with the labeled-TMBP. **b** Porous silicon chip after washings in demi-water

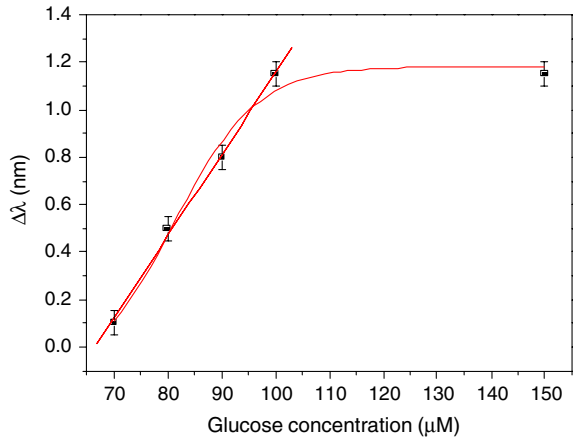

Fig. 5 Dose–response curve for PSi DBR optical sensor exposed to several concentration of glucose

continuous detection of the level of glucose in diabetic patients.

In conclusion, we have reported an effective methodology for the covalent immobilization of proteins from extremophiles on a nanostructured material, such as porous silicon wafer, that can be utilized as general platform for the development of very stable protein-based arrays for analyses of high social interest.

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