Nanostructured silicon-based biosensors for the selective identification of analytes of social interest

2006 J. Phys.: Condens. Matter 18 S2019

(http://iopscience.iop.org/0953-8984/18/33/S17)

INSTITUTE OF PHYSICS PUBLISHING JOURNAL OF PHYSICS: CONDENSED MATTER

J. Phys.: Condens. Matter **18** (2006) S2019–S2028 doi:10.1088/0953-8984/18/33/S17

Nanostructured silicon-based biosensors for the selective identification of analytes of social interest

Sabato D'Auria[1,4], Marcella de Champdoré[1], Vincenzo Aurilia[1], Antonietta Parracino[1], Maria Staiano[1], Annalisa Vitale[1], Mosè Rossi[1], Ilaria Rea[2], Lucia Rotiroti[2], Andrea M Rossi[3], Stefano Borini[3], Ivo Rendina[2] and Luca De Stefano[2]

[1] Institute of Protein Biochemistry, CNR, Via Pietro Castellino, 111 80131 Naples, Italy
[2] Institute for Microelectronics and Microsystems, CNR—Department of Naples, Via Pietro Castellino 111, 80131 Naples, Italy
[3] Istituto Nazionale di Ricerca Metrologica—INRIM, Via Strada delle Cacce 91, 10100 Turin, Italy

E-mail: s.dauria@ibp.cnr.it

Received 13 January 2006
Published 4 August 2006
Online at stacks.iop.org/JPhysCM/18/S2019

Abstract

Small analytes such as glucose, L-glutamine (Gln), and ammonium nitrate are detected by means of optical biosensors based on a very common nanostructured material, porous silicon (PSi). Specific recognition elements, such as protein receptors and enzymes, were immobilized on hydrogenated PSi wafers and used as probes in optical sensing systems. The binding events were optically transduced as wavelength shifts of the porous silicon reflectivity spectrum or were monitored via changes of the fluorescence emission. The biosensors described in this article suggest a general approach for the development of new sensing systems for a wide range of analytes of high social interest.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

For many decades, scientists have recognized the power of incorporating biological principles and molecules into the design of artificial devices. Biosensors, an amalgamation of signal transducers and biocomponents, play a prominent role in medicine, food, and processing technologies. Compactness, portability, high specificity, and sensitivity represent some reasons why biosensors are considered as holding promise, with high potential for replacing current analytical practice [1].

[4] Address for correspondence: Institute of Protein Biochemistry, Italian National Research Council, Via Pietro Castellino, 111-80131 Naples, Italy.

Fluorescence detection is the dominant analytical approach in medical testing, biotechnology, and drug discovery. Starting in the 1980s the first chemically synthesized fluorescence probes for specific analytes became available. However, the development of probes for complex analytes present in biological specimens cannot be approached via chemical synthesis alone [2–4]. In fact, the number of potential ligands specifically recognized by different proteins is very large and ranges from small molecules to macromolecules (including proteins themselves). The advantages of using proteins as components of biosensors are many and include relatively low costs in design and synthesis, the fact that proteins are, at least in general, soluble in water, and finally, with the progress of molecular genetics, the possibility of improving/changing some of the properties of the proteins by genetic manipulation [5, 6].

Recently, a lot of experimental work exploiting the properties of porous silicon (PSi) in chemical and biological sensing has been reported [7, 8]. PSi is an almost ideal material as a transducer due to its porous structure, with a hydrogen terminated surface, having a specific area of the order of 200–500 m^2 cm^{-3}, so a very effective interaction with several adsorbates is ensured. Moreover, PSi is an available and low cost material, completely compatible with standard IC processes, and a useful component of so-called smart sensors [9]. The PSi is fabricated by the electrochemical etching of a silicon (Si) wafer in a hydrofluoridic acid solution. It is well known that the porous silicon 'as etched' has an Si–H terminated surface due to the Si dissolution process [10]. PSi optical sensors are based on changes of photoluminescence or reflectivity upon exposure to the target analytes [11, 12], which replace the air in the PSi pores. The optical changes depend on the chemical and physical properties of the bound analyte. As a consequence, these devices cannot be used for the identification of analytes in a complex mixture. In order to enhance the sensor selectivity, it is possible to utilize PSi wafer as substrate for the covalent immobilization of biomolecules, such as proteins, enzymes, and antibodies, that are able to recognize target analytes in a complex matrix [13–20].

In this paper, we report new findings of relevance to the design of advanced biosensors obtained by a multidisciplinary group of scientists, for the optical detection of analytes of social interest such as glucose, ammonium nitrate, and glutamine.

2. Glucose detection

Close control of blood glucose is essential to avoid the long term adverse consequences of elevated blood glucose, including neuropathies, blindness, and other consequences [21]. Non-invasive measurements of blood glucose have been a long-standing research target because they would allow the development of a variety of devices for diabetic health care, including continuous painless glucose monitoring, control of an insulin pump, and warning systems for hyperglycaemic and hypoglycaemic conditions. Hypoglycaemia is a frequent occurrence in diabetics, and can result in coma or death. The acute and chronic problems of diabetics and hypoglycaemia can be ameliorated by continuous monitoring of blood glucose. At present, the only reliable method for measuring blood glucose is via a finger prick and subsequent glucose measurement, typically using glucose oxidase. This procedure is painful and even the most compliant individuals, with good understanding and motivation for glucose control, are not willing to prick themselves more than several times per day. These medical needs have fostered intensive efforts to develop sensors for glucose [22, 23]. The absence of a suitable non-invasive glucose measurement has fostered decades of research, little of which has however resulted in simpler and/or improved glucose monitoring. Included in this effort is the development of fluorescence probes specific for glucose, typically based on boronic acid chemistry [24, 25]. An alternative approach to glucose sensing using fluorescence is based on proteins which bind glucose. Optical detection of glucose appears to have had its origin

in the promising studies of Schultz and co-workers [26, 27], who developed a competitive glucose assay which does not require substrates and does not consume glucose. This assay used fluorescence resonance energy transfer (FRET) between a fluorescence donor and an acceptor, each covalently linked to concanavalin A (ConA) or dextran. In the absence of glucose the binding between ConA and dextran resulted in a high FRET efficiency. The addition of glucose resulted in its competitive binding to ConA, displacement of ConA from the labelled dextran, and a decrease in FRET efficiency. These results generated considerable enthusiasm for fluorescence sensing of glucose [28]. The glucose–ConA system was also studied by other labs. They identified several problems, namely that the system was only partially reversible upon addition of glucose, and that it became less reversible with time and showed aggregation [29]. For this reason, the use of other glucose-binding proteins as sensors was explored by several research groups [30–35]. If a reliable fluorescence assay for glucose could be developed, then the robustness of lifetime-based sensing [36] could allow development of a minimally invasive implantable glucose sensor, or of a sensor which uses extracted interstitial fluid. The lifetime sensor could measure glucose concentration through the skin [37] using a red laser diode or light emitting diode (LED) device as the light source. These devices are easily powered with batteries and can be engineered into a portable device. An implantable sensor can be expected to report on blood glucose because tissue glucose closely tracks blood glucose with a 15 min time lag [38].

Glucose oxidase (GO) (EC 1.1.3.4) from *Aspergillus niger* catalyses the conversion of β-D-glucose and oxygen to D-glucono-1,5-lactone and hydrogen peroxide. It is a flavoprotein, highly specific for β-D-glucose, and is widely used to estimate glucose concentration in blood or urine samples through the formation of coloured dyes [39]. Because glucose is consumed, this enzyme cannot be used as a reversible sensor. We extended the use of GO under conditions where no reaction occurs. In particular, in order to prevent glucose oxidation, we removed the FAD cofactor which is strictly required for the reaction.

The absorbance spectrum of apo-GO shows the characteristic shape of the coenzyme-free proteins, with an absorbance maximum at 278 nm due to the aromatic amino acid residues. The absence of absorption at wavelengths above 300 nm indicates that the FAD has been completely removed. The fluorescence emission spectrum of apo-GO at room temperature upon excitation at 298 nm displays an emission maximum at 340 nm, which is characteristic of partially shielded tryptophan residues. The addition of 20 mM glucose to the enzyme solution resulted in a quenching of about 18% of the tryptophanyl fluorescence emission (data not shown). This result indicates that the apo-GO is still able to bind glucose. The observed fluorescence quenching may be mainly ascribed to the tryptophanyl residue 426. In fact, as shown by x-ray analysis and molecular dynamics simulations, the glucose-binding site of GO is formed by Asp 584, Tyr 515, His 559 and His 516. Moreover, Phe 414, Trp 426 and Asn 514 are in locations where they might form additional contacts with glucose [40].

The intrinsic fluorescence of proteins is usually not useful for clinical sensing because of the need for complex or bulky light sources and because of the presence of numerous proteins in most biological samples. ANS is known to be a polarity-sensitive fluorophore which displays an increased quantum yield in low polarity environments. Additionally, ANS frequently binds to proteins with an increase in intensity. We examined the effects of GO on the emission intensity of ANS. Addition of apo-GO to an ANS solution resulted in an approximate 30-fold increase in the ANS fluorescence intensity. Importantly, the intensity of the ANS emission was sensitive to glucose, decreasing by approximately 25% upon glucose addition. The ANS was not covalently bound to the protein. That addition of glucose results in a progressive decrease in the ANS fluorescence intensity suggests that the ANS is being displaced into a more polar environment upon glucose binding. The decreased ANS intensity occurred with a glucose-

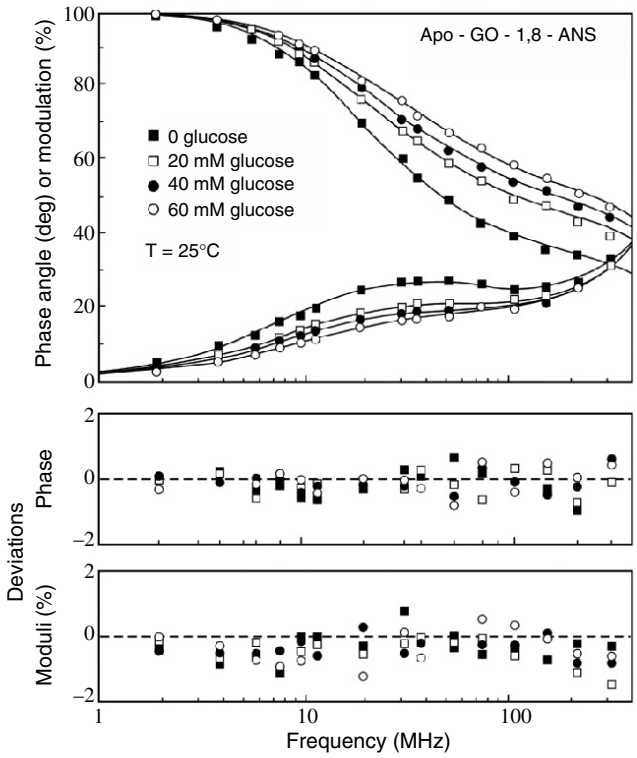

Figure 1. Frequency domain intensity decays of 1,8-ANS-glucose oxidase at different concentrations of glucose. Excitation was at 335 nm while the emission was observed through interference filter 535/50 nm with two Corning 3–71 cut-off filters.

binding constant near 10 mM, which is comparable to the K_D of the holo-enzyme. The fact that the binding affinity has not changed significantly may imply that the binding is still specific to glucose. Intensity decays of ANS-labelled apo-GO in the presence of glucose were studied by frequency domain fluorometry. Addition of glucose shifts the frequency responses to higher frequencies, which is due to a decreased ANS lifetime (figure 1). The shorter lifetime of ANS apo-GO in the presence of glucose is again consistent with the suggestion that glucose displaces the ANS to a more polar environment. The mean lifetime decreases by over 40% upon addition of glucose. These results demonstrate that apo-glucose oxidase, when labelled with suitable fluorophores, can serve as a protein sensor for glucose.

3. Explosive detection

Organophosphorous pesticides, nerve agents and nitro-aromatic compounds are very dangerous substances which can be used against civil or military objectives. Recently, Bourne and colleagues have demonstrated the mechanism of inhibition, at molecular level, of the acetylcholinesterase (AChE) with several inhibitor molecules [41]. The peripheral anionic site on AChE, located at the active centre gorge entry (figure 2), encompasses overlapping binding sites for allosteric activators and inhibitors; yet, the molecular mechanisms coupling this site to the active centre at the gorge base to modulate catalysis remain unclear. The peripheral site

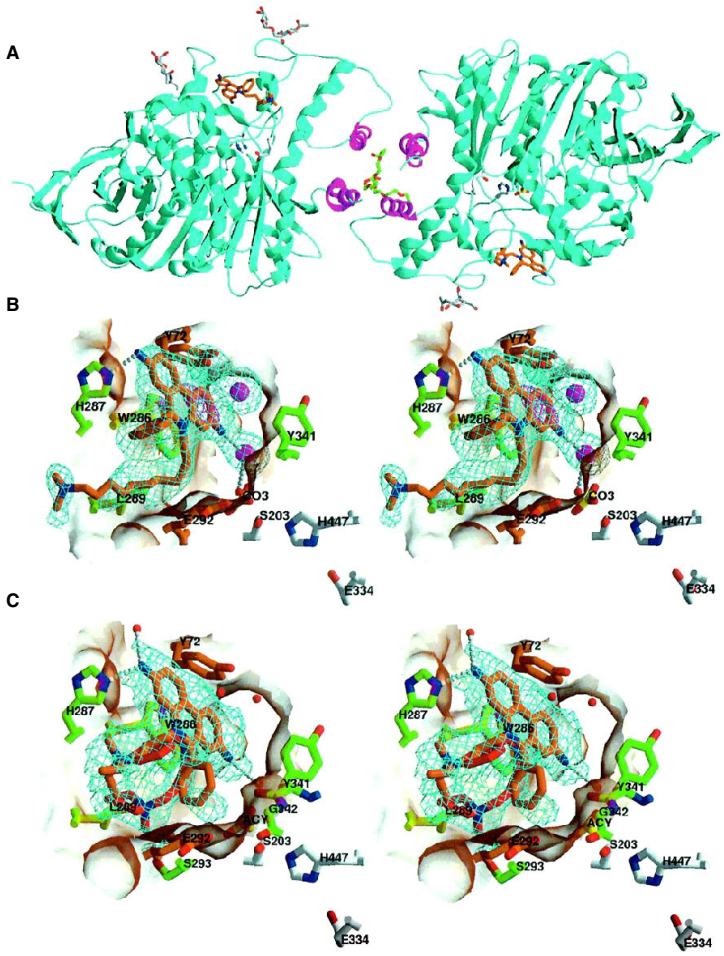

Figure 2. Structures of the DI–mAChE and PI–mAChE complexes. (A) Ribbon diagram of the mAChE dimer (cyan, with the four-helix bundle in magenta) bound to DI (orange bonds, blue nitrogen and red oxygen atoms). The carbohydrate moieties linked to residues Asn 350 and Asn 464 in both subunits are displayed as grey bonds and coloured spheres. The side chains of the catalytic triad residues, Ser 203, Glu 334, and His 447, are shown as white bonds in the two dimer subunits. The PEG molecule bound at the centre of the four-helix bundle and the carbonate molecule bound to Ser 203 are shown as grey (green) and light grey (yellow) bonds, respectively. (B) Close-up stereo view of the DI molecule (coloured as in A) bound to the PAS, with the 2.35 Å resolution omit $F_o - F_c$ electron density map contoured at 3.5σ (cyan) and 7.5σ (blue); the coordinates of this region were omitted and the protein coordinates were refined by simulated annealing before the phase calculation. The interacting side chains of mAChE residues His 287 and Leu 289, located in the loop region connecting helices $\alpha^3_{6,7}$ and $\alpha^4_{6,7}$, and of residues Trp 286 and Tyr 341 at the gorge entrance, are displayed as grey (green) bonds; those of mAChE residues Tyr 72 and Glu 292, whose respective mutations as methionine and lysine in BgAChE abolish PI binding (see figure 6), are highlighted in light grey (orange). The chloride ions and solvent molecules are shown as pink and red spheres, respectively. The catalytic triad residues, Ser 203, Glu 334, and His 447, and the carbonate molecule (bottom) are shown as white and light grey (orange) bonds, respectively. Hydrogen bonds between mAChE and the DI molecule are shown as white dotted lines. (C) Close-up stereo view of the PI molecule (coloured as for DI) bound to the PAS, with the 2.35 Å resolution omit $F_o - F_c$ electron density map contoured at 3.5σ (cyan). The PI phenyl and diethylmethylammonio moieties, which show alternative positions in the structure, are displayed as red and orange bonds. The mAChE side chains interacting with the PI molecule are displayed as grey (green) and light grey (orange) bonds as in (B). The mAChE molecular surfaces buried at the DI–mAChE (B) and PI–mAChE (C) complex interfaces are displayed in transparency.

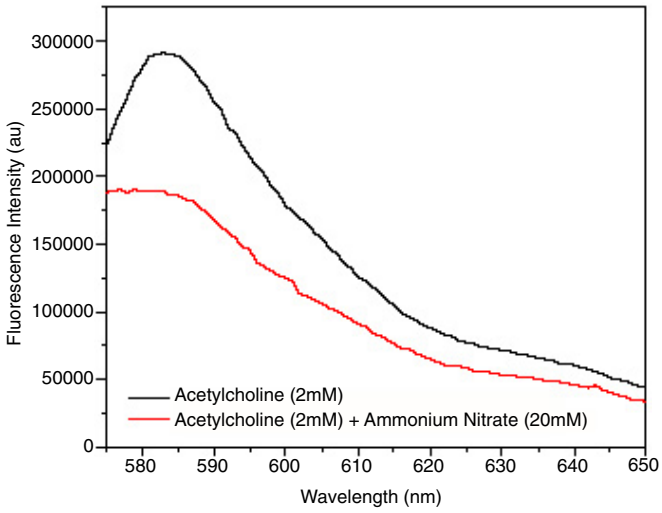

Figure 3. Fluorescence emission spectra of AChE immobilized on Psi, in the absence and in the presence of ammonium nitrate.

has also been proposed to be involved in heterologous protein associations occurring during synaptogenesis or upon neurodegeneration [42].

A crystal form of mouse AChE, combined with spectrophotometric analyses of the crystals, allowed us to obtain unique structures of AChE with a free peripheral site, and as three complexes with peripheral site inhibitors: the phenylphenanthridinium ligands, decidium and propidium, and the pyrogallol ligand, gallamine, at 2.20–2.35 Å resolution [43]. Comparison with structures of AChE complexes with the peptide fasciculin or with organic bifunctional inhibitors unveiled new structural determinants contributing to ligand interactions at the peripheral site, and permitted a detailed topographic delineation of this site [44]. These structures provide templates for designing compounds directed to the enzyme surface that modulate specific surface interactions controlling catalytic activity and non-catalytic heterologous protein associations. Since several dangerous substances inhibit AChE activity, first responders and personnel at risk for exposure to these compounds need sensor systems that operate in real time and are reliable, cost-effective, compact, portable, and sensitive [45].

AChE was immobilized on a porous silicon chip by applying 50 μl of a 1 mg ml^{-1} solution prepared in 100 mM PBE buffer at pH 6.5 to each chip. The chip was incubated at 4 °C overnight. After the reaction, the excess of reagent was removed and the wafers were extensively washed at room temperature with PBE buffer. The AChE activity was monitored by using the Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit, by following the fluorescence variations at 590 nm.

In figure 3, we report the fluorescence emission in the absence and in presence of ammonium nitrate. As we can see, the presence of ammonium nitrate results in a quenching of the fluorescence emission of about the 39%, indicating the effectiveness of the optical biosensor for the detection of the basic compound of common explosives.

4. Glutamine detection

Glutamine (Gln) is an important energy and nitrogen source used in the culturing of eukaryotic cells [46, 47]. Since the catabolism of glutamine leads to the build-up of ammonia which may

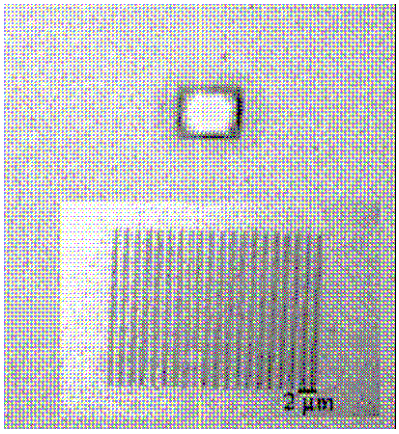

Figure 4. Optical microscope image of a PSi chip spotted by a labelled protein after electronic beam irradiation under laser light excitation. The bright area due to the protein fluorescence is clearly evident. The inset shows the nanometric resolution of the pattern produced.

become toxic to the growing culture and thus inhibit growth [48, 49], monitoring glutamine concentrations throughout the growth cycle is an important process. Biosensors available for glutamine are based on immobilized glutaminase [50, 51]. Complications in the determination of glutamine using this type of enzymatic biosensor arise due to the high degree of chemical similarity to glutamate as well as other amino acids. Consequently, additional proteins and electrodes must be added to take into account the interfering signals.

The *Escherichia coli* periplasmic space contains a diverse group of binding proteins whose main function is to present various molecules, such as sugars, amino acids, peptides, and inorganic ions, for transport into the cell [52]. Glutamine-binding protein (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 L-glutamine across the cytoplasmic membrane [53, 54]. Of the naturally occurring amino acids, only glutamine is bound by GlnBP, with a K_d of 3×10^{-7} M [54]. The protein consists of two similar globular domains linked by two peptide hinges, and x-ray crystallographic data indicate that the two domains undergo large movements upon ligand binding. The changes in global structure of GlnBP following the Gln binding make it a good candidate for serving as the basis for new biosensor design.

We detected the molecular binding between the GlnBP from *Escherichia coli* and Gln by means of an interferometric microsensor based on the utilization of unmodified porous silicon as solid support for the chip preparation. The sensor operates by measurement of the Fabry–Perot fringes in the wavelength reflection spectrum from the porous silicon layer. The ligand binding was detected as a fringe shift in wavelength, corresponding to a refractive index change. We developed a three-step procedure for the Gln detection. In particular, firstly we registered the optical spectrum of the porous silicon layer as etched; then we repeated the measurement after the GlnBP absorption on the chip surface, and, finally, a Gln solution was spotted on it. Figure 3 shows the shifts induced by each step in the fringes, due to protein–ligand interaction. Figure 4 shows how effective the linking between the protein and the PSi surface is after electron beam irradiation: the bright area is emitted as a spot on an irradiated PSi area and photographed by an optical microscope under laser (Ar^+) excitation after several rinses in demi-water. The real nanometric resolution of this functionalizing method is clearly visible. The graft effect is due to the desorption of surface hydrogen atoms which is provoked by the

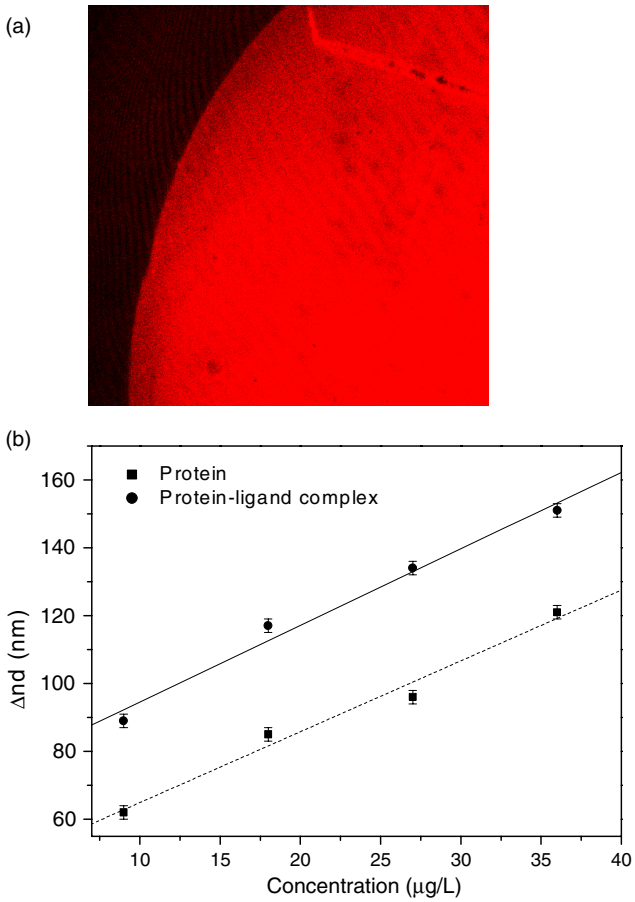

Figure 5. (a) Confocal microscopy image of the PSi chip infiltrated with the labelled GlnBP after dialysis in demi-water. (The fluorescence emitted by rhodamine-labelled GlnBP covalently bounded to the photochemically modified PSi surface is registered by a confocal microscope under laser beam (He–Ne) exposure.) (b) Dose–response curve as a function of the concentration of the protein and the Gln–GlnBP complex.

electron beam. The naked surface is thus covered by silicon radicals which strongly react with the proteins. In figures 5(a) and (b) the features of an optical biosensor are presented. In particular, the fluorescence emitted by rhodamine-labelled GlnBP immobilized on the photochemically modified PSi surface is shown in figure 5(a). We also measured the change in the optical path of the PSi layer due to the protein concentration before and after the interaction of the ligand. Figure 5(b) shows the dose–response curve as a function of the concentration of GlnBP and the complex GlnBP–Gln. In this curve, each point represents the average of three independent measurements and the error bars represent the standard deviations. In the protein concentration range investigated, the sensor exhibits a linear response ($R^2 = 0.99$) and it is possible to determine the sensitivity of the optical interferometer to the organic matter by estimating the slopes of the two curves: $s_{\text{prot}} = 21(1)$ nm μg^{-1} l and $s_{\text{comp}} = 23(1)$ nm μg^{-1} l for the protein and complex, respectively. As expected, the relative distance between the two curves is constant, the protein–complex ratio being always 1:1.

5. Conclusions

This paper reviews examples of optical biosensors for the detection of analytes of clinical, industrial, and home security interest. The biosensors described, based on porous silicon nanotechnology, may represent a model for designing advanced optical arrays (lab-on-a-chip) for simultaneously determining analytes of social interest.

Acknowledgments

This work was supported by CRdC-ATIBB POR UE-Campania Mis 3.16 activities (SD, MR) and by the CNR Commessa Diagnostica avanzata ed alimentazione (SD).

References

[1] Wolfbeis O S 2000 *Anal. Chem.* **72** 81R–89R
[2] Lakowicz J R 1995 *Proc. SPIE* **2388** 598
[3] Spichiger-Keller U E 1998 *Chemical Sensors and Biosensors for Medical and Biological Applications* (New York: Wiley–VCH) p 313
[4] Verkman A S, Sellers M C, Chao A C, Leung T and Ketcham R 1989 *Anal. Biochem.* **178** 355–61
[5] Tsien R Y, Rink T J and Poenie M 1985 *Cell Calcium* **6** 145–57
[6] Romoser V A, Hinkle P M and Persechini A 1997 *J. Biol. Chem.* **272** 13270–4
[7] Dancil K-P S, Greiner D P and Sailor M J 1999 *J. Am. Chem. Soc.* **121** 7925–30
[8] Arrand H F, Loni A, Arens-Fischer R, Kruger M G, Thoenissen M, Lueth H, Kershaw S and Vorazov N N 1999 *J. Lumin.* **80** 119–23
[9] Gao J, Gao T and Sailor M J 2000 *Appl. Phys. Lett.* **77** 901–3
[10] De Stefano L, Moretti L, Rossi A M, Rocchia M, Lamberti A, Longo O, Arcari P and Rendina I 2004 *IEEE Trans. Nanotech.* **3** 49–54
[11] De Stefano L, Moretti L, Rendina I, Rossi A M and Tundo S 2004 *Appl. Opt.* **43** 167–72
[12] Mulloni V and Pavesi L 2000 *Appl. Phys. Lett.* **76** 2523
[13] D'Auria S, Di Cesare N, Staiano M, Gryczynski Z, Rossi M and Lakowicz J R 2001 *Anal. Biochem.* **303** 138–44
[14] D'Auria S and Lakowicz J R 2001 *Curr. Opin. Biotechnol.* **12** 99–104
[15] D'Auria S, Rossi M, Gryczynski I, Gryczynski Z and Lakowicz J R 2000 *Anal. Biochem.* **283** 83–8
[16] D'Auria S, Di Cesare N, Gryczynski Z, Gryczynski I, Rossi M and Lakowicz J R 2000 *Biochem. Biophys. Res. Commun.* **274** 727–31
[17] D'Auria S, Rossi M, Hermann P and Lakowicz J R 2000 *Biophys. Chem.* **84** 167–76
[18] Staiano M, Bazzicalupo P, Rossi M and D'Auria S 2005 *Mol. BioSyst.* **1** 354–62
[19] Borini S, D'Auria S, Rossi M and Rossi A M 2005 *Lab Chip.* **5** 1048–52
[20] Scognamiglio V, Staiano M, Rossi M and D'Auria S 2004 *J. Fluoresc.* **14** 491–8
[21] The Diabetes Control and Complications Trial Research Group 1993 *N. Engl. J. Med.* **329** 977–86
[22] Roe J N and Smoller R 1998 *Crit. Rev. Ther. Drug Carrier Syst.* **15** 199–241
[23] Wang J, Chatrathi M P, Tian B and Polsky R 2000 *Anal. Chem.* **72** 2514–8
[24] James T D, Limaane P and Shinkai S 1996 *Chem. Commun.* 281–8
[25] Tong A-J, Yamauchi A, Hayashita T, Zhang Z-Y, Smith B D and Teramae N 2001 *Anal. Chem.* **73** 1530–6
[26] Schultz J, Mansouri S and Goldstein I J 1982 *Diabetes Care* **5** 245–53
[27] Meadows D and Schultz J S 1988 *Talanta* **35** 145–50
[28] Russell R J and Pishko M V 1999 *Anal. Chem.* **71** 3126–32
[29] Lakowicz J R and Maliwal B P 1993 *Anal. Chim. Acta* **271** 155–64
[30] Tolosa L, Gryczynski I, Eichhorn L R, Dattelbaum J D, Castellano F N, Rao G and Lakowicz J R 1999 *Anal. Biochem.* **267** 114–20
[31] D'Auria S, Herman P, Rossi M and Lakowicz J R 1999 *Biochem. Biophys. Res. Commun.* **263** 550–3
[32] Marvin J S and Hellinga H W 1998 *J. Am. Chem. Soc.* **120** 7–10
[33] Giuliano K A and Taylor D L 1998 *TIB Tech.* **16** 135–40
[34] Wilson G S and Hu Y 2000 *Chem. Rev.* **100** 2693–704
[35] Schalkhammer T, Lobmaier C, Ecker B, Wakolbinger W, Kynclova E, Hawa G and Pittner F 1994 *Sensors Actuators* B **18/19** 587–91

[36] Szmacinski H and Lakowicz J R 1995 *Sensors Actuators* B **29** 15–24
[37] Bambot S B, Rao G, Romauld M, Carter G M and Lakowicz J R 1995 *Biosens. Bioelectron.* **10** 643–52
[38] Velho G, Froguel P, Thevenot D R and Reach G 1988 *Diab. Nutr. Metab.* **3** 227–33
[39] Menstein D J, Pai E F, Schopfer L M and Massey V 1986 *Biochemistry* **25** 6807–16
[40] Hecht H J, Kalisz H M, Hendle J, Schmid R D and Schmburg D 1993 *J. Mol. Biol.* **229** 153–72
[41] Bourne Y, Kolb H C, Radic Z, Sharpless K B, Taylor P and Marchot P 2004 *Proc. Natl Acad. Sci. USA* **101** 1449–54
[42] Bourne Y, Radic Z, Kolb H C, Sharpless K B, Taylor P and Marchot P 2005 *Chem. Biol. Interact.* **157/158** 159–65
[43] Bourne Y, Taylor P, Radic Z and Marchot P 2003 *EMBO J.* **22** 1–12
[44] Tai K, Shen T, Henchman R H, Bourne Y, Marchot P and McCammon J A 2002 *J. Am. Chem. Soc.* **124** 6153–61
[45] White B J, Legako J A and Harmon H J 2003 *Biosens. Bioelectron.* **18** 729–34
[46] Thomas J N 1986 *Mammalian Cell Technology* ed W G Thilly (Boston, MA: Butterworth) pp 109–30
[47] Zielke H R, Zielke C L and Ozand P T 1984 *Fed. Proc.* **43** 121–5
[48] Ozturk S S and Palsson B O 1990 *Biotechnol. Prog.* **6** 121–8
[49] Oh G S, Izuishi T, Inoue T W and Yoshida T J 1996 *J. Ferment. Bioeng.* **81** 329–36
[50] Sorochinskii V V and Kurganov B I 1997 *Appl. Biochem. Microbiol.* **33** 515–29
[51] Madaras M B, Spokane R B, Johnson J M and Wookward J R 1997 *Anal. Chem.* **69** 3674–8
[52] Higgins C F 2001 *Res. Microbiol.* **152** 205–10
[53] Nohno T, Saito T and Hong J-S 1986 *Mol. Gen. Genet.* **205** 260–9
[54] Weiner J H and Heppel L A 1971 *J. Biol. Chem.* **246** 6933–41