

New Emergent Nanotechnologies in Medical and Biochemical Applications: Advanced Fluorescence Protein-Based Nanosensors

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Abstract: In this review we explore the advantages deriving from the use of either enzymes or binding proteins to develop non-consuming substrate fluorescence biosensors. We report on a novel approach to address the consumption of substrate by enzyme-based biosensors, namely the utilization of apo-enzymes as non-active forms of proteins, which through still able to bind the ligand yet cannot transform it into product. We also report recent studies in which fluorescence labelling of a naturally thermostable binding protein by a fluorescent probe allows a quantitative monitoring of glucose. Finally, we will illustrate a novel methodology based on the utilization of porous silicon chips that allows a nanotechnological approach to the realization of protein arrays for analyses of high medical and biochemical interest.

Keywords: Porous silicon, proteins, diabetes, fluorescence, biosensors.

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 [1]. Fluorescence detection is the dominant analytical tool in medical testing, biotechnology and drug discovery. Starting in 1980s fluorescence probes for specific analytes become available [2-4]. Some of these sensing fluorophores are relatively simple, as illustrated by quinoline probes which are collisionally quenched by chloride [5,6]. However, the molecular complexity of the sensors quickly increases if one requires analyte binding to cause a spectral change. For example, the fluorophores specific for calcium are structurally complex and only a few display spectral shifts upon binding calcium [7]. As consequence, the development of specific sensors for biochemically relevant analytes is even more challenging. In fact, it is difficult to imagine how one would design a fluorescent probe which specifically binds pyruvate, lactate, or creatinine. Even a suitable structure could be designed and synthesized, there is no guarantee that the final molecule will display a spectral change, adequate water solubility, and a suitable affinity constant.

To circumvent these difficulties modern biotechnology has resorted to the idea of using proteins and enzymes as components of sensors for biochemical analytes [8]. Detection of living cells of Ca^{++} dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence [9]. The idea is to exploit the extremely wide range of selective affinities sculpted into the various proteins by biological evolution. The number of potential ligands specifically recognized by different proteins is very large and

ranges from small molecules to macromolecules (including protein 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 progresses of molecular genetics, the possibility of improving/changing some of the properties of the proteins by genetic manipulation. Many of the ligands that are important in clinical medicine and in the food control industry are relatively small (up to 1000 daltons). In these cases the enzymes appear to be the class of proteins endowed with the highest specificity and affinity. Other classes of proteins, such as receptors, transporters, antibodies etc., often present lower specificity although they offer other advantages such as the fact that they can specifically recognize a wide range of much larger ligands.

In this article we will briefly review the most recent and advanced applications of fluorescence-protein sensors for the sensing of analytes of social interest, such as lactate and glucose. In particular, we will show the use of a coenzyme-depleted lactate dehydrogenase for a non-consuming lactate sensor, and the use of an inactive form of glucose oxidase and the use of a thermostable sugar-binding protein as specific probes for the design of an advanced non-consuming glucose biosensor for diabetic patients. Finally, we will show a very recent generally applicable technique for nanopatterning of proteins on a porous silicon surface that allows the design of nano-arrays to sense analytes of high medical and biochemical interest.

2. A PROTEIN BIOSENSOR FOR LACTATE

Measurements of blood lactate are available in predicting multiple organ failure and death in patients with septic shock. Lactate acidosis is also known to accompany decreased tissue oxygenation, hypovolemic, left ventricular failure, and drug toxicity. While blood lactate is a useful diagnostic indicator, its use is hindered by the time required for a lactate determination [10-12]. Even under favourable

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conditions, a lactate measurement takes 30 min or longer, which is too long for many clinical decisions. Lactate determinations are typically performed by enzymatic oxidation to pyruvate by lactate dehydrogenase or lactate oxidase, followed by detection of NADH or H₂O₂, respectively [13,14]. Recently, an alternative way for measuring lactate using the lactate dehydrogenase (LDH) from beef heart has been reported [15]. The tryptophan fluorescence of LDH was shown to be sensitive to micromolar additions of lactate, resulting in a fluorescence decrease of about 30 %. However, a clinical useful sensor requires that the wavelength be long enough to allow the use of simple excitation sources. Within the past several years UV output near 370 nm has become available from light-emitting diodes (LED), and laser diodes as short as 399 nm have been reported [16,17]. LEDs are also known to be useful for nanosecond lifetime measurements because of the capability of high-frequency modulation. Hence, we labelled the LDH with a fluorophore suitable for LED excitation. LDH, when noncovalently labelled with 8-anilino-1-naphthalene sulfonic acid (ANS) displayed a decrease in the ANS emission intensity upon binding lactate [18]. This decrease occurs without the consumption of lactate. The addition of lactate results in 40 % decrease of the ANS-LDH fluorescence emission. The use of the above described device for measuring the effect of the lactate on the ANS-labelled LDH results in a compensation angle change of about 6°. While the range seems small, the compensation angles are readily measured to about 0.1°, so that a 6° change corresponds to an accuracy of 2 % in the lactate concentration. Despite of the obtained results, we believe that the ANS-LDH system represents only the first step toward the development of a useful point-of-care lactate sensor. In fact, the LDH used in these experiments was only marginally stable. The utilization of LDH from thermophilic sources should result in a long-term stable sensor.

3. GLUCOSE SENSING

There is considerable medical interest in measurements of blood glucose [19]. Close control of blood glucose is necessary to avoid the long term health effects of diabetes which include blindness and neuropathies. As a consequence there is a substantial world-wide effort to develop non-invasive and minimally invasive methods for frequent and/or continuous monitoring of glucose in blood [20-21]. A wide variety of methods have been tested, including optical rotation, near-infrared absorbance, Raman scattering, as well as the design and synthesis of glucose-specific fluorescence probes and resonance energy transfer between proteins which bind glucose and acceptor-labelled glucose analogues [22-26]. Proteins which bind glucose have also been used, such as hexokinases, glucose oxidase, glucose-galactose-binding protein, as probes to monitor the concentrations of glucose [27-30]. The use of protein-based sensors depends however on protocols to enhance the protein stability such as the introduction of changes in the protein amino acid composition leading to enhanced protein structural stability [31]. An alternative method is to use naturally thermostable enzymes and proteins isolated from thermophilic microorganisms. These macromolecules have intrinsically stable structural features [32-35] and they can be considered as ideal probes for the development of innovative sensing systems [36].

In the following sections, we will describe the use of inactive form of a mesophilic glucose oxidase as non-consuming sensor, and of a sugar-binding protein from a thermophilic organism for glucose sensing.

3.1. Glucose Oxidase for Glucose Sensing

Glucose oxidase (GO) (EC 1.1.3.4) from *Aspergillus niger* catalyzes the conversion of β -D-glucose and oxygen to D-glucono-1,5-lactone and hydrogen peroxide. It is a flavo-protein, 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. Because glucose is consumed, this enzyme cannot be used as a reversible sensor. In order to prevent glucose oxidation, we removed the FAD cofactor that is required for the reaction. The coenzyme-depleted enzyme can still bind glucose with an affinity comparably to the holo-enzyme. Additionally, the tryptophan fluorescence of the apo-GO was sensitive to glucose binding, resulting in a decrease of emission intensity of about 25 %. However, the intrinsic fluorescence intensity from proteins is usually not useful for clinical sensing because of the need for complex or bulky light sources as well as for the presence of numerous proteins in most biological samples. In an attempt to obtain a glucose sensor with longer excitation and emission wavelengths we labelled GO with 8-anilino-1-naphthalene sulfonic acid (ANS) [37]. Fig. 1 shows the effect of the addition of glucose on emission intensity spectra of ANS-labelled GO. In Figs. 2A,B are depicted the glucose effect on the maximum emission intensity and on mean lifetime decay of the ANS-labelled enzyme, respectively. The results show that the glucose affects the fluorescence features of the ANS-labelled GO. In particular, the glucose addition results in a decrease of the intensity and the mean-lifetime of 25 % and over 40 %, respectively, indicating the potential usefulness of the apo-GO as a glucose sensor.

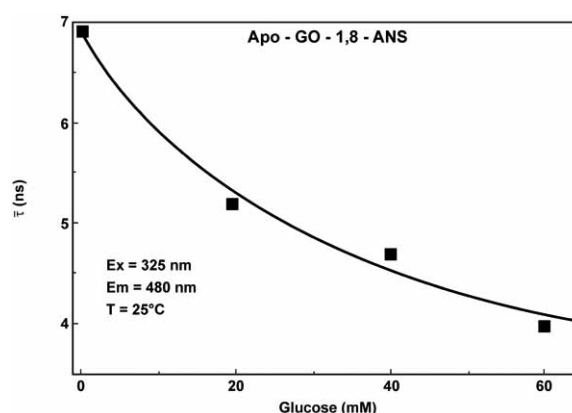


Fig. (1). Effect of glucose on the mean decay time of 1,8-ANS labelled apo-glucose oxidase.

3.2. Thermostable Protein for Glucose Sensing

A thermostable sugar-binding protein from the archaeon *Pyrococcus horikoshii* (Ph-SBP) was studied for the sensing of glucose [38]. Ph-SBP is a monomer of 55 kDa that binds glucose molecules. The protein possesses a typical α/β secondary structure organization and the interaction with glucose does not modify the secondary structure content of the protein (Figs. 3a,3b). The fluorescence emission spectrum of Ph-SBP at room temperature upon excitation at 295 nm dis-

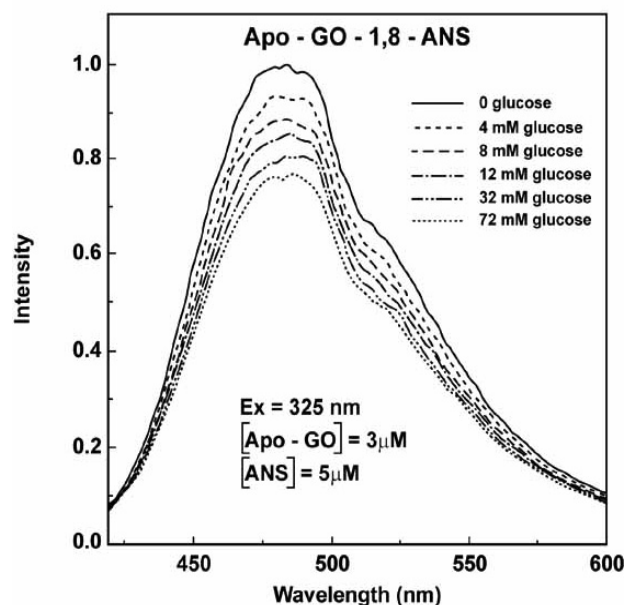


Fig. (2A). Emission spectra of 1,8-ANS labelled apo-glucose oxidase in the presence of different amount of glucose. Excitation at 325 nm.

plays an emission maximum at 340 nm, which is characteristic of partially shielded tryptophan residues. The addition of 10 mM glucose to the protein solution results in quenching of the tryptophanyl fluorescence emission by about 18 % (data not shown). This result indicates that the Ph-SBP is able to bind glucose. The ability of Ph-SBP to bind glucose as well as its high stability in a wide range of temperatures prompted us to investigate the possible utilization of Ph-SBP as a probe for the development of a substrate non-consuming fluorescence protein biosensor for glucose. The protein was labelled with the cysteine-reactive fluorescence probe 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium

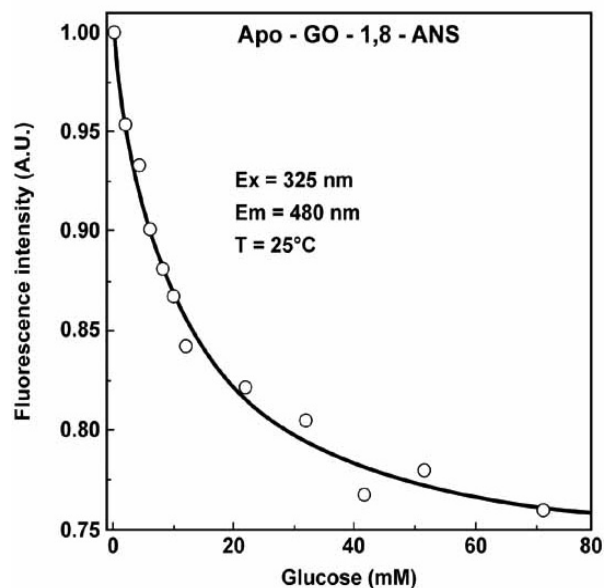


Fig. (2B). Glucose-dependent emission intensity of 1,8-ANS bound to apo-glucose oxidase. Excitation at 325 nm; Emission at 480 nm.

In Fig. 4 are shown the emission fluorescence spectra of the covalently labelled IAANS-Ph-SBP. The intensity of the IAANS emission was sensitive to the addition of glucose. In the inset, the effect of glucose on the emission maximum is shown. IAANS is known to be a molecule sensitive to its local environment [39]. The result obtained (a decrease in the emission intensity) suggests that the binding of glucose to the IAANS-Ph-SBP displaces the IAANS into a more polar environment as a result of a conformational change of the protein.

From a practical point of view, to detect biomolecular interactions, one of the partners, the sensor molecule, should

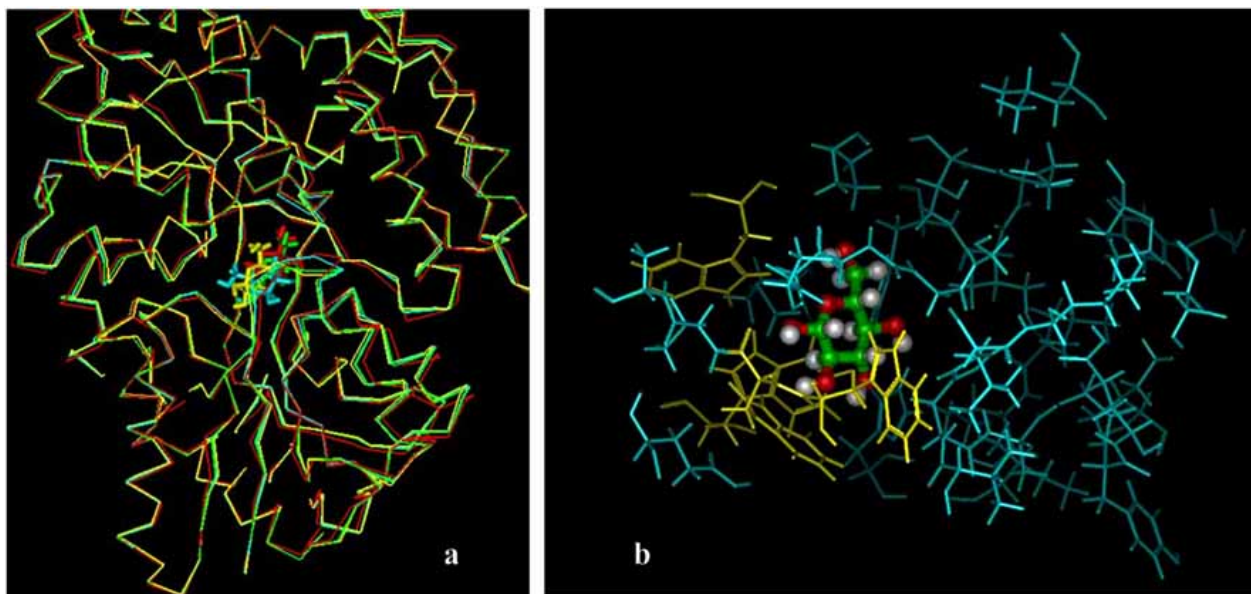


Fig. (3). 3D structure of Ph-SBP with glucose. a) Global view of the different complexes simulated with glucose into the binding cavity of the protein. The colours correspond to different positions of the sugar in the binding site. b) Close-up view of the complex between glucose and Ph-SBP with the lower interaction energy.

be immobilized on a sensor surface. The counterpart molecule, the analyte, is usually dissolved in the liquid phase and binds the immobilized sensing molecule. We have immobilized the IAANS-Ph-SBP on a reactive aldehyde silylated slide. The aldehyde on the silylated slides reacted readily with primary amines on the protein forming a Schiff's base linkage. The immobilized IAANS-Ph-SBP was tested for its capacity to bind glucose. In Fig. 5 are shown the results of front-face fluorescence measurements. The addition of 10 mM glucose to the immobilized IAANS-Ph-SBP results in the quenching of the emission intensity by about 20 % and in a small blue-shift of the emission maximum. Even if the visualization of the front-face emission spectra may suggest a weak emission fluorescence signal from the protein sample, it is important to state that the front face measurements are fully reproducible. In addition, these results are consistent with those obtained on the IAANS-Ph-SBP shown in Fig. 4, and indicate that the process of immobilization does not change the properties of IAANS-Ph-SBP. The results shown above demonstrate that the thermostable Ph-SBP can serve as a probe for the development of a non-consuming glucose biosensor.

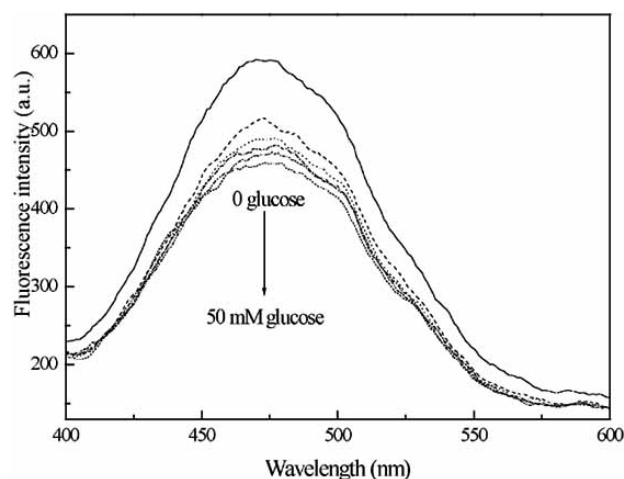


Fig. (4). Emission fluorescence spectra of covalently labelled IAANS-Ph-SBP. Excitation was at 370 nm and temperature was set at 25 °C. The inset shows the effect of glucose on the fluorescence emission maximum.

4. IMMOBILIZATION OF MACROMOLECULES ON A SOLID SUPPORT: NANOPATTERNING ON POROUS SILICON

The realization of a biosensor requires the immobilization of the sensing system, *i.e.* the macromolecule, on a solid support. A new technique of nanodeposition of proteins on a porous silicon surface was investigated by authors and is presented here [40]. The immobilization of biomolecules on a solid substrate and their localization in “small” regions are major requirements for a variety of novel intriguing applications, such as bioelectronics, biosensing, and other emerging interdisciplinary fields. In particular, scaling down to submicrometre resolution opens the door to the fabrication of completely new nanodevices, *e.g.* molecule-based transistors and nanosensors.

Porous Silicon (PS) is a silicon nanosponge with three main advantages for the fabrication of biodevices: the large

specific surface (of the order of $200 \text{ m}^2 \text{ cm}^{-3}$) [41], the biocompatibility [42], and the compatibility with standard silicon technology (electronics). In fact, in recent years a lot of work has been devoted to investigating chemical and biological applications of PS technology [43-48]. The technique of protein nanopatterning described here is based on the electron irradiation of fresh PS in a Scanning Electron Microscope (SEM). The nanopatterning process involves three steps:

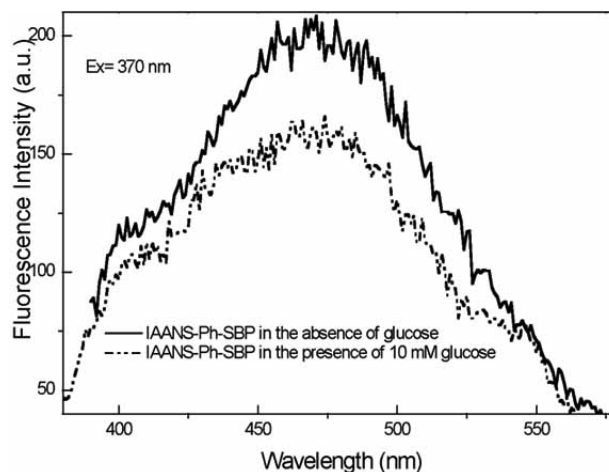


Fig. (5). Fluorescence front-face measurements of immobilized IAANS-Ph-SBP in the absence and in the presence of glucose. Excitation was at 370 nm. The spectra are corrected for the dilution factor.

- (i) Anodization of a single crystal Si wafer in HF-EtOH solution, producing a PS layer;
- (ii) Electron irradiation of PS in the SEM;
- (iii) Addition of protein solution.

After rinsing in deionized water and drying under a nitrogen stream, the specific binding of proteins to the PS irradiated regions was observed. This is immediately visible in the optical microscope image of Fig. 6a, and it is demonstrated in Fig. 6b, where the fluorescence spectra acquired from irradiated and non-irradiated areas of the same PS sample are shown, after the addition of the rhodamine-labelled glutamine-binding protein solution. As a confirmation of this evidence, in Fig. 7 the results of the Fourier Transform Infrared (FTIR) microscopy characterization, performed on a PS sample exposed to the glucose-binding protein solution, are reported. The so-called “Amide I” band centred at about 1650 cm^{-1} , typical signature of the peptide bond, is easily detected in the spectrum of the irradiated area, although no Attenuated Total Reflection (ATR) approach was used to increase the instrument sensitivity, as normally required for similar measurements on solid substrates. This observation testified that a huge amount of protein was selectively bound to the PS regions modified by the electron beam. The strong interaction between proteins and irradiated PS was explained on the basis of a previously described mechanism [49,50]. The irradiated surface, able to bind alkenes through the cleavage of C=C double bonds, can react with proteins, for instance through the peptide bond. In Fig. 6a the optical microscope image of protein patterned areas obtained by electron irradiation at various SEM conditions is shown. The dif-

ferent amount of bound proteins is clearly visible by the contrast between different regions. Moreover, the image in the inset, taken at higher magnification, shows the submicrometre lines filling one of these areas. A nanopatterning method which is controlled by means of the electron beam conditions, such as the energy and the dose, has been developed. By investigating the effect of various parameters on the nanopatterning process, a nice tunability of the amount of bound proteins was found. A monotonic increase of the intensity of the fluorescence signal, coming from the fluorophore-labelled proteins, versus the electronic dose employed for PS irradiation, was observed. Furthermore, it was verified that the incubation time, i.e. the period during which the sample is kept in contact with the protein solution, strongly influenced the amount of molecules binding to the material. Varying this time frame from 5 minutes up to 2 hours, a steep increase in the fluorescence was observed, but even after just a few minutes at room temperature the signal arising from the irradiated patterns was easily detected. Within this study of the parameters affecting the nanopatterning process, the most interesting result concerned the behaviour of the amount of bound proteins versus the energy of the electrons hitting the material during the irradiation step. The Fig. 8 shows the fluorescence spectra acquired from regions of the same sample, irradiated at various accelerating voltage values, while the other SEM parameters (beam current and electronic dose) were kept constant. It can be noted that the fluorescence signal became more intense when the energy was augmented from 10 keV to 15 keV, but it started lowering under a further increase of the value of this parameter. This is due to the fact that the penetration depth of the electrons in the sample increases with the energy, so that, the higher the accelerating voltage, the greater the volume of PS modified by the incident beam, until the electrons become so fast that they go beyond the porous layer with minimum interaction. This interpretation is confirmed by a previous investigation [50]. Since proteins selectively bind to irradiated PS, the resolution of this technique is strictly connected to the resolution of standard EBL. As a demonstration of this, the fluorescence spectra coming from two equal areas, one filled by the scanning beam following submicrometre lines (as the pattern of the inset of Fig. 6a), and the other fully filled were compared. The lines are optically visible and the fluorescence intensity is almost proportional to the surface fraction directly scanned by the electron beam. Moreover, it is worth noting that PS is a convenient substrate for the EBL technique, because the proximity effect, a key-factor limiting the resolution of the technique, is strongly reduced due to the low density of the sponge-like material [51]. Finally, to demonstrate the sensing capabilities of our system, the functionality of the immobilized biomolecules was investigated. Glucose-binding proteins were grafted to Electron Beam-Activated Porous Silicon (EBAPS), and a solution of fluorescent glucose was dosed on the sample subsequently. The fluorescence spectra shown in Fig. 9 clearly demonstrated that proteins were able to bind the target molecules. Thus, the biomolecules immobilized on a porous silicon surface retained their functionality and their biological activity, so that many sensing applications are easily at hand. Interestingly, the amount of bound glucose increased with the dose employed to create EBAPS patterns, as in the case of binding proteins. This suggested that almost all the grafted bio-

molecules were participating in the detection of the analyte. The main advantage of this technology resides in the high resolution (submicrometre) coupled with the 3-dimensionality of the patterns. This permits localization of a large amount of biomolecules on a small area in the xy plane, and may allow the fabrication of highly sensitive nanobiosensors.

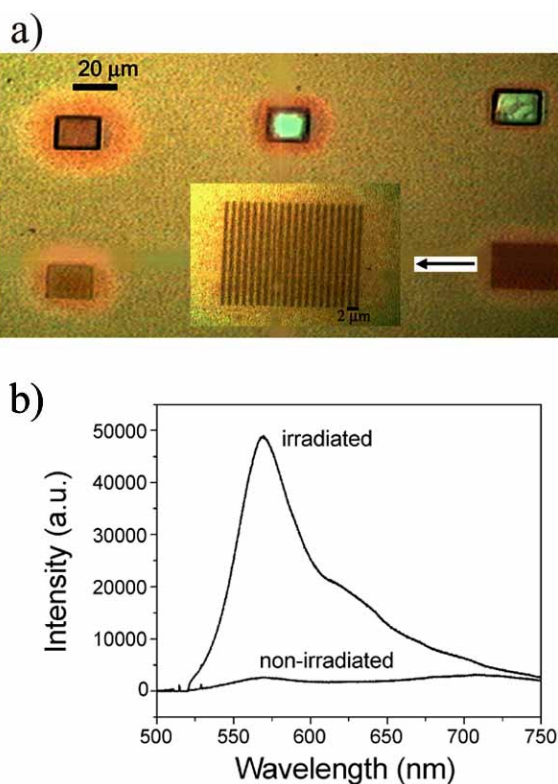


Fig. (6). a) Optical microscope image of protein patterns created on a porous silicon surface by electron beam irradiation at various conditions and after exposure to a glutamine-binding protein solution for an incubation time of 1 hour at 37 °C. In the inset, the magnification of one pattern is shown, revealing the submicrometre resolution of the technique. b) Fluorescence spectra obtained from two regions of the same porous silicon sample. One region was irradiated by an electron beam with energy of 15 keV and a dose of 140 mC cm⁻². After the irradiation, the sample was exposed to a rhodamine-labelled glutamine-binding protein solution for 1 hour at 37 °C. The peak of the irradiated region is about 30 times higher than the peak of the nonirradiated region.

5. CONCLUSIONS

The possibility of using non-active apo-enzymes or proteins belonging to the “binding-protein” family for the design of reversible biosensor greatly expands the range of analytes which can be selectively sensed. The showed spectral changes occurring upon ligand binding are still somewhat small and this is an area that requires improvement and further studies. Exploring other polarity sensitive probes or the use of RET methodologies appears to be a promising avenue in this direction. Although the work with apo-enzymes is, so far, at a more advanced stage, it is possible that other type of proteins will soon enter this field. For in-

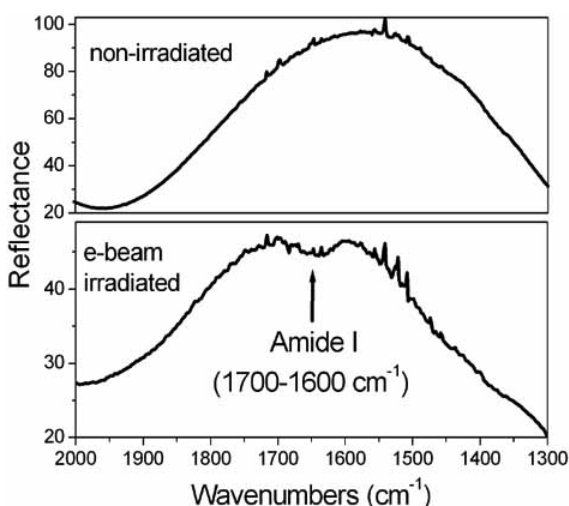


Fig. (7). FTIR spectra acquired from two regions of the same porous silicon sample. One region was irradiated by the electron beam with energy of 15 keV and a dose of 140 mC cm⁻². After the irradiation, the sample was exposed to a glucose-binding protein solution for 2 hours at 37 °C.

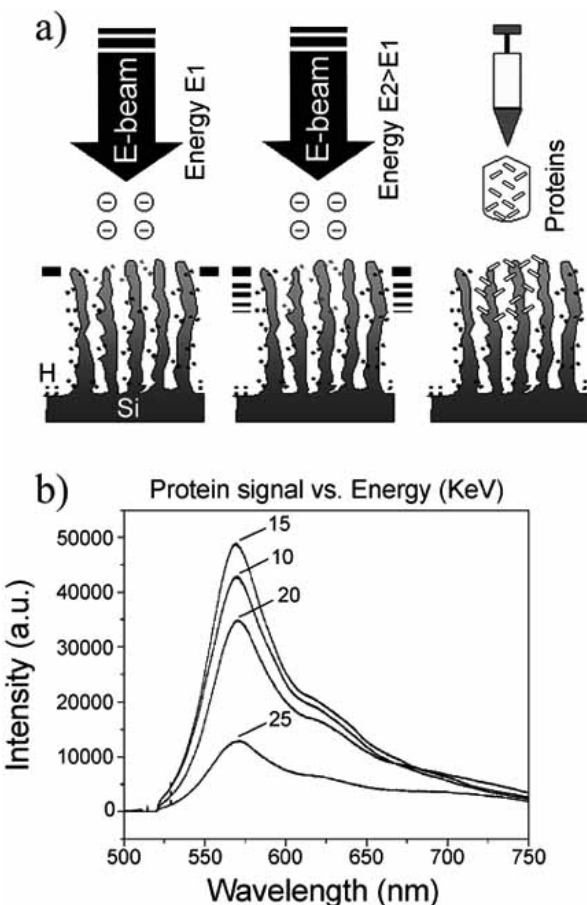


Fig. (8). Schematic of the 3D nanopatterning process driven by the electrons hitting the silicon nanosponge at various energy values (a). The electrons provoke a local desorption of hydrogen (H) from the surface, leaving dangling bonds (DB) able to bind proteins. In (b), fluorescence spectra acquired from different regions of the same sample are reported. The sample was irradiated with an electronic dose of 140 mC cm⁻², and exposed to a rhodamine-labelled glutamine-binding protein solution for 1 hour at 37 °C.

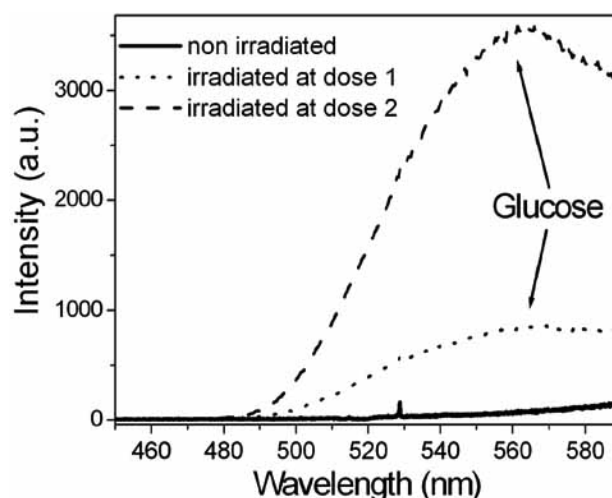


Fig. (9). Fluorescence spectra acquired from different regions of the same sample, activated with different electronic doses (D1 = 70 mC cm⁻², D2 = 140 mC cm⁻²) and functionalized with glucose-binding proteins. After rinsing in deionized water and drying under a nitrogen stream, a droplet of fluorescent glucose solution was dosed on the sample. After rinsing and drying, the presence of fluorescent glucose is detected only on the EBAPS regions.

stance the example of the naturally thermostable sugar-binding protein from the archaeon *Pyrococcus horikoshii* shows that sensors based on receptors could be developed. In addition, we believe that in the next few years two factors will greatly promote the role that rationale gene design/manipulation will have in improving the properties of proteins to be used as sensors. One is the improvement and simplification of the techniques for the manipulation of genes. The second is the accumulation of more information on the three dimensional structure of proteins. Finally, the use of nanotechnological approaches for the design and realization of nano-arrays for analyses of social interest will definitely boost the field of biosensing.

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