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Porous Silicon-based Electrochemical Biosensors

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1. Introduction

There is a growing need of highly efficient compact devices for a wide range of applications in several fields. Among the candidate materials, porous silicon (PSi) has attracted an increasing research interest, apart from its obvious potentially straightforward integration with standard Si technologies, thanks to its unique properties, and its present applications span from biomedicine (Anglin et al. 2008) to biosensing, from photonics (Huy et al. 2009) to photovoltaic devices (Xiong et al. 2010).

After its discovery (Uhlir 1956), porous silicon hasn't attracted much attention until the discovery of its room temperature luminescence properties (Canham 1990). However, the porous silicon-based photonics with all-silicon light-emitting devices never showed, to date, high enough efficiency for real applications. Nevertheless, many other applications have since been the object of much research, thanks to the many advantages of porous silicon: the flexibility of its formation process (Föll et al. 2002), the extensive tailoring of its structural properties (Lehmann et al. 2000), the very large specific surface (Halimaoui 1993) and also its biocompatibility, mandatory for both drug delivery devices and several biosensing applications (Low et al. 2009; Park et al. 2009). The deep knowledge of silicon chemistry is easily applicable to porous silicon (Buriak 2002; Salonen and Lehto 2008) and allows functionalization of the internal pore surface for the chemical bonding of the biological molecules of interest or for a better stabilization of the structure. The relevance for the field of PSi biosensors of a well controlled surface chemistry has been detailed by Lees and coworkers and by Kilian and coworkers (Lees et al. 2003; Kilian et al. 2009) and will be treated more deeply later in this article.

The very large internal surface of porous silicon proved to be a great advantage since, like other porous materials, it allows the bonding of active molecules over a large surface in a small volume (e.g. a 20 µm thick PSi sample with a specific surface area of 500 m^2/cm^3 may offer 1 m^2 of developed surface with only 1 cm^2 of external surface), with a sensible increase of the efficiency of the devices. Moreover, the large internal surface is important when there is the need of dispersing the active molecules, as happened in the case of laser dye dispersed in a PSi matrix (Setzu et al. 1999). Differently from other materials, however, PSi may be easily prepared either in powder or wafer, depending on the specific application. This allows for the fabrication of devices that can be dispersed in a given medium or that can be reusable. Devices integrating PSi layers with specific enzymes or with molecules with

specific target allow the realization of label free biosensors. Examples of this kind of devices have been demonstrated, for instance, for DNA sensing (Rong et al. 2008) and for triglycerides quantitative measurements (Setzu et al. 2007).

Porous silicon was successfully used in the development of a quite large variety of new biosensors, mainly using optical detection (Chan et al. 2001; Jane et al. 2009). It is surprising that porous silicon electrochemical sensors didn't get as much attention as the optical sensors. This is likely due to the fact that much research efforts have been devoted to the development of optical PSi devices in the field of optoelectronics. Then, a natural transfer of this knowledge to the field of biosensing has occurred when PSi-based biosensors become an interesting research field. However, the general field of electrochemical sensors (Bakker and Telting-Diaz 2002; Privett et al. 2008) is the most developed sensor branch and also PSi electrochemical sensors have been developed showing interesting characteristics and sensitivity properties.

Several reviews describe the state of the art of PSi biosensors (Anglin et al. 2008; Jane et al. 2009; Kilian et al. 2009) mainly based on optical signal transduction. The remarkable development of optical PSi biosensor has been triggered by the ability to modulate the porous silicon refractive index in the etch direction, and therefore to tailor the optical properties of the devices to one's needs, that has stimulated the research in the field of signal transduction by optical means. Optical transduction through either Fabry-Perot fringes, microcavity resonators or rugate filters has been widely investigated (Lin et al. 1997; Chan et al. 2001; Sailor 2007). There are several examples of quantitative determination of a given DNA strain by using optical detection on a single or multiple-layer PSi sample (Chan et al. 2000; De Stefano et al. 2007). Other examples show in-body detection of drug release (Anglin et al. 2008). Optical detection may be extremely useful when there is the need of a very high sensitivity for small amount of molecules to be detected, since the refractive index of the porous layer is highly affected by a change in the refractive index of the liquid in the pores (Anderson et al. 2003). The measured sensitivity of the optical biosensors strongly depends on the chosen optical structure and on the analyte (Haes and Van Duyne 2002; DeLouise et al. 2005).

It is, however, rather surprising that very little research has been made in the field of electrochemical porous silicon-based sensors even if electrochemical sensors have several important advantages: low cost and high sensitivity, together with a low power requirement and relatively simple detection instruments. Moreover they can be miniaturised more easily than optical biosensors. All these considerations make particularly worthwhile to review the advantages of PSi electrochemical biosensors. This is the main aim of the present chapter.

2. Porous silicon formation, oxidation, functionalisation, and biomolecules immobilisation

2.1 Formation process and main properties of porous silicon

Porous silicon samples are mainly produced by an electrochemical etch in the dark of a bulk single crystalline silicon substrate (Lehmann 1996). Fig. 1 shows a typical electrochemical cell used for PSi formation (Fig. 1a). A PSi sample is also shown (Fig. 1b). There are also various alternative preparation methods (Kolasinski 2005), i.e. chemical vapour etching (Ben Jaballah et al. 2005), metal-assisted etching (Harada et al. 2001; Chattopadhyay et al. 2002), and stain etching (Steckl et al. 1993; Ünal et al. 2001).
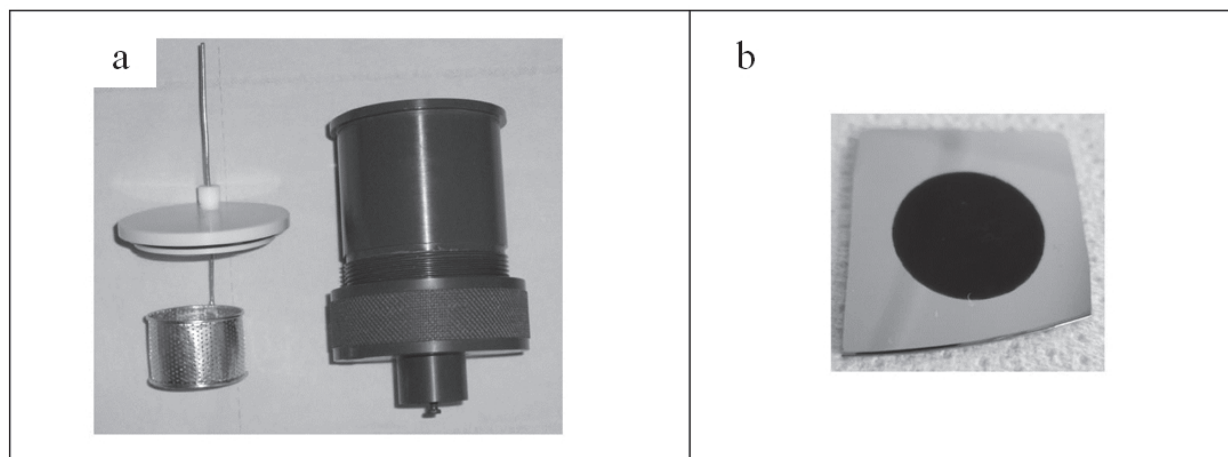
Fig. 1. Electrochemical cell used of PSi formation (a) and a PSi sample (b).

These methods give, at present, less reproducible results with respect to electrochemical etch, even though stain etch PSi is already commercially available. The etching solutions are prepared using HF, ethanol and pure water in different concentrations. The concentration of HF in the solution is one of the parameters controlling the structural properties of the samples and gives different porosities and pores' densities for a given current density used in the fabrication process. The HF concentration is also a fundamental parameter for the porosity range available. Halimaoui (Halimaoui 1993) studied the PSi layer porosities as a function of the applied current density for different HF concentrations, and observed that the porosity range between the lowest and highest current densities available for the porous layer formation varied for different HF concentrations. It has also been demonstrated that the PSi characteristics depend on the HF concentration of the etching solution (Dian et al. 2004; Kumar et al. 2009) from pores shape to density. In particular, Dian and coworkers showed that, for the same formation current density, varying the HF concentration leads to layers with different characteristics and porosities whose variations may reach about 30% in their experimental conditions. Kumar and coworkers studied the variations of the physical and electronic properties of PSi layers prepared using etching solutions with various HF contents by means of a combination of volumetric sorption isotherms, visual colour observation, photoluminescence, scanning electron microscopy, and Raman spectroscopy.

2.2 PSi layers morphology and design

Critical parameters for the defining of the PSi layers pores morphology are the doping type and the doping level of the crystalline silicon substrates used for the preparation of the samples (Föll et al. 2002). These parameters affect the kind of porosity, starting from the pores' diameter that can span from nanopores (a few nm) to mesopores (a few tens of nm to a few hundreds of nm) up to macropores (a few µm).

Only the nanoporous p- and p^+-type porous silicon show room temperature photoluminescence (Cullis et al. 1997). p^+ and n^+-type PSi are mesoporous and suitable for immobilisation of bio-macromolecules with a few nm diameter, while p-type PSi, whose pore diameter is of the order of a few nm, is suitable only for very small molecules. Macroporous n-type PSi may accommodate larger molecules, whose size depends on the pores' diameter. It can be prepared with pores in the 100 nm – few µm range, depending on
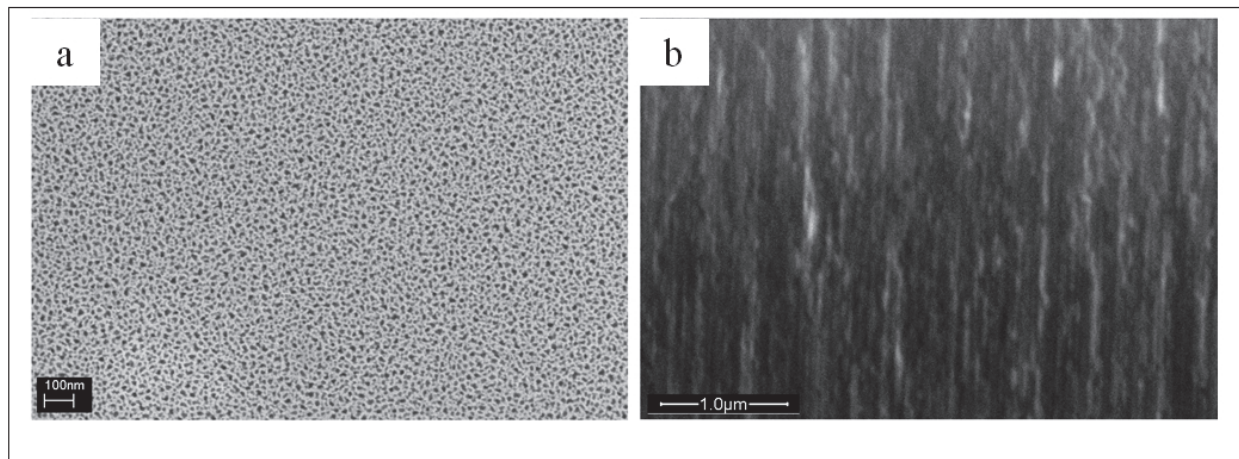
Fig. 2. SEM micrographs of *n*⁺-PSi sample. Top view (a) and the side view (b) of the nano-pores.

the formation condition (Gruning and Lehmann 1996; Ouyang and Fauchet 2005). The pores' diameter can be further modified after formation by means of one or more of the several available techniques able to enlarge the pores' diameter to better adapt to the size of the molecule of interest. Tinsley-Bown and co-workers (Tinsley-Bown et al. 2000) described a method based on immersing the porous layers in an ethanol-rich alkali (KOH) solution, whose effect on the pores' diameter may be controlled by the immersion time. Hamm and coworkers (Hamm et al. 2003) used a similar method by immersing the PSi samples in a NaOH etching (0.1 and 1 M) solution. To avoid problems due the hydrophobic nature of the PSi walls, an ethanol drop is put on the samples' surface before the immersion in the NaOH solution. These methods add a significant flexibility in the tailoring of the PSi layers structural properties in view of the realization of biosensors. More details about the dependence of the kind, shape and size of the PSi pores on the substrate doping may be found in the scientific literature (Smith and Collins 1992; Lehmann et al. 2000; Föll et al. 2002).

A relevant characteristic of porous silicon is that once the porous layer is formed it will play no longer any role in the ongoing formation process: the electrochemical etch is essentially an interface process taking place only at the porous/crystalline interface (Chazalviel et al. 2000). Since the structural and optical features of the formed layers depend on the formation parameters, and in particular on the formation current density, it is possible to design structures well tailored on the needs of the application. In particular, knowing that higher formation current densities lead to layers with higher porosities and lower refractive index, PSi multilayers with an in-the-depth modulation have been realised. A periodic variation of the formation current density will lead to a periodic variation of the porosity in the formation direction and then to a corresponding periodic refractive index variation (Setzu et al. 2000), leading to the realization of 1D photonic bandgap in the formation direction.

2.3 Oxidation of PSi surface

Surface chemistry of PSi is a feature of fundamental interest (Kilian et al. 2009). Chemical modifications at the air-solid interfaces strongly affect the biosensing performance (Dancil et al. 1999). Indeed, freshly prepared PSi is unstable due to the presence of highly reactive silicon hydride (Si-H$_x$, x = 1, 2 and 3) species that are very reactive both in air and in water

(Anderson et al. 2003). Thus, in order to use PSi as a matrix for a biosensing device, a stable surface must be obtained. Several procedures addressed to this purpose have been reported. The most common is to grow an oxide layer on the PSi surface - to avoid spontaneous oxidation either in air or in water media – through a thermal (Reddy et al. 2003), or a chemical (ozone) treatment (Dancil et al. 1999). Oxidation treatment can be followed by a silanisation step to introduce suitable functional groups (Kilian et al. 2009). The biomolecule needed for biosensing can be adsorbed either after the oxidation (physical adsorption) or the silanisation (chemical adsorption) steps. Whatever the kind of interactions between the PSi and the biomolecule, the oxidation step affects the operative behaviour of potentiometric biosensors. The formation of the SiO_2 layer during thermal or ozone oxidation cannot be controlled; therefore, it is not possible to obtain a reproducible surface in different PSi wafer samples preparation. And reproducibility, clearly, is a crucial step in the fabrication of a biosensor.

Anodic oxidation is a technique commonly used to stabilize the very reactive surface of fresh porous silicon (Bsiesy et al. 1991; Petrova-Koch et al. 1992; Cantin et al. 1996). Compared to other oxidation techniques, it allows the obtainment of highly reproducible oxidised samples. Recently, we reported a study where PSi layers were oxidised through this electrochemical procedure (Salis et al. 2010). Anodic oxidation is realised through a controlled procedure where a given current intensity is made to flow into an electrochemical cell until a fixed oxidising potential or a given oxidation time is reached. A typical anodic oxidation curve is shown in Fig. 3.
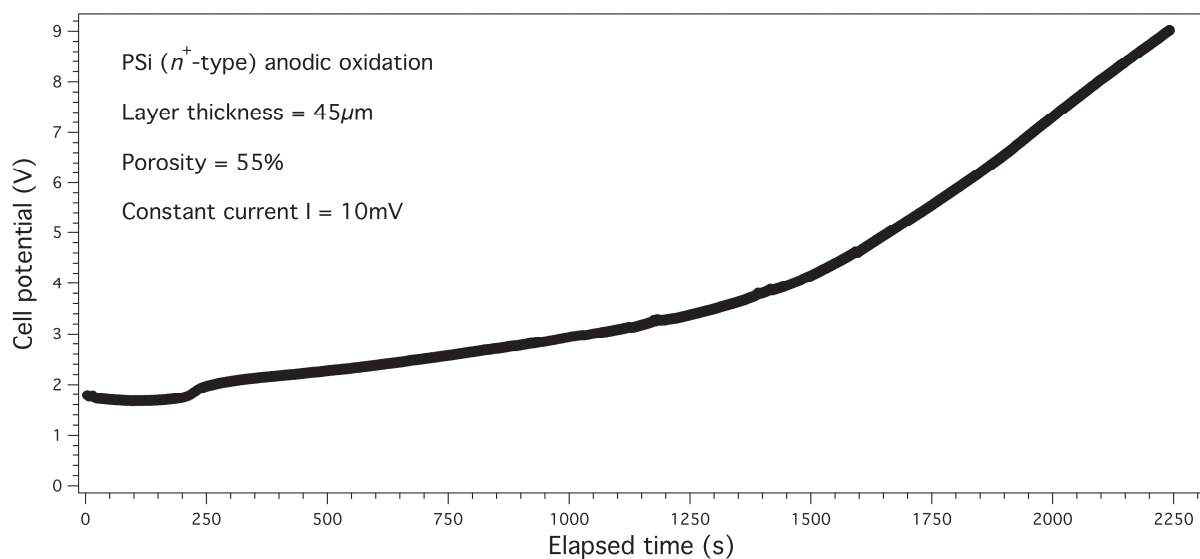

Fig. 3. A typical anodic oxidation curve.

2.4 PSi surface functionalisation and biomolecules immobilisation

As reported above, the surface of fresh PSi is almost completely covered by highly reactive hydride species (Petrova-Koch et al. 1992). Indeed, when PSi is put in contact with alkaline solutions, and even with buffer solutions at physiological pH, it dissolves giving orthosilicic acid $(Si(OH)_4)$ (Anderson et al. 2003). Neglecting this phenomenon can lead to confuse a signal drift, due to the oxidation phenomenon, with the biosensing signal (Lin et al. 1997; Janshoff et al. 1998; Dancil et al. 1999). Besides oxidation, a strategy to improve PSi stability

is the modification of the surface by mean of functionalising agents. This is usually done as a preliminary step which ends with the biomolecule immobilisation.

Xia and coworkers published a study where porous silicon surfaces were bio-functionalised by a simple three-step method (Xia et al. 2006). A scheme of the functionalising procedure is shown in Fig. 4. First the hydrogen-terminated porous silicon was oxidised and amino-silanised in a one-pot reaction by 3-aminopropyl(triethoxyl)silane (APTES) with the aid of an organic base, diisopropylethylamine. Then, the primary amine reacted with a two homobifunctional cross-linker, bis(N-succinimidyl)carbonate. By modulating the reaction conditions, a high surface coverage of linking groups, succinimidyl ester, could be obtained. A similar functionalisation could be done by using (N,N'-bis(p-maleimidophenyl)methylene instead of bis(N-succinimidyl)carbonate. Succinimidyl ester is an amino-reactive group, therefore mouse monoclonal antibody bearing amino groups was grafted (Xia et al. 2006). An enzyme (Horseradish peroxidise) linked immunosorbent assay was used to evaluate the surface density of antibody. The other linker, (N,N'-bis(p-maleimidophenyl)methylene, after reflux in acetonitrile with surface amines, resulted in maleimide-terminated surfaces. Then a reduced urokinase bearing accessible thiol groups was grafted and its enzymatic activity determined.
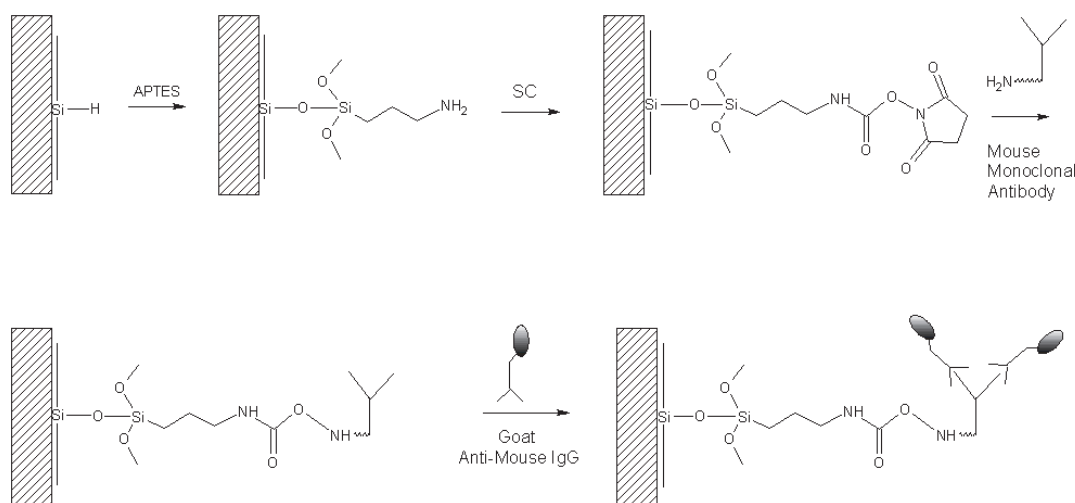

Fig. 4. An example of PSi functionalisation and enzyme immobilisation. Adapted from (Xia et al. 2006).

Another procedure was followed by Fernandez and coworkers (Fernandez et al. 2008). PSi surface was firstly oxidised then modified with APTES and with the bifunctional reagent gluharaldehyde. This procedure allowed to immobilise covalently *Pseudomonas cepacia* lipase as confirmed by means of FTIR spectroscopy.

Although these procedures confer a high degree of chemical stability to PSi surface, they may have some drawbacks. As will be seen below, the presence of silica layers at solid/liquid interface confers sensitivity to pH variations. The functionalisation steps, which substitutes silica with other chemical groups may lead to the loss of pH sensitivity and hence to the impossibility to use it for the realisation of potentiometric biosensor (Hamann and Lewis 2006). This is not, in any case, a problem for other types of signal transduction.

As noted earlier, all porous materials have a large specific surface that can reach very high area values, such as 2000 m^2/g e.g. in case of activated carbon (Kaneko et al. 1992). This high value, considering an activated carbon density of about 0.5 g/cm^3 (it depends on the

characteristics of the activated carbon), is analogous to that obtainable with porous silicon, whose specific surface may reach more than 900 m^2/cm^3 in the case of *p*-type nanoporous silicon (Halimaoui 1993). This large specific surface area allows the immobilisation of a large amount of active molecules in a very small space. The choice of the pore's morphology best adapted to the size of the molecule to be loaded into the PSi matrix is crucial for the optimisation of the device's properties. Karlsson and coworkers (Karlsson et al. 2003) studied the penetration depth of human serum albumin as a function of the PSi pores' size and demonstrated that there is a threshold pore diameter for the pores' filling. This diameter is related not only to the original molecule's size but also to the immobilisation method, that can help or impede the molecule's penetration within the pores by modifying the molecule's shape and/or the chemical environment.

DeLouise and coworkers (DeLouise and Miller 2004) studied the enzyme immobilisation capacity of porous silicon samples using glutathione-*s*-transferase (GST) to quantify the amount of enzyme bound to the PSi pores' surface. Using a simple geometric model of the columnar PSi pores they estimated the total available internal surface and the maximum amount of enzyme that can be immobilised onto this surface by simply assuming that the GST molecules, considered as spheres, will form a compact monolayer coverage. This model showed to be quite effective in estimating the minimum pores' depth (and then internal surface) needed to accommodate a given number of enzyme's moles dissolved into the immobilisation solution. Another point of interest in the immobilisation of molecules within the PSi pores is the fact that it is possible to distribute the molecules in such a way that the interaction between them may be reduced to a minimum. This can be a key point when these interactions may impede the molecule's activity relevant for the application being studied. For instance, this is the case for laser dyes, where their ability to emit light significantly depends on a sufficient dispersion of the molecules and is quenched when the molecules aggregate. Setzu and coworkers (Setzu et al. 1999) showed how the inclusion of a laser dye (Rhodamine 6G) into a porous silicon microcavity was an effective method to improve significantly its emission efficiency, demonstrating at the same time the good dispersion of the dye molecules.

The insertion of active molecules in nano- or mesoporous structures also showed an improvement of enzyme stability in several materials thanks to the spatial confinement and to the interaction with the pores' walls (Sotiropoulou et al. 2005; Kima et al. 2006). This enhanced stability is relevant for increasing shelf life, allowing the reusability of the devices and improving the biosensor-related measurement reliability.

3. Electrochemical biosensors

There are two main types of electrochemical transduction in biosensors: potentiometry and amperometry/voltammetry. They have been used for the analysis of different kinds of substances as shown in table 1.

3.1 PSi-based potentiometric biosensors

Potentiometric biosensors use porous silicon as an electrode of an electrochemical cell (Thust et al. 1996). The PSi wafer has the function both to immobilise the biological element and to be sensitive to the chemical variations produced by the immobilised biomolecule in the electrolyte solution, transducting them in a detectable electric signal. The measured parameter is the potential difference between the cathode (i.e. a platinum electrode) and the

anode (PSi electrode) of the electrochemical cell. This voltage is affected both by the chemical composition of the electrolyte solution (i.e. pH) and on the semiconducting properties of PSi, which depend on the flat-band potential (V_{fb}). In the absence of an electric field, in a semiconductor V_{fb} is related to the Fermi level of the semiconductor E_F, by:

$$E_F = eV_{fb} \tag{1}$$

where e is the charge of the electron. V_{fb} depends on the phenomena occurring at the PSi surface. Thus, when the semiconductor constitutes one of the electrodes of an electrochemical cell, the measured cell voltage is related to V_{fb}. Besides, the principle of the potentiometric transduction is based on the presence of silicon oxide at the solid/liquid interface. Indeed, in oxidised samples the porous silicon surface is covered by a silica layer which is in direct contact with the electrolyte solution. The silica layer chemically adsorbs water producing silanol (Si-OH) groups. According to the 'site binding model' of the electrical double layer at the oxide/water interface (Yates et al. 1974), silanol groups display an amphoteric behaviour being differently charged according to the pH of the electrolyte solution adsorbing/desorbing H⁺ ions onto the surface of oxidised silicon.

Type of transduction	Type of PSi	Biological element	Analyte	Reference
Potentiometry (C-V)	p-type	Penicillase	Penicillin	(Thust et al. 1996)
Potentiometry (C-V)	p-type	*Porcine pancreatic* lipase	Triglycerides	(Reddy et al. 2001; Reddy et al. 2003)
Potentiometry (C-V)		Lipase Urease	Triglycerides Urea	(Basu et al. 2005)
Potentiometry (OCP)	n⁺-type	*Candida rugosa* lipase	Triglycerides	(Setzu et al. 2007)
Amperometry	p-type	Cholesterol oxidase, Biliriubin oxidase, Glutamate oxidase	Cholesterol/ bilirubin	(Song et al. 2007)
Cyclic Voltammetry	p-type	Glutamate oxidase	ALT/AST	(Song et al. 2009)
Cyclic Voltammetry	p-type	Laccase/Azurin	-	(Ressine et al. 2010)
Cyclic Voltammetry	p-type	DNA	tDNA	(Jin et al. 2010)
Conductivity	p-type	Tyrosinase	Catechol	(Tembe et al. 2008)

Table 1. Type and principle of functioning of developed electrochemical biosensors.

The site binding model is commonly applied to metal oxide sites which may either bind with a hydrogen ion at the surface,

$$MOH + H^+ \rightleftharpoons MOH_2^+$$
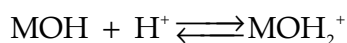
with equilibrium constant K_{a1}, or the oxide sites may release a hydrogen ion,

$$MOH \rightleftharpoons MO^- + H^+$$
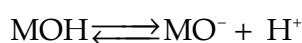
with equilibrium constant K_{a2}.

Indeed, silicon oxide surface in contact with aqueous solutions is positively charged, negatively charged or neutral depending on the pH of the solution and on the point of zero charge (pH$_{pzc}$=1/2(pK_{a1} + pK_{a2})) of the solid surface.

Nernst law predicts an ideal shift of surface potential of 59 mV per pH unit at 298 K.[1] In fact, the slope of potential/pH curves is usually lower (Madou et al. 1981; Nakato et al. 1987; Reddy et al. 2003) since a dependence $\approx$ 30 mV/pH for oxidised silicon was found. This seems to be a general phenomenon for semiconductors in contact with electrolyte solutions whose surface potential shows a linear dependence on pH. Deviations from the Nernst law are often observed. For example, for TiO$_2$ the dependence of flat-band potential on pH is 50 mV/pH unit (van de Lagemaat et al. 1998), for 6H-SiC is 40 mV/pH unit, for GaP is 37 mV/pH unit. On these bases, PSi-based potentiometric biosensors can detect substances which produce a change of pH of the electrolyte solution in contact with the electrode surface. This has been first hypothesised about 15 years ago (Thust et al. 1996).

Thust and coworkers immobilised penicillinase, an enzyme sensitive to penicillin, on oxidised *p*-type PSi through physical adsorption. To characterise the electrochemical properties of their penicillin biosensor, capacitance-voltage (C-V) measurements were carried out. The response of the sensor to penicillin was explained by the pH change of the electrolyte near the silica surface which originates from the enzymatic reaction where penicillin G is hydrolysed to liberate H$^+$. Depending on the resulting pH value of the electrolyte solution, the position of the C-V curve shifts along the voltage axis. As shown in Fig. 5, the shifts were evaluated at 60% of the maximum capacitance value.

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[1] The surface potential drop across the interface is described by the equation (Yates et al. 1974):

$$2.303\Delta pH = -\frac{e\Psi_0}{kT} - \frac{1}{2}\ln\left[\frac{(v+u)\alpha_-}{(v-u)\alpha_+}\right] \tag{1}$$

Where ΔpH= pH-pH$_{pzc}$, Ψ_0 is the surface potential, e = 1.602$\times$10^{-19} C, k is the Boltzmann constant, T is the absolute temperature, and the parameters inside square brackets are functions of the surface charge σ_0 that we indicate as $f(\sigma_0)$. After few passages we can write:

$$\Psi_0 = 2.303\frac{kT}{e}(pH - pH_{pzc}) - \frac{2.303}{2}\frac{kT}{e}\log f(\sigma_0) \tag{2}$$

At *T*=298K

$$\Psi_0 = 0.059[pH - pH_{pzc} - \frac{1}{2}\log f(\sigma_0)] \tag{3}$$

Calculating the derivative of surface potential respect to pH

$$\frac{d\Psi_0}{dpH} = 0.059\left(1 - \frac{dpH_{pzc}}{dpH} - \frac{1}{2}\frac{d(\log f(\sigma_0))}{dpH}\right) \tag{4}$$

Since pH$_{pzc}$ depends on the intrinsic acid-base properties of the oxide surface (K_{a1} and K_{a2}), it can be considered constant with pH. Thus, only if σ_0 is independent by pH the difference of potential between the surface of the oxide and the electrolyte solution follows the Nernst law, and a slope ($d\Psi_0/dpH$)) of 59 mV is obtained. The results of Yates et al. showed that σ_0 strongly depends on pH, hence the third term of equation (4) is not generally zero. Moreover σ_0 is different for different oxides depending on the difference ΔpK (=pK_{a1}-pK_{a2}) and on the concentration and the type of the supporting electrolyte.
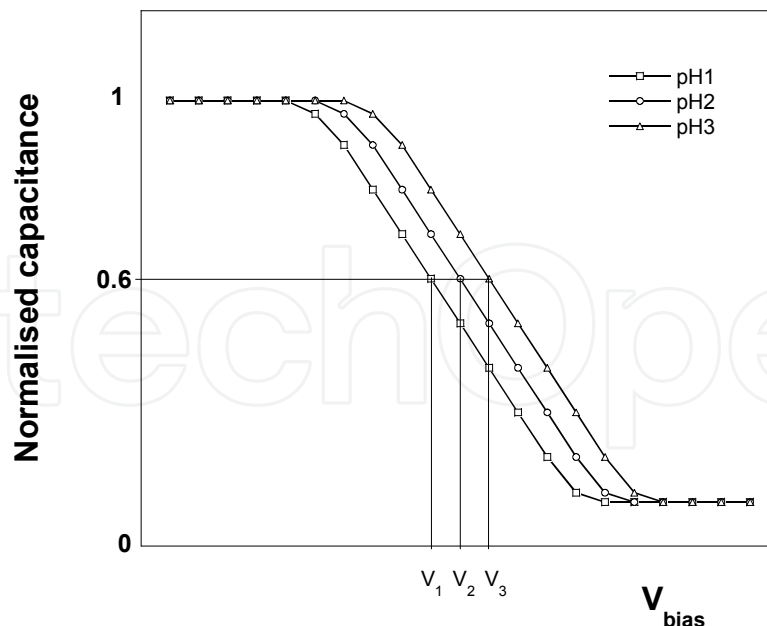
Fig. 5. Typical behaviour of the C-V curves for different pH values. Increasing the pH of the solution, causes a shift of the C-V curves to higher values along the potential axis. Adapted from (Thust et al. 1996).

Following this pioneering work, Chadha and coworkers published a series of papers aimed to the development of a potentiometric PSi-based biosensor for the analysis of triglycerides. The active biomolecule was a *Porcine pancreatic* lipase that catalyses the hydrolysis of tributyrin, thus producing butyric acid whose dissociation causes a pH decrease (Reddy et al. 2001; Reddy et al. 2003). Although the authors claimed that the lipase was immobilised on the PSi surface, it is hard to believe that this would happen in the stated experimental conditions, since the lipase solution was added to the tributyrin emulsion and then dropped on the electrode surface for the measurement. It is more likely that in this configuration the lipase acts as a homogeneous biocatalyst producing butyric acid which promotes the pH shift that is detected by the electrochemical cell and read as a potential change. In a successive work this system was used also for the analysis of urea by means of an urease enzyme instead of the lipase (Basu et al. 2005).

Finally, the system was implemented since *Pseudomonas cepacia* lipase was effectively immobilised through a preliminary chemical modification of the surface of oxidised PSi (APTS, glutaraldehyde). All steps were confirmed by means of FTIR spectroscopy (Fernandez et al. 2008).

Most of the works described used *p*-type PSi whereas only our group focused the attention on n^+-type PSi (Setzu et al. 2007). This kind of substrate was used for the realisation of a potentiometric biosensor for triglycerides quantification. The working configuration for this sensor is an electrochemical cell where the anode is a PSi sample loaded with *Candida rugosa* lipase which was physically adsorbed into the mesopores. The electrodes configuration used for the potentiometric measurement is shown in Fig. 6.

In this configuration the PSi sample was used as an electrode, while a platinum electrode was used as the second electrode. Moreover, a reference electrode (saturated calomel electrode) was used to take into account properly the variations of the cell's open circuit potential (*OCP*). The emulsion was kept under constant stirring. The hydrolysis of an
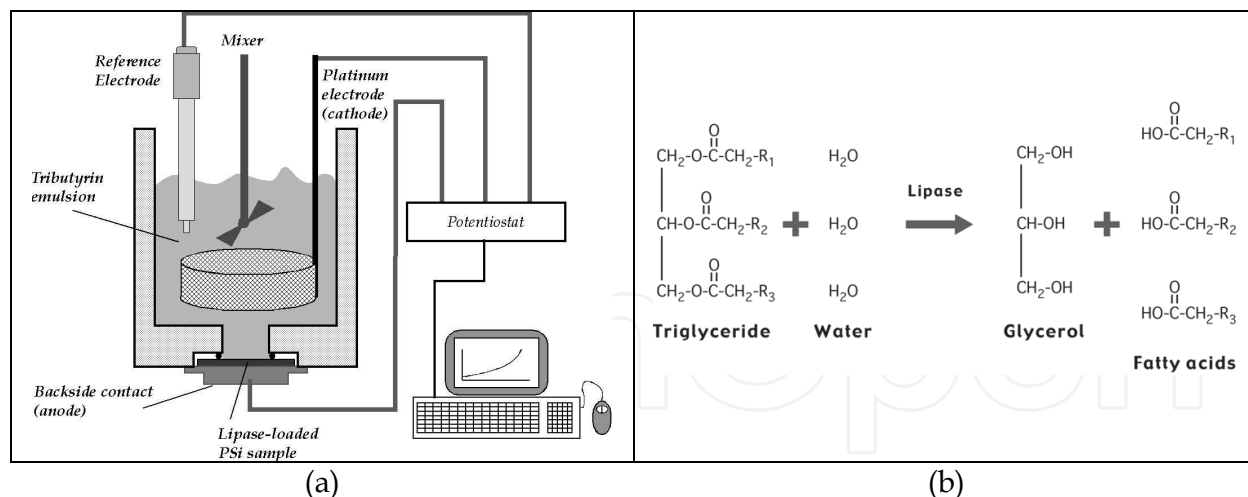
Fig. 6. PSi-based Potentiometric biosensor setup (a). Lipase catalysed hydrolysis of triglycerides (b).

aqueous emulsion of tributyrin was used to ascertain the activity of the immobilised lipase. The butyric acid formed, as a result of the biocatalyst action, produces a pH change that is measured by the variation of the open circuit potential at the electrodes of the electrochemical cell (Okorn-Schmidt 1999). The open-circuit potential, also referred to as equilibrium potential or rest potential, is the overall voltage generated in the electrochemical cell measured with respect to the potential of a reference electrode. This voltage is measured in the open circuit configuration using a high-impedance voltmeter, so that only a negligible current can flow through the cell. The *OCP* is an easily measurable electrochemical parameter, but at the same time is a complex quantity since it is the sum of all possible potential drops in the system. Indeed, in such experimental setup additional potential drops (V_0) are involved in addition to the flat band potential, V_{fb}, for example potential drops at electrolyte/reference electrode and semiconductor/metal interfaces.

The absolute value of *OCP* gives no useful information about interface reactions. On the contrary, *OCP* variation is a good parameter to investigate interfacial phenomena. Indeed, the only contribution to *OCP* that can vary as a consequence of a reaction at the electrode surface is V_{fb}, since all other possible potential drops are, in first approximation, not affected by interface modifications. The study of the time evolution of the *OCP* is then equivalent to the study of the time evolution of the flat-band potential. This type of biosensor is in principle re-usable, thus allowing a significant cost reduction while maintaining high detection efficiency.

3.1.1 Comparison of PSi triglycerides biosensors with others literature detectors

It is interesting to compare the performances of PSi biosensors with other kinds of sensors described in the scientific literature. We will do here a brief comparison with a few literature examples of devices using completely different methods, with no pretention of exhaustivity and limited to triglycerides biosensors, to better understand how PSi bisensors place themselves with respect to other devices for the same applications. The literature values are to be compared to those reported for PSi. The PSi potentiometric detectors show, in the paper of Setzu and coworkers (Setzu et al. 2007), a linear regime, dependent on the amount of lipase immobilised within the PSi pores, up to about 10 mM. In the paper of Reddy and co-workers (Reddy et al. 2003) the authors show a calibration curve up to a few tens of mM.

These results can also be compared with the human blood normal triglycerides level which is below 1.69 mM (150 mg/dL) in the normal regime and above 5.56 mM (500 mg/dL) for a very high risk.

| Method	Sensitivity	Detection range	Reference
Potentiometric - Immobilised lipase in PSi	-	<15mM	(Setzu et al. 2007)
Potentiometric, PSi electrode, lipase in solution	30mV/pH unit	5-40mM	(Reddy et al. 2003)
Impedimetric - Polyanilyne nanotubes	2.59×10^{-3} $K\Omega^{-1}$ mg^{-1} dL	25–300 mg dL^{-1}	(Dhand et al. 2009)
pH measurements – lipase on silica beads - tributyrin	0.478 pH/mM	<4 mM	(Pijanowsk et al. 2001)
pH measurements – lipase on silica beads - triacetin	0.022 pH/mM	<30 mM	(Pijanowsk et al. 2001)
Potentiometric - EISCAP	500 µM	8mM	(Fernandez et al. 2009)
Micromechanical	10 µM	500µM	(Fernandez et al. 2009)
ENFET – lipase on magnetic nanoparticles	-	5-30mM	(Vijayalakshmi et al. 2008)

Table 2. Comparison between sensitivity and detection range for several methods of triglycerides analysis.

Dhand and coworkers (Dhand et al. 2009) using a polianilyne nanotube film obtained a linear response range of 25–300 mg dL^{-1}, a sensitivity of 2.59×10^{-3} $K\Omega^{-1}$ mg^{-1} dL, with a response time of 20 s and regression coefficient of 0.99. Pijanowsk and coworkers studied a pH detector based on silica gel beads immobilised with a lipase in a microreactor (Pijanowsk et al. 2001). The authors tested three different methods for immobilising the lipase and compared the detector sensitivity on three different triglycerides. The higher sensitivity was obtained for tributyrin with 0.478 pH/mM for concentration < 4 mM, while the widest linear range, up to 30 mM, was obtained using triacetin but with a significantly lower sensitivity (0.022pH/mM). Fernandez and coworkers compared two kind of Si-based biosensors (Fernandez et al. 2009).

The first one was an EISCAP (electrolyte–insulator–semiconductor capacitor) sensor built depositing silicon nitride on *p*-type Si substrate and used for potentiometric transduction, while the second was a Si-based micromechanical sensor whose detection method is based on the measurement of the resonance frequency of a cantilever immersed in a solution containing the enzyme substrate. While the EISCAP sensor showed a sensitivity of about 500µM and a linear range up to about 8 mM, the micromechanical sensor had a sensitivity of about 10 µM and a detection range tested up to 500 µM. Vijayalakshmi and co-workers (Vijayalakshmi et al. 2008) used an ion-selective field effect transistor (ISFET) for detection of pH changes induced by lipase immobilised onto magnetic $NiFe_2O_4$ nanoparticles dispersed in a triglyceride's solution obtaining a linear measurement range of 5–30 mM. These results, summarised in Table 2, evidence that the reported values for PSi biosensors compare well with literature results even with completely different systems.

3.2 Other types of PSi-based electrochemical biosensors

PSi can be used for the realisation of amperometric and voltammetric biosensors, besides potentiometric ones. In this case the immobilised enzyme catalyses a redox reaction involving analyte oxidation/reduction which produces a flux of electrons measured, in terms of current intensity, by the electrodes of the electrochemical cell. In these systems, PSi is usually one of the involved electrodes. In fact, due to its low conductivity properties, the PSi surface has to be modified using a conducting material as gold (Ressine et al. 2010), platinum (Song et al. 2007), or a conductive polymer (Jin et al. 2010; Zhao and Jiang 2010). In that case the main function of PSi is to act as a high surface area substrate to enhance the sensitivity of biosensing. Indeed, it has been reported that porous electrodes can increase biosensor sensitivity in comparison with flat surface electrodes. Song and coworkers (Song et al. 2006) conducted a series of experiments using a planar silicon electrode and a porous silicon electrode. Cholesterol oxidase was covalently immobilised on each electrode by silanisation. The calculated effective surface area and sensitivity of the porous electrode were about 3.1-fold larger than those of the planar electrode. Successive works of the same group were devoted to develop an electrochemical biosensor array system for the diagnosis and monitoring of liver diseases (Song et al. 2007; Song et al. 2009). This biosensor array was able to simultaneously detect four different biomarkers of liver disease, namely: cholesterol, bilirubin, alanineaminotransferase (ALT) and aspartateaminotransferase (AST) levels in aqueous fluid samples. The aim of the work was to design a miniaturised measurement system using electrodes processed by porous silicon and array technology. Enzymes (cholesterol oxidase, bilirubin oxidase and glutamate oxidase) were covalently immobilised to Pt thin-film electrodes based on the PSi substrate. The immobilisation procedure involved few steps, namely: Pt oxidation; silanisation of hydroxilated Pt film electrode with APTES (aminopropyltriethoxysilane); silanised surface activation with glutaraldehyde; covalent binding of enzymes through reaction of amino groups of lysines with aldehydic group of activated electrode surface (Fig. 7). All the immobilised enzymes catalyse the oxidation of their respective analytes yielding hydrogen peroxide. This molecule is oxidised at the Pt thin film electrode, according to:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$
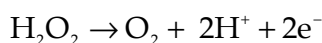
The control of the level of these analytes in human serum is important in the diagnosis and monitoring of liver diseases and myocardial infarction.
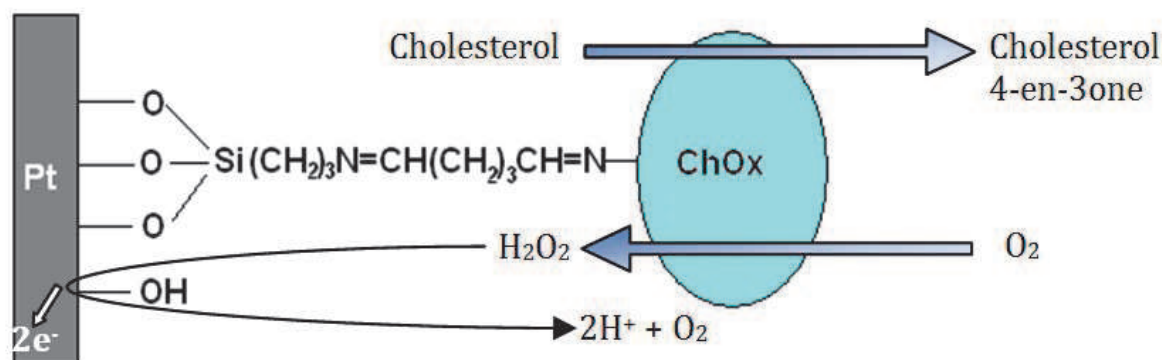

Fig. 7. Schematic illustration of electron transfer mechanism in silanised Pt thin film electrode with Cholesterol Oxidase (ChOx) immobilisation. Adapted from (Song et al. 2007).

Ressine and coworkers fabricated a novel three-dimensional chip with high surface area from double side porous silicon wafers by a sequential two-step anodic dissolution process (Ressine et al. 2010). Double-sided three-dimensional porous silicon chips, 6 mm × 6 mm, covered with a 40 nm gold (nano)layer, were fabricated from a porous silicon wafer. These devices, characterised by scanning electron microscopy along with electrochemical characterisation, showed ample conductivity, mechanical stability, and high surface area (10 times higher electrochemically active surface area compared with the geometric area). The three-dimensional gold coated silicon chips were further modified with thiol layers, followed by immobilisation of a simple copper-containing redox protein, azurin, or a complex multicopper redox enzyme, laccase. The bioelectrochemical studies showed very high surface concentrations of azurin and laccase, *i.e.* close to the theoretical monolayer coverage. However, direct electron transfer reactions between the biomolecules and gold surfaces were observed only for a small percentage of the immobilised redox protein and enzyme, respectively. Thus, highly efficient oxygen-bioelectroreduction on laccase-modified 3D thiol-gold-porous silicon chips (as compared to planar laccase-modified gold electrodes, $42 \, \mu A/cm^2$ versus $7 \, \mu A/cm^2$, respectively) was obtained only in the presence of an efficient soluble redox mediator. The bioelectrochemical studies provided unequivocal evidence of efficient O_2-bioelectrocatalysis by laccase in the three-dimensional chip structure.

Jin and co-workers fabricated and electrochemically characterised a label-free DNA sensor based on a porous silicon substrate (Jin et al. 2010). A *p*-type silicon wafer was electrochemically anodised in an ethanolic hydrofluoric (HF) solution to construct a PS layer on which polypyrrole (PPy) film was directly electropolymerised. To achieve direct electropolymerisation of PPy on PSi substrate without pre-deposition of any metallic thin-film underlayer, a low resistivity wafer was used. The rough surface of the PSi layer allowed for a strong adsorption of the PPy film. Intrinsic negative charge of the DNA backbone was exploited to electrostatically adsorb 26 base pairs of probe DNA (pDNA) into the PPy film by applying positive potential. The pDNA was designed to hybridise with the target DNA (tDNA) which is the insertion element (*Iel*) gene of *Salmonella enteric serovar Enteritidis*. Dependence of peak current around 0.2 V versus Ag/AgCl on tDNA concentration and incubation time were shown from the cyclic voltammograms of PS/PPy + pDNA + tDNA substrates. Scanning electron microscopy (SEM) image of the cross-section of a PS/PPy + pDNA + tDNA multilayered film showed successful direct electropolymerisation of PPy for a nano-PS DNA biosensor.

Tembe and coworkers developed a conductivity-based catechol biosensor using porous silicon as the immobilisation matrix for enzyme tyrosinase (Tembe et al. 2008). The enzyme was immobilised in an electrochemically etched surface of *p*-type silicon. The presence of enzyme in a porous structure and the retention of enzyme activity were confirmed by scanning electron microscopy and spectrophotometric studies, respectively. The principle of the sensor is based on the change in the conductivity of the tyrosinase-entrapped porous silicon matrix. When the entrapped tyrosinase interacts with catechol, the change in the current voltage (I-V) characteristics is obtained, which is proportional to analyte concentration.

4. Conclusions

In this chapter an overview of the recent advances on the use of porous silicon as a matrix for the realisation of biosensors is given. The main advantages of PSi biosensors, both with

electrochemical or optical transduction, are the large developed surface, the relative simplicity of the fabrication process, the easy integration with standard technology, the wide range of possible applications. We focused here our attention on electrochemical (particularly potentiometric) biosensors since, despite their easy realisation, for both potentiometric and amperometric/voltammetric biosensors, few research efforts have been devoted to these devices compared with optical biosensors. Nevertheless, there is a number of promising works concerning PSi-based electrochemical biosensors now available in the literature.

The key points discussed here concern the techniques used for PSi formation, the importance of surface stabilisation by oxidation techniques, the different strategies for enzyme/biomolecule immobilisation, and the realisation of the electrochemical biosensors. Concerning the last point, we showed that the realisation of potentiometric biosensors is based on the oxidation of the PSi pores' walls needed for both stabilising the PSi layers and confer them pH sensitivity. The transduction is due the formation of and acidic/basic substance as the result of the reaction catalysed by a suitable enzyme immobilised on the PSi surface.

Amperometric/voltammetric PSi-based biosensors, instead, measure the current intensity produced by the action of a redox reaction on the analyte of interest by an enzyme immobilised on the electrode surface. The importance of PSi is due the sensitivity enhancement due to the higher surface area of PSi compared to flat electrodes. The main difficulty in these biosensors is due to the low conductivity of PSi compared to metal electrodes. This can be overcome by coupling PSi with a Pt, Au, or a conductive polymer thin film to enhance its conductivity.

In conclusion, porous silicon constitutes an interesting matrix for the realisation of biosensing devices. Due to the recent growing interest it is very likely that previously neglected electrochemical transduction will reach a similar level of development as that obtained with optical transduction.

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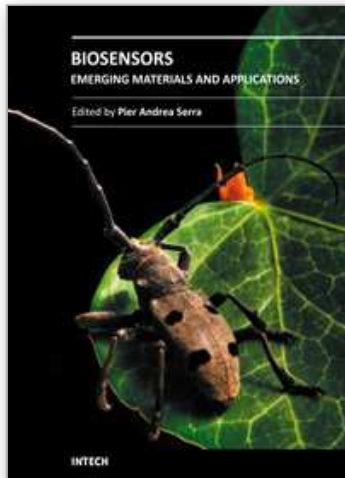
Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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