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Hybrid Film Biosensor for Phenolic Compounds Detection

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1. Introduction

Phenolic compounds are industrial chemicals widely used in the manufacture of products. Most of them are generated artificially and are found in the wastewaters from chemical plants, exhaust gases from incinerators, the side stream smoke from cigarettes. They are easily adsorbed in humans, regardless of their form. High levels of phenols have been shown to have detrimental effects on animal health, and some phenolic compounds are reportedly carcinogenic (N. Li et al., 2005) and allergenic (Haghighi et al., 2003), and due to their toxic effects, their determination and removal in the environment are of great importance.

Biosensors can make ideal sensing systems to monitor the effects of pollution on the environment, due to their biological base, ability to operate in complex matrices, short response time and small size. The determination of phenol and its derivative compounds is of the environmental greatness, since these species are released into the environment by a large number of industries, *e.g.* the manufacture of plastics, dyes, drugs, antioxidants and waste waters from pulp and paper production. This group of biosensors is of great interest because of their application in food and pharmaceutical industry.

Among enzymes, laccases and tyrosinases (Duran et al., 2002) or horse-radish peroxidase (Freire et al., 2001) as well as polyphenol oxidase are groups of enzymes that catalyze the transformation of a large number of phenolic compounds. The mechanism for tyrosinase, laccase and peroxidase in the electrochemical biosensors are different. Enzyme molecules are re-reduced by phenolic compounds after they were oxidized by oxygen (for tyrosinase and laccase) or hydrogen peroxide (for peroxidase) on the surface to the electrode. The tyrosinase biosensors are applicable to the monitoring of phenolic compounds with at least one free *ortho*-position. On the other hand, the laccase biosensor can detect phenolic compounds with free *para*- and *metha*- position with a complicated catalytic cycle. Horseradish peroxidase (HrP) based biosensors are most sensitive for a great number of phenolic compounds since phenols can be act as electron donors for peroxidase (Yang et al., 2006).

As seen, phenolooxidases have wide substrate specificity and a great potential for the determination of phenolic compounds. Furthermore, fungal laccases catalyze demethylation reactions an important and initial step of the biodegradation process of the lignin polymer

chain, and subsequently decompose the lignin macromolecule by splitting aromatic rings and C–C bonds in the phenolic substructures (Freire et al., 2001).
In enzymatic devices, efforts have been concentrated on the control over enzyme activity, which is highly dependent on the interface between the nanocomposite and the enzyme. Such control has led to immobilization techniques suitable for anchoring the enzyme close to electrode with preservation of biological activity. In these type of devices, where preservation of the enzyme activity at the nanocomposite/enzyme interface is the key for designing efficient electrode, charge transfer between enzyme and electrode should be fast and reversible. This charge transfer may be also optimized with some mediating particles being used in conjunction with the biological molecules at the electrode surface. Conducting polymers are often used as support materials for amperometric biosensors (Kuwahara et al., 2005). The interlaced polymer is expected to facilitate the electron transfer as well enhancing the sensor sensitivity.
It is essential for the sensitivity of the system that the recognition units have optimized surface density, good accessibility, long-term stability and minimized non-specific interactions with compounds other than the analyte. Such model molecular assemblies can be prepared by Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) techniques in which we have to the moment successful experience (Cabaj et al., 2010a; Cabaj et al., 2010b), layer-by-layer (LbL) or by employing self-assembly monolayers (SAMs) as well as electrodeposition. Construction of novel phenol detecting biosensor is challenge for new technologies and the key problem is modification of electrode by enzyme using thin film preparation methods.

2. Hybrid thin film fabrication

Proteins are usually adsorbed on the surface of a solid substrate in a disordered orientation and are apt to suffer from conformational change, which renders the functional sites inaccessible to interacting molecules and substrates for enzymatic reactions (Kumada et al., 2007; Nakanishi et al., 2001). The extent of the conformational change of proteins in their adsorbed state differs with the type of protein, the kinds/states of the surface including hydrophobicity/hydrophilicity, and with the environmental conditions, such as temperature, pH, and ionic strength of the solution.
Most immobilization methods developed thus far involve modification or coating of the surface with appropriate substances to change the surface property and/or provide functional groups for the binding of protein (Zhu & Snyder, 2003). On the other hand, immobilization of proteins on a bare surface with no modification utilizes specific interactions between the protein and the surface, which includes immobilization on the Au surface *via* thiol (sulfhydryl) groups and necessitates using an affinity peptide that is specific to the particular surface. Conceptual schemes for the various immobilization methods are summarized in Table 1.

2.1 Non-covalent immobilization

2.1.1 Physical adsorption

Extensive work in area of biosensors is mainly connected with immobilization of protein in miniaturized structures, which may also contain hybrid materials for enhancing sensitivity and selectivity (Siqueira et al., 2010). Many biosensing devices integrate biomolecules with metal nanoparticles (Debabov 2004), carbon nanotubes, solid matrices (S.N. Kim et al., 2007)

and polymers (Hammond 2004). Proteins can be immobilized in a variety of structures as in transmembrane pores, and phospholipids Langmuir-Blodgett/Langmuir-Schaefer films, layer-by-layer (LbL) structures (Cabaj et al., 2010a; Cabaj et al., 2010b). For electrical detection, in particular, mediators or conducting matrices may be used to enhance conduction.

Immobilization method	**Surface for immobilization**	**Capturing mechanism**	*Ref.*
Physical adsorption	Polystyrene, nitrocellulose, LB/LS, LbL films	Physical adsorption	Cabaj et al., 2010a; Cabaj et al., 2010b; Nakanishi et al., 2008
Immobilization using hydrogel	Glass plate	Entrapment in gel	Nakanishi et al., 2008
Immobilization using coiled coil interaction	Octadecyltrichlorosilane (OTS) coated surface covalently bound with artificial polypeptide containing Leu zipper	Coiled coil association of a heterodimeric Leu zipper pair	Nakanishi et al., 2008
Immobilization on Au surface	Au surface	Chemisorption of SH-groups on Au	Nakanishi et al., 2008
Glutatione/S-Transferase-mediated immobilization	Polystyrene coated with protein followed by covalently coupling with glutathione	Affinity between glutathione and S-Transferase	Nakanishi et al., 2008
Silane coupling method	Glass modified with bifunctional silane coupling reagents containing aldehyde	Schiff's base linkage between aldehyde and amino groups	Nakanishi et al., 2008
Immobilization using polystyrene (PS)-affinity peptide	Hydrophilic polystyrene	Affinity of the affinity peptide to PS surface	Nakanishi et al., 2008

Table 1. Various immobilization methods

Direct adsorption of biomolecules on a solid surface may also lead to the rapid, simple immobilization. For example, a gold surface can directly adsorb biomolecules *via* strong electrostatic interaction and thiol-based chemical binding under ambient conditions, and a SiO_2 surface can directly adsorb biomolecules mainly by van der Waals force and hydrogen bonding. The atomically flat sapphire (Al_2O_3) substrate with a hydrophilic surface may be used for hydrophilic adsorption (Yoshida et al., 1998).

However, such a direct adsorption of biomolecules on the surface of metals or metal oxides is usually non-specific and the orientation of the adsorbed structures remains largely random (D.C. Kim et al., 2009). As direct adsorption may result in significant conformational change in the native structure of proteins and severe loss of biological activity.

2.1.2 Layer-by-layer method

The technique of production of sensing structures in the form of thin films has to comply with three requirements: molecular recognition, signal transduction and signal detection by the abiotic component. That is the key problem in construction of miniaturized sensing devices. Nanofilms produced with LbL and LB methods may give a satisfactory results in controlling of layer architecture.

In the LbL method, solid supports are immersed into aqueous solutions of materials to immobilize. Alternating layers of positively and negatively charged materials are adsorbed in a sequence, which may lead to multilayers. The procedure of the film preparation is depicted in Figure 1.

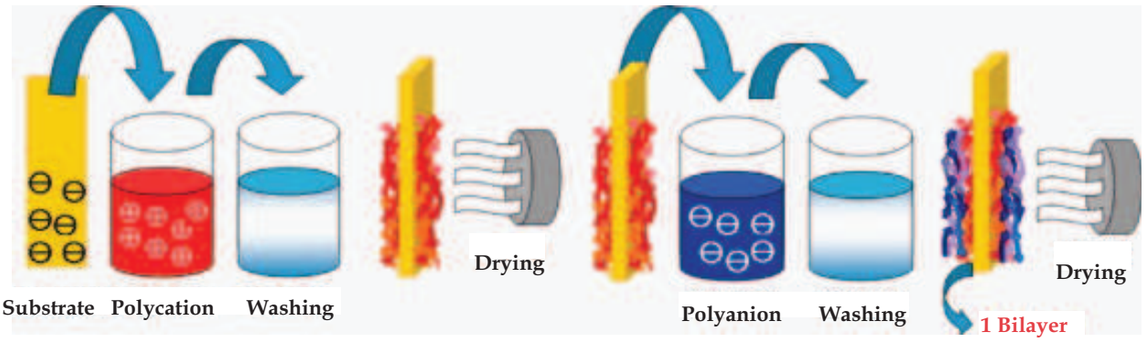


Fig. 1. Schematic procedure for fabrication of LbL films. The substrate is alternately dipped into solutions containing polycations and polyanions to produce bilayers (Siqueira et al., 2010)

The amount of material adsorbed on the support and the surface topography depend on various parameters, including concentration of material to be adsorbed, pH, ionic strength. The obtaining films are thermally stable and resist to washing in aqueous solutions. In order to sensors fabrication, the LbL layers have been prepared of proteins, nucleic acids and polysaccharides (Siqueira et al., 2010; Liu et al., 2008).

Deposition of a large number of layers can be performed by using a mechanical robot (Portnov et al., 2006) or a simple fluidic system. The main drawbacks of LbL deposition are sensitivity of the receptor containing multilayer to variations of ionic strength, generally leading to destabilization of an assembly and an influence of the drying process on the multilayer structure (Lourenco et al., 2007). LbL deposition was used *i.e.* to incorporate Glucose oxidase (GOx) into a multilayer of poly(styrene sulfonic acid)/polypyrrole (PSS/PPY) on the surface of *in-situ* polymerized PPY (Ram et al., 2000).

2.1.3 Langmuir–Blodgett technique

On the contrary to LbL procedure, the LB film-forming materials are obligatory insoluble in water. LB films are obtained by transferring a Langmuir layer from air/water interface onto the solid support *via* vertical dipping into subphase. Multilayers can be deposited by repeating the solid immersion/withdrawal steps.

Langmuir–Blodgett technique was widely used at the end of 1980s–beginning of 1990s as a relatively simple technology to get highly ordered organic films with artificial or natural receptors. However the system assembled by external forces does not lead to highly stable and defect free films. Therefore this technique was used mostly in combination with other immobilization procedures. Indeed, phospholipids matrices have been used to host biocatalyst molecules that are then co-deposited onto the solid supports as LB films (Cabaj et al., 2010a; Cabaj et al., 2010b; Girart-Ergot et al., 2005) , as shown in Figure 2.

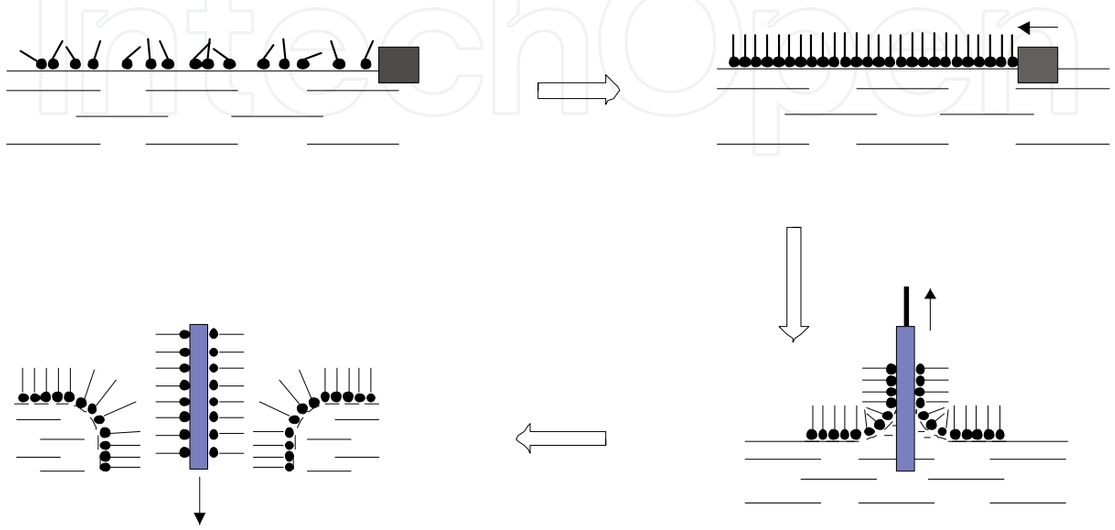


Fig. 2. Schematic path of LB technique

Hydrophobic derivatives of (poly(3- dodecylthiophene), poly(3-hexylthiophene)) were used in a mixture of stearic acid and galactose oxidase (Sharma et al., 2004), laccase, tyrosinase or GOx (J.R. Li et al., 1989; Singhal et al., 2002) to be deposited by Langmuir–Blodgett technique. During the last years the application of Langmuir–Blodgett approach was almost completely replaced by the technologies of self-assembly based on layer-by-layer deposition or on binding of thiols to some metals, modification by silanization and subsequent covalent immobilization.

As proteins are not ideal amphiphilic molecules, the techniques need to be adapted, either by chemical methods (*e.g.*, derivatization methods (Riccio et al., 1996) or varying the subphase composition (Erokhin et al., 1995) or by applying some mimetic systems of biological membranes, due to preserve their native structure and function in monolayer. But the LB technique has been found to be a suitable approach for the development of protein nanostructured matrixes. This technique has been utilized for production to sensitive elements based on protein molecules (Cabaj et al., 2010a; Cabaj et al., 2010b), and more recently, for fabrication of protein-based templates to be employed in nanocrystallography (Pechkova & Nicolini 2004). Generally, this method not only does not destroy the protein structure and function, but also provides new useful properties, such as protein thermal and temporal stability and film anisotropy (Sivozhelezov et al., 2009). The proteins in solutions start to denaturate at 60-70°C where as the secondary structures of proteins in the LB film is slightly affected only till 200°C (Sivozhelezov et al., 2009). This property is not possible to find in chaotically oriented layers. Furthermore, due to this procedure it is possible to control the order of the protein structures, as follows from improved properties of protein crystals and improved helical content of proteins when LB technique is applied.

The quality of protein-monolayer formation at the air-water interface is related to the degree of preservation of the native properties of all proteins. The magnitude of the electrostatic forces maintaining the protein structure is comparable with that of the surface tension. Proteins tend to form stable monolayers at the air-water interface because of their mixture of hydrophilic and lipophilic groups. Often spreading species such as proteins at the air-water interface can effect the conformation of the molecule such as causing unfolding. For example insulin or ovalbumin unfold completely whereas myoglobin and cytochrome C are only partially unfolded (Birdi 1999). This again is thought to be a function of the ratio of polar to non-polar amino acids residues. Highly polar proteins such as xanthine oxidase do not form stable monolayers (Erokhin et al., 1995). In all these circumstances the convenient matrix may be required. For instance, according to Girart-Ergot et al. (2005) enzyme bioactivity in mixed lipid LB films is preserved due to the lipid molecular assembly protects the enzyme, positioning the polypeptide moiety in such a way as to allow the recognition and signal events. In fact, phospholipids have been used as protecting agents for several types of material, not only for membrane cell proteins (Caseli et al., 2002) but also for polysaccharides (Pavinatto et al., 2007) and synthetic polymers (Caseli et al., 2001).
The lipid fraction of biological membranes is mainly composed of phospholipids with different chain lengths and ionic character. Therefore, phospholipids are widely used as mimetic systems in studies involving the cross resistance to drugs.

2.1.4 Langmuir–Schaefer technique

Langmuir-Schaefer (Fig. 3) as well as Langmuir-Blodgett techniques represent a "classical" tool for biofilm engineering, recently improved and extended to a wide variety of proteins (Nicolini 1997). It is also very useful in deposition other than protein rigid layers.
The method implies horizontal touching with the substrate of the preformed monolayer. For protein layers it is not necessary to use a grid as a separator because the layer is rather soft, but is important to compensate the charge of the molecules at the water surface before deposition (Nicolini 1996).
Moreover, it is seemed that LS methodology in case of deposition of tyrosinase is more effective than vertical process (Cabaj et al., 2010b).

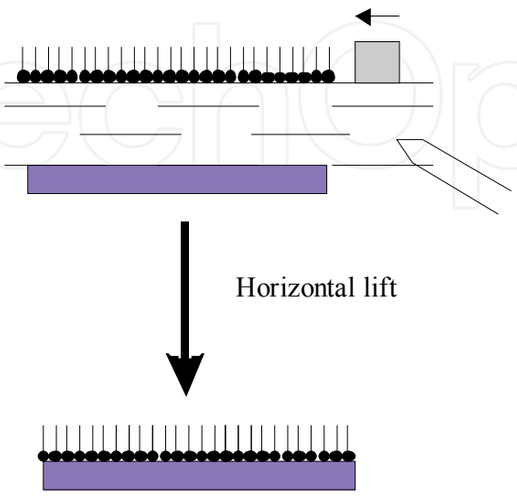


Fig. 3. Langmuir-Schaefer technique

2.2 Covalent immobilization

The covalent immobilization of functional, biological molecules onto a defined and also conductive surface provides the basis for sophisticated biomolecular architectures with numerous applications for *in vitro* studies on the behavior of biological structures such as proteins and cells, for implant or for biosensor devices. In recent years, much work has been devoted to the development of new techniques for protein immobilization on different substrates.

Among the chemical immobilization methods, the following techniques can be distinguished:

- covalent attachment to a water-insoluble matrix,
- cross-linking with the use of a multifunctional and low molecular weight reagent,
- *co*-cross-linking with other neutral substances, for instance proteins.

Cross-linking can be described as a technique, in which an enzyme is linked to the supporting polymeric material by the mean of chemical bonding. The particular method utilizes bifunctional reagents, *i.e.* glutaraldehyde. The main disadvantages of this technique include: enzyme damage, limited diffusion of the substrate, poor mechanical strength (Eggins 2002).

Covalent coupling with the electrogenerated polymer can be defined as an immobilization method which employs formation of bond between a functional group of the enzyme and the support polymeric matrix . The coupling of molecules to the polymer surface is usually carried out by water-soluble carbodiimide or *N*-hydroxysuccinimide, or the mixture of both reagents. However, the presence of additional chemicals in such a solution may lead to partial denaturation of the enzyme as well as damages within the polymer structure. Among others, nucleophilic groups of the enzyme amino acids, which do not play a major role in the catalytic mechanism, seem to be the best targets. The most common nucleophilic groups used for coupling are: NH_2, COOH, OH, SH, C_6H_4OH and imidazole. One of the biggest advantages of this method is that the enzyme will not be released from the support when performing the catalysis (Klis et al., 2007).

Analyte	**Polymer/monomer/ way of polymerization**	**Receptor**	**Type of transducing**	*Ref.*
Boronic acid	Poly(aniline boronic acid)/electrochemical polymerization	Glycoproteins	Photometric	Anzenbacher et al., 2004
Na^+, K^+, NH_4^+	Overoxidized polypyrrole	Valinomycin	Amperometric	Izaoumen et al., 2005
Cyclodextrin	Poly(3-methylthiophene)/ electrochemical polymerization	Dopamine neurotransmitters	Amperometric	Bouchta et al., 2005
Atrazine	Polypyrrole/ electrochemical	Tyrosinase	Amperometric	Gerard et al., 2002
Phenolic compounds	LB film of bis(thiophene)carbazole	Laccase	Photometric	Cabaj et al., 2008

Table 2. Selected examples of sensors with receptors covalently immobilized to conducting polymers

Chemical bonding of receptors by covalent reaction between functional groups of receptor and *i.e.* polymer provides the strongest immobilization. Various derivatives of pyrrole and thiophene modified by ionophore units were reported (Table 2). These derivatives were electropolymerized mostly in organic solvents. Low solubility of modified monomers in aqueous media and often low conductivity of resulting polymer can be crucial for biomolecule immobilization. Suitable derivatives are pyrrole and thiophene bearing carboxyl function at the β-position (Rahman et al., 2003; G. Li et al, 2000; Cha et al., 2003; Freitas et al., 2005; Kong et al., 2003; Kuwahara et al., 2005; Peng et al., 2005) or *N*-substituted pyrrole (Culvo-Munoz et al., 2005).
Besides PPY, such polymers as polythionine are found to be convenient for the immobilization due to the presence of free amino functions in the polymer structure and reversible and stable behavior of the polymer film in biological compatible media (Ferreira et al., 2006). A sensor based on tyrosinase linked to electroactive poly(dicarbazole) *via* carboxy group was presented in Cosnier et al. (2001). Various strategies to preparation of polymer with synthetic (polyalkyl ether, crown ether, pyridyl-based ligands) and biological receptors are reviewed in McQuade et al., 2000.

3. Methods of detection

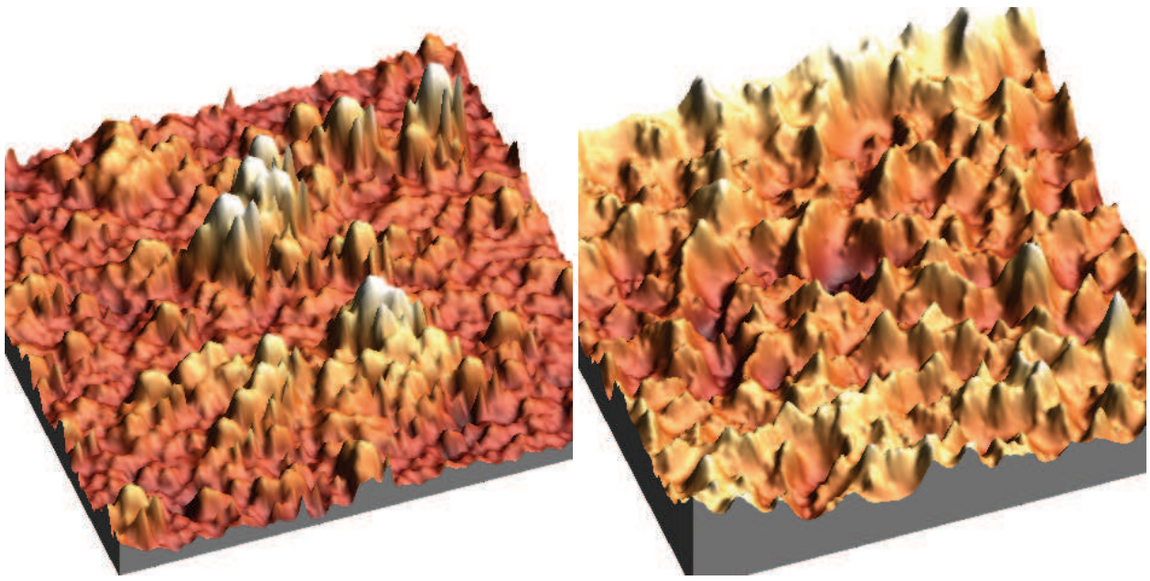
The choice method of detection is based mainly on the type of material employed for producing the sensing units and on the analyte to be detected. Electrochemical methods normally require electroactivity either of the film itself or induced by the analytes. Using electrochemistry may be advantages because distinct experimental procedures can be used, including amperometric measurements and cyclic voltammetry (Siqueira et al., 2010).
Electrical impedance spectroscopy, in turn, offers advantage in being in principle applicable to any type of molecule immobilized in the sensing structure as well as to any type of analyte. In this technique, used in characterization of dielectric properties of materials, the interaction between external field with the dipole moments of the dielectric material is measured as a function of frequency (Neto et al., 2003).
The optical methods are generally more restrictive with regard to the number of system for which they can be used. They include UV-vis absorption or fluorescence spectroscopies, where the former may be used when the analyte or a mediator absorbs in the UV-vis region. If fluorescence measurement are used, fluorescent chromophores must be incorporated into the system, and there are some important advantages: simple optical monitoring may suffice for detection, thus making it possible to produce low-cost sensors. Furthermore, sensing based on fluorescence is usually highly efficient.
Detecting of phenolic compounds is mostly electrochemical process (S. Yang et al., 2006; Shan et al., 2003; Serra et al., 2003), rarely UV absorption (Cabaj et al., 2010a; Cabaj et al., 2010b).

4. Imaging of surface structure

The morphology of the deposited multilayered structures is characterized at nanometer level by AFM (atomic force microscopy) that is a tool with different possibilities and limits. Multilayered structures are possible to form because the technique gives the possibility to form multilayered architectures controlled at molecular level.

AFM can image biological samples under aqueous conditions with high resolution in three dimensions without the use of any probes. AFM has been successfully used to image isolated phase separated bilayers and peptide–lipid domains in supported bilayers. Also monolayers containing glycosphingolipids and cholesterol have been imaged (Yuan & Jhnston 2000) as well as phenoloxidases or glucose oxidase mixed (with linoleic acid, phospholipids) LB/LS films (Kuwahara et al., 2005).

The phenoloxidases (laccase, tyrosinase) and glucose oxidase hetero layers were visualized by contact mode AFM (Fig. 4). The enzyme molecules were fairly well deposited onto solid substrate. Immobilized phenoloxidases as well as glucose oxidase (Sołoducho & Cabaj 2010) were observed as an aggregated pattern in solid-like state with keeping their characteristic random cloud-like or island structure. The heterogeneous films roughness was found relatively high (especially in case of tyrosinase film) for an LB film, which indeed shows that the enzymes were transferred. The roughness of linoleic acid - laccase film was measured as 7.17 nm (similar results was found for film of lipase (Baron et al., 2005)), when the roughness value of tyrosinase film was found as 19 nm. To compare, the roughness of glucose oxidase LB film has been measured as 0.38 nm (Singhal et al., 2003) or 1.9 for LS films. These obtained values were attributed to the immobilization process of comparatively large molecule aggregates of enzymes (laccase, tyrosinase, glucose oxidase) incorporated to LB/LS films. This leads to conclude that there is sometime formation of an agglomerate of enzymes rather than an organized monolayer at the air/water interface. The AFM results showed that the effect could be also associated with changes in the enzyme conformations A monolayer rearrangement, such as two-dimensional formation or hindered molecular orientation, might take place during the phase transition behaviour resulted in the molecular aggregates on the protein layer.



a) b)

Fig. 4. AFM topography images of a) linoleic acid – laccase LB film, b) phospholipids – glucose oxidase LS film (Sołoducho & Cabaj 2010)

5. Biomolecules for phenolic compounds detection

Biosensors based on the coupling of a biological entity with a suitable transducer offer an effective route for detection of phenolic compounds. For phenolic compounds determination biosensors modified with tyrosinase, peroxidase, laccase and polyphenol oxidase have been reported. Electrodes modified with these enzymes have the advantage that the detection of phenolic compounds can be carried out between -0.2 and 0.05 V *versus* SCE and the interface is minimized. The mechanism for tyrosinase, laccase and peroxidase in the electrochemical biosensors is different (S. Yang et al., 2006).

5.1 Horseradish peroxidase

Peroxidases are enzymes of the class EC 1 .l 1. defined as oxidoreductases using hydroperoxides as electron acceptor. It has been found that most of the peroxidases: plant peroxidases, cytochrome c peroxidase, chloroperoxidase, lactoperoxidase, etc. are heme-proteins with a common catalytic cycle (Everse et al., 1991). Horseradish peroxidase (HrP) has been most thoroughly studied and frequently used to exemplify the peroxidase reaction cycle:

Native peroxidase(Fe^{3+}) + H_20_2 → Compound - I+ H_20	(la)
Compound - I + AH_2 → Compound - II + AH*	(lb)
Compound - II + AH_2 → Native peroxidase(Fe^{3+})+ AH* + H_20	(lc)(Ruzgas et al., 1996)

The first reaction (la) involves a two-electron oxidation of the ferriheme prosthetic group of the native peroxidase by H_20_2 (or organic hydroperoxides). This reaction results in the formation of an intermediate, compound-I, consisting of oxyferryl iron (Fe^{4+}=O) and a porphyrin π cation radical. In the next reaction (1b), compound-I loses one oxidizing equivalent upon one-electron reduction by the first electron donor AH_2 and forms compound-II (oxidation state +4). The later in turn accepts an additional electron from the second donor molecule AH_2 in the third step (lc), whereby the enzyme is returned to its native resting state, ferriperoxidase.

The oxidation products formed during the peroxidase reaction depend on the nature of the substrate. Electron donors such as aromatic amines and phenolic compounds are oxidized to free radicals, AH* (reactions (lb) and (1c)) (Ruzgas et al., 1996). The following discussion about the bioelectrochemistry of peroxidases is directed for a more detailed presentation of the electrochemical reactions of peroxidase providing the basis for amperometric peroxidase-modified electrodes. Horseradish peroxidase based biosensors are most sensitive for a great number of phenolic compounds since phenols can be act as electron donors for peroxidase (S. Yang et al., 2006).

5.2 Laccases

Laccase (EC 1.10.3.2) is defined as a blue oxidase capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water by a multicopper system. Laccases belong to a large group of the multicopper enzymes, which includes among others ascorbic acid oxidase and ceruloplasmin (Hublik & Schinner 2000).

Laccase demonstrates a broad substrate specificity (Vianello et al., 2007). It catalyses the oxidation of such diverse compounds as: *o*, *p*-diphenols, aminophenols, polyphenols,

polyamines, lignin, some inorganic ions, aryl diamines, benzenthiols, phenothiazines (Vianello et al., 2007). The substrate for laccase is also molecular oxygen, hence the enzyme plays a role of terminal electron acceptor in a four electron process in which water is the final product. Moreover, this blue oxidase is known to demethylate lignin and methoxyphenol acids (Vianello et al., 2007).

Typical laccase reaction (Fig. 5) leads to conversion of the phenolic substrate into aryloxyradical by the mean of a one-electron oxidation process. During the second stage of the oxidation the active species can be converted into a quinone. In the next step, both the quinone and the free radical product undergo a non-enzymatic coupling reactions leading to the polymerization resulting in dimers, oligomers and polymers (Duran et al., 2002; Riva 2006). Products of the oxidative coupling reactions result from either C-O and C-C coupling of the phenolic reactants or N-N and C-N coupling of the aromatic amines. The particular reaction is known as the detoxification of phenolic contaminants (Hublik & Schinner 2000).

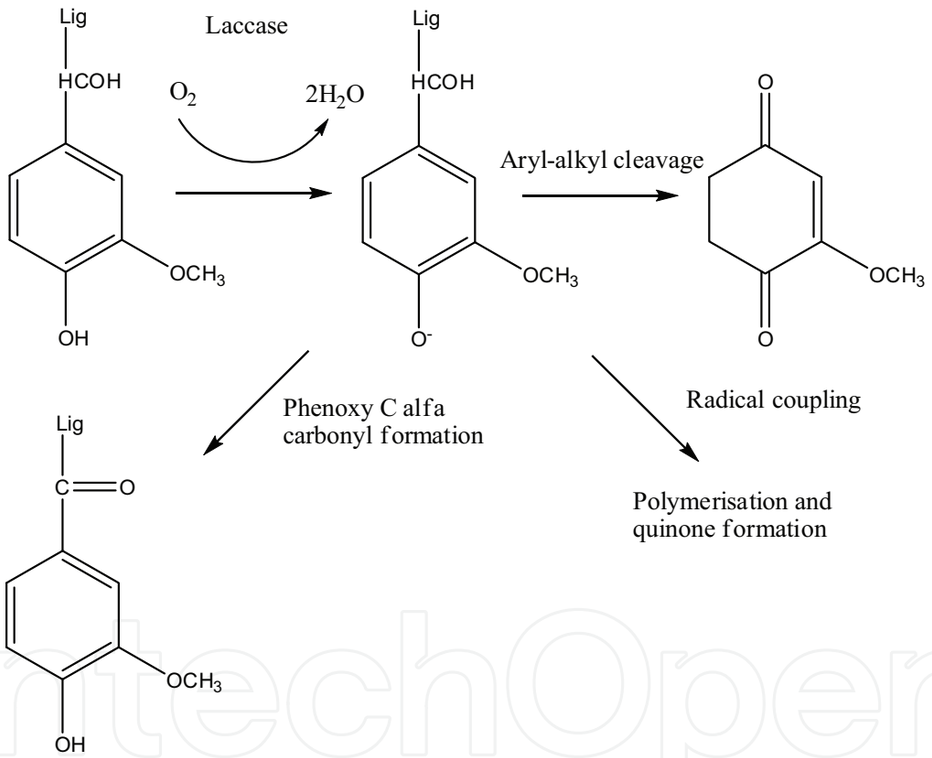


Fig. 5. Laccase-catalysed oxidation of phenolic groups (according to Archibald et al. (1997))

5.3 Tyrosinases

Tyrosinase (monophenol monooxygenase, EC 1.14.18.1) is a binuclear copper containing metalloprotein which catalyses, in the presence of molecular oxygen, two different reactions: (1) the transformation of -mono-phenols into *o*-diphenols (monophenolase activity) and (2) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity, Fig. 6).

This enzyme, found in prokaryotes as well as in eukaryotes, is involved in the formation of pigments such as melanins and other polyphenolic compounds. Each of the two copper ions is bounded by three conserved histidines residues (Lerch 1988).

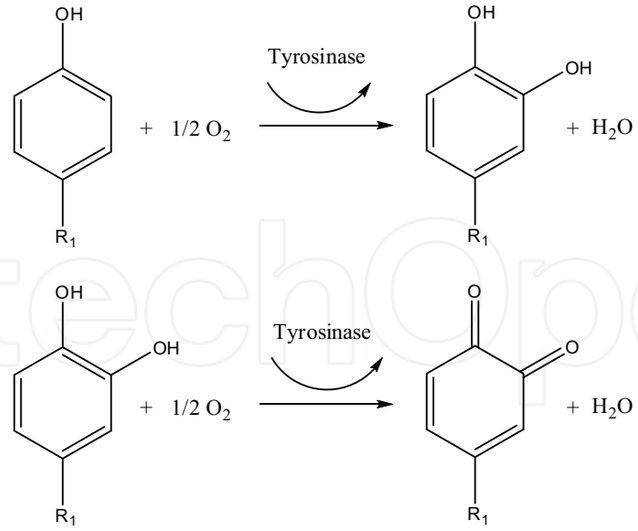


Fig. 6. Tyrosinase-catalysed oxidation of phenolic compounds

6. Biosensors for phenolic compounds

Electrochemical biosensors are rather cheap, simple to fabricate, and reusable. They have high stability and sensitivity. This kind of sensors can potentially be used for other species with the necessary modifications. Many phenolic compounds are successfully detected using electrochemical sensors as most sensors are oxidized at readily accessible potentials.

6.1 Horseradish peroxidase electrodes for phenolic compounds detection

Many different methods such as covalent immobilization (Imabayashi et al., 2001), sol-gel derived matrix (Rosatto et al., 2002), recently LbL assembly was employed for modification of electrodes (S. Yang et al., 2006). And still the combination of oxidoreductases and amperometric electrodes is the most commonly studied biosensor concept (Fig. 7).

Imabayaschi et al. (2001) reported also the HrP biosensor constructed by enzyme covalently immobilized on the mercaptonic acid self-assembled monolayer (SAM) on the gold electrode. The most simple electrode for the detection of peroxide consists of a layer of peroxidase molecules adsorbed onto the electrode surface. If the electrode is placed into a sample and poised at a potential more negative than 0.6 V *vs*. SCE then a proportionality between the registered reduction current and the peroxide concentration is observed. This phenomenon was observed for horseradish peroxidase adsorbed on carbon black, graphite, carbon fibers, gold, and platinum electrodes (Ruzgas et al., 1996). The electrode current is due to an electrochemical reduction of compound-I and –II (Fig. 8).

The response of the peroxidase biosensors to phenolic compounds is based on the double displacement or "ping-pong" mechanism in which two substrates, H_2O_2 and the electron-donating phenolic compounds are involved. At the electrode surface, peroxidase molecules are oxidized by H_2O_2 followed of its reduction by phenolic compounds. In the last reaction, the phenolic compounds are mainly converted into quinones or free radical products, which are electroactives and can be electrochemically reduced on the electrode surface. The reduction current is proportional to the phenolic compounds concentration in the solution, as long as the H_2O_2 concentration is not limiting (S. Yang et al., 2006).

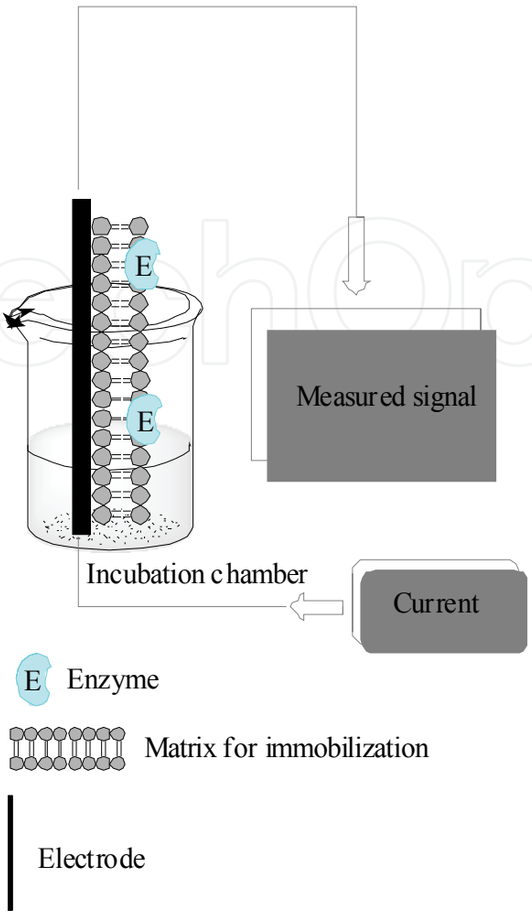


Fig. 7. Simplified biosensor system for detection of phenolic compounds

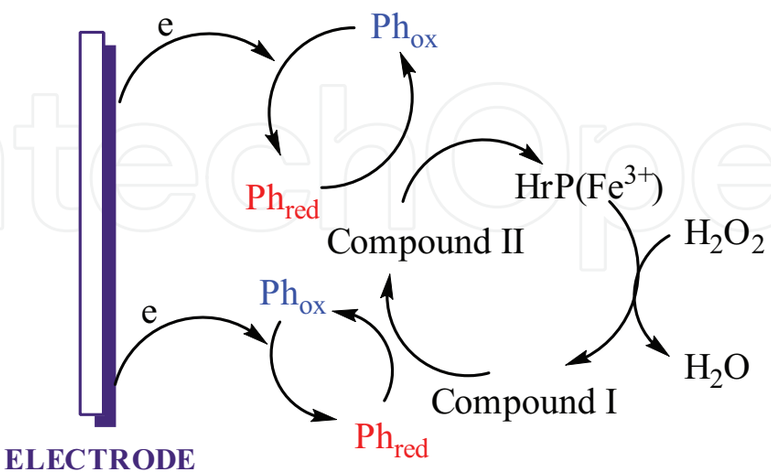


Fig. 8. Scheme of the reactions occurring at the surface of the electrode modified with horseradish peroxidase; Ph_{ox} and Ph_{red} are the oxidized and reduced forms of the phenolic compounds, respectively

Oxidation of phenolic compounds by horseradish peroxidase in solution can be enhanced by nitrogen ligands such as ammonium ions as wel as aromatic amines (*i.e. o*-dianisidine, benzidine, *o*-aminophenol, *p*-aminophenol, *p*-cresol, aniline) (Ruzgas et al., 1996). These observations gave the possibility to correlate the increase of the reduction current of peroxidase electrode with the concentration of aromatic amines.

The monitoring of the enzyme reaction is accomplished by the electrode reduction of the phenoxy radicals formed, the current being proportional to the concentration of phenolic compounds as long as the H_2O_2 concentration is not limiting. Therefore, an excess of H_2O_2 should be added to the working solution in order for the biosensor to be able to respond to the phenolic compounds (Serra et al., 2001). However, it is well known that the presence of a high concentration of H_2O_2 causes inhibition of the activity of peroxidase (Scheller et al., 1997). Moreover, H_2O_2 is unstable in solution. These facts cause some difficulties in the practical use of the peroxidase biosensor for the detection of phenolic compounds. In order to improve this type of detection, there are developed composite multienzyme systems.

Serra et al. (2001) reported the sensing system for phenolic compounds where horseradish peroxidase is mixed with glucose oxidase (GOx). In this biosensor, GOx was responsible for generating *in situ* H_2O_2 needed for the enzyme reaction with the phenolic compounds. For the sensor design, matrices of graphite and Teflon were selected. The enzymatic electrodes were constructed by simple physical inclusion of the enzymes (HrP, GOx) into the bulk of graphite-Teflon pellet with no covalent attachments.

Serra et al. described also the three enzyme system with HrP, GOx and tyrosinase to monitor possibly large number of phenolic compounds (Serra et al., 2001).

6.2 Laccase and tyrosinase electrodes for phenolic compounds detection

Laccase and tyrosinase are both copper containing oxidases catalyzing the oxidation of phenolic compounds in the presence of oxygen. In these reactions oxygen is reduced directly to water on the surface of enzymatic electrode (Fig. 9).

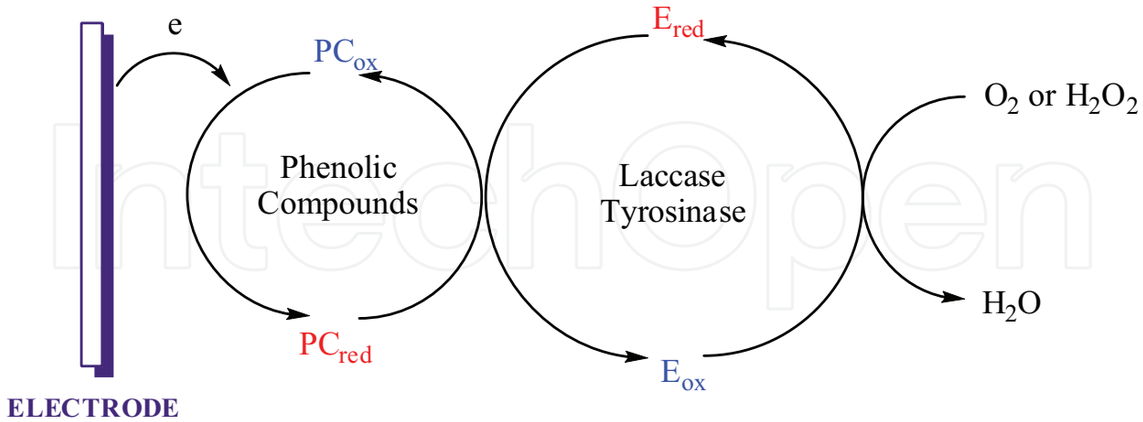


Fig. 9. Mechanism of the reactions on the laccase/tyrosinase biosensor. PC: phenolic compound; E: enzyme; *red* and *ox* are the reduced and oxidized forms

For the design of biosensor different methods of enzyme immobilization were employed. They include the modification of solid graphite (Ortega et al., 1994), incorporation of enzyme into carbon paste, immobilization on surface of different membranes (Yarpolov et

al., 1995), Langmuir-Blodgett hybrid films (Cabaj et al., 2010a; Cabaj et al., 2008; Sołoducho & Cabaj 2010). The most sensitive biosensors are based on tyrosinases (Psrellada et al., 1997), however, in order to low stability of this class of enzymes, these devices usually present short life-times (Vianello et al., 2004). Alternatively to tyrosinases – laccases are often used.

Laccase is one of the best candidate for use in analytical systems for determination of chlorinated and polyphenolic compounds (Freire et al., 2002), mainly due to its great stability (Gianfreda et al., 1999), wide range of detectable phenolic compounds (Freire et al., 2002) and it is not susceptible to product inhibition. Laccase can act over the phenolic compounds that are not reactive with the other mentioned enzymes (Freire et al., 2002). In addition, one of the most important point in using laccase is its sensitivity for phenolic compounds, such as chloroguaiacols and guaiacols, which are considered very toxic (Freire et al., 2002). The enzymatic oxidation of phenolic compounds and anilines by laccases generate radicals that react with each other to form dimers, oligomers or polymers covalently coupled by C–C, C–O and C–N bonds. In the case of substituted compounds, the reaction can be accompanied by partial demethylations and dehalogenations (Claus 2004).

However, an exhaustive overview in the basic aspects of immoblilization of laccase and tyrosinase has been reported. Whereas, to retain enzyme's specific biological function, their immobilization on solid matrix is a key factor in preparing biosensors. So far several immobilization strategies have been commonly used to immobilize small molecules onto appropriately functionalized glass slides, including covalent immobilization with Staudingeer ligation (Kohn et al., 2003). The immobilization methods for laccase or tyrosinase such as physical adsorption (Cabaj et al., 2010a; Cabaj et al., 2010b), covalent attachment (Vianello eta al., 2004), incorporation within carbon paste (Duran et al., 2002), immobilization in polymer films (Timur et al., 2004), entrapment in some sol–gel matrices (Duran et al., 2002) have been also reported in the literature. Vianello et al. (2004) presented a high-sensitivity flow biosensor based on a monomolecular layer of laccase immobilized on a gold support. This biosensor detects phenols in the low micromolar range, *i.e.* below European Community limits (Vianello et al., 2004).

Laccase belongs to the restricted groups of redox enzymes that show efficient direct heterogeneous electron transfer at electrodes (Ghindilis et al., 1997). When laccase was adsorbed on graphite electrode, bioelectrocatalytic reduction of oxygen occurs and is observed as a reduction current caused by direct (mediatorless) electron transfer from the electrode to the immobilized laccase and then further to molecular oxygen in solution. Oxygen is reduced to water in a four-electron transfer mechanism. In this mechanism the electron donor (substrate) penetrates the active site of the enzyme where it is oxidized in a single electron oxidation step often producing electrochemically active compound (possibly a radical) that it turn can be re-reduced at the electrode surface in a mediated electron transfer step. This creates an electron-shuttle process between the electrode and the laccase providing the basis for reduction of molecular oxygen by mediated electron transfer at the enzymatic electrode (Haghighi et al., 2003).

In particular, several biosensors based on tyrosinase were developed for determination of phenols (Mai Anh et al., 2002; Dzyadevych eta al., 2002). The tyrosinase was immobilized on an electrode's surface as a thin film or in a membrane on a Clark oxygen electrode (Macholan 1990), chemically bonded to a solid graphite electrode (Cosnier & Innocent 1993) or controlled-pore glass (Zachariah & Mottola 1989) and using electropolymerization of an amphilic pyrrole derivative–enzyme mixture (Cosnier & Popescu 1996). Tyrosinase was also adsorbed on the surface of phospholipids Langmuir-Schaefer film (Cabaj et al., 2010b).

Tyrosinase-based electrochemical biosensors suffer from low enzyme stability and significant inhibition of the enzyme by reaction products; both these factors deteriorate electrode characteristics in phenol determination. In the case of HrP, the limitation is the necessity of hydrogen peroxide presence to complete the biocatalytic cycle.
Nevertheless, tyrosinase- and laccase-based amperometric biosensors have proved to be very useful for the determination of phenols and substituted phenols at low levels (Ghindilis et al., 1997).

7. Modification of electrodes by conducting polymers

Conducting polymers have found increased applications in various industries. Some of the main classes of conducting polymers that are available for various applications include polyacetylene, polyaniline (PANI), polypyrrole (PPY), polythiophene (PTH), polyethylenedioxythiophene (PEDOT), poly(paraphenylene), poly(paraphenylenevinylene), polyfluorene, polycarbazole, and polyindole (PI), etc. Conducting polymers exhibit intrinsic conductivity when the conjugated backbone of the polymer is oxidized or reduced (Bredas & Street 1985). Apart from its conductivity, the change of electronic band in the conducting polymer affects the optical properties in the UV-visible and near IR region. The changes in conductivity and optical properties make them candidates for use as optical sensors or element able to modify enzymatic electrodes.
Synthetic and biological receptors can be used to manipulate the sensitivity of a conducting polymer for different analytes (Adhikari & Majumdar 2004; Ahuja et al., 2007). Some conducting polymers that have been modified with various receptors are listed in Table 3. To immobilize the receptor, it is bonded to the polymer matrix through covalent or noncovalent interaction. Physical adsorption (Lopez et al., 2006), the Langmuir-Blodgett technique (Cabaj et al., 2010ab), layer-by layer deposition technique (Ram et al., 2000), and mechanical embedding method (J. Kan et al., 2004) are used to bind the receptor to the matrix through noncovalent bonding. Gerard et al. (2002) have discussed the advantages and limitations of these techniques.

8. Future of biosensing

Despite the rapid progress in biosensor development, *i.e.* clinical applications of biosensors are still rare, with glucose monitor as an exception. Sensors, biosensors have a number of disadvantages compared to classical chemical monitoring methods, however, they ensue a number of requirements of current and emerging environmental pollution or medical monitoring that chemical methods fail to address. Ongoing developments in material technology, computer technology, and microelectronics are expected to help to omit many of these problems. It is expected that progress in the development of tools and strategies to identify, record, store, and transmit parameter data will help in expanding the scope of the use of sensors on a broader scale (Blasco & Pico 2009).
The proceeding researches have to be carried out in all the building blocks of biosensors, which include transducers, recognition molecules, immobilization strategies, as well as transduction mechanisms. Particular attention should be also given to the control of molecular architectures afforded by thin film forming methods (LB/LS, LbL, electrodeposition), especially when biomolecules could be combined with layers of polymers or metallic nanoparticles (Au). Relevantly, the success of LB, LbL films for

Analyte	Receptor	Conducting polymer	Type of immobilization	Type of transduction	*Ref.*
H_2O_2	Horseradish peroxidase	PANI/ polyethylene terephthalate	Physical adsorption	Optical	Caramori & Fernandes 2004; Borole et al., 2005; Fernandes et al., 2005
H_2O_2	Horseradish peroxidase	PEDOT/PSS (poly(styrene-sulfonate))	Physical adsorption	Amperometric	Asberg & Inganas 2003
Hydro-quinone	Laccase	Poly-*o*-phenylenediamine	Physical adsorption	Amperometric	Pałys et al., 2007
Catechins	Laccase	Poly (tertthiophene)	Covalent immobilization	Amperometric	Rahman et al., 2008
Phenol	Tyrosinase	PEDOT	Physical adsorption	Amperometric	Vedrine et al., 2003
Catechol	Tyrosinase	Poly(dicarbazole)	Physical adsorption	Amperometric	Cosnier et al., 2001
Catechol	Tyrosinase	Poly(1,8-diaminocarbazole)	Covalent immobilization	Amperometric	Skompska et al., 2007

Table 3. Examples of conducting polymer-based biosensors for phenol compounds detection

biosensing can be attributed to the fact that entrained water remains activity of biomolecules. When extremely high sensitivity is required, the optical methods (luminescence) are favorable. Also important is the possibility of integrating sensing molecules with silicon-based technology, a path to accomplish low-cost biosensors with scalable production (Siqueira et al., 2010).

New devices based on microelectronics and related (bio)-micro-electro-mechanical systems and (bio)-nano-electro-mechanical systems are expected to provide technological solutions (Bezbaruah & Kalita 2010). Miniaturized sensing devices, microfluidic delivery systems, and multiple sensors on one chip are needed. High reliability, potential for mass production, low cost of production, and low energy consumption are also expected and some progress has already been achieved in these areas (Farre et al., 2007).

A wireless sensor network comprising spatially-distributed sensors or biosensors to monitor environmental conditions will contribute enormously towards continuous environmental monitoring especially in environments that are currently difficult to monitor such as coastal areas and open seas (Farre et al., 2009). Blasco and Pico (2009) expect that such a network can provide appropriate feedback during characterization or remediation of contaminated sites. The laboratory-on-a-chip is another concept that is going to impact future sensor

technology. These chips involve microfabrication to achieve miniaturization and/or minimization of components of the analytical processes. It has been suggested that nanoscale and ultra-miniaturized sensors could dominate the production lines in the next generation of biotechnology-based industries (Farre et al., 2007).

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This book is a collection of contributions from leading specialists on the topic of biosensors for health, environment and biosecurity. It is divided into three sections with headings of current trends and developments; materials design and developments; and detection and monitoring. In the section on current trends and developments, topics such as biosensor applications for environmental and water monitoring, agro-industry applications, and trends in the detection of nerve agents and pesticides are discussed. The section on materials design and developments deals with topics on new materials for biosensor construction, polymer-based microsystems, silicon and silicon-related surfaces for biosensor applications, including hybrid film biosensor systems. Finally, in the detection and monitoring section, the specific topics covered deal with enzyme-based biosensors for phenol detection, ultra-sensitive fluorescence sensors, the determination of biochemical oxygen demand, and sensors for pharmaceutical and environmental analysis.

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