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Application of Conducting Polymers in Electroanalysis

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1. Introduction

Electroanalytical methods or electroanalysis have several advantages over other analytical methods. They offer competitive characteristics such as low cost, fast response, easy automation, miniaturization, portability, the ability to monitor in-situ and in real time, and no need for sample manipulation. They are based on the control of electrical signal and it correlation to analyte concentration. Thus several possibilities of electrochemical transducer have been envisaged but the most commonly used have been divided into the following five categories.

Conductimetric: when a potential is applied across two plates (usually a sine wave voltage), the current that passes through the solution is measured and the conductivity is determined from the current and potential according to the generalized Ohm's law.

Potentiometric: when the potential is measured between the sensing and the reference electrodes under no current flow.

Amperometric: when a constant potential is maintained between a sensing and reference electrodes and current generated by electro-activity of some species at the surface of sensing electrode is measured versus the time.

Voltammetric: which is similar to amperometric, but in this case the potential is varied with time and the current is monitored versus this variation of the potential.

Impedometric: which is based on the simulation of electrochemical cell to electrical circuit, consists of the application of small magnitude perturbation of this circuit with alternative potential, and measuring its impedance. So electrochemical parameters, such as double layer capacitance, resistance solution and impedance of faradaic process, can be determined to characterize the electrode surface and to monitor any change in its state.

In the majority of these approaches the electrode can act as source or captor of electrons transferred from an electroactive molecule located in the interfacial region between the electrode and the solution.

However, this process should not be regarded as perfect. The inertia of the electrode depends on the medium and the applied potential. Many compounds provide similar electrochemical signal (for example; many neurotransmitters exhibit similar values of peaks potential of oxidative process in voltammetric analysis). Some analytes have irreversible and non reproducible oxidoreduction activities at the surface of conventional electrodes, etc... Thus, the electrode must be modified by some recognizing inorganic,

organic, or biological components to detect the analyte with required sensitivity, selectivity and reproducibility.

Development of such so-called heterogeneous electrodes is therefore carried out in order to produce micro-extraction and pre-concentration of analyte, increase the active surface area and working potential window, achieve a good electrical conductivity and inertia, and / or to immobilize biological and chemical recognizing elements. Development of these modified electrodes should be reproducible and controllable in order to be easily transferred to industrial mass production. Also the new surface should be homogeneous, ordered and free of defects to enhance the electron charge transfer and the accessibility of the analyte to recognizing elements or specific sites in the modifier.

Conducting polymers (CPs), which combine the electronic characteristic of metals and inorganic semiconductors, possess the attractive advantage of having easy synthesis control over the properties of the polymeric exposed surface such as structure, morphology and thickness. The use of these polymers represents the most important development in the preparation of modified electrodes to make sensors and biosensors. Their synthesis can be achieved by two conventional methods.

Firstly, chemical synthesis: by adding oxidative agents to a dissolved corresponding monomer in adequate solvent. Thus the electrode must be modified by each one of the popular techniques for depositing controlled thickness film, such as spin-coating, Langmuir-Blodgett-technique or layer-by-layer.

Or secondly by electrochemical method, which has the advantage of combining the synthesis and modification steps in one procedure. So the monomer is electrochemically oxidized at a controlled potential giving rise to free radicals. They are absorbed through the surface of the electrode and subsequently undergo a wide variety of reactions leading to the polymers network. However, the advantage of combining synthesis and modification in a unique procedure should not be considered as exclusively for conducting polymers, because other non-conducting polymers have the same plus point. The difference is that conducting polymers can achieve multilayer and controlled thickness thanks to their intrinsic conductivity.

In addition, these materials were considered as multifunctional in electro-analytical methods development. So they were used as receptors, transducers, immobilization matrix, and/or as anti-fouling and protective materials.

Modification of conventional electrodes with these materials was shown to be an outstanding approach to producing electro-analytical devices able to respond to practically every analytical demand. This approach, and those which were then derived from it, was used to control analytes of interest in environment, agroalimentation, clinical, security terms etc…

Several reviews were published to describe and characterize the advantageous use of conducting polymers especially in the biosensors area (Bartlett & Cooper 1993; Santhanan, 1998; McQuade et al., 2000; Gerard et al., 2002; Cosnier, 2003; 2007; Rahman et al., 2008; Lange et al., 2008; Peng et al., 2009; Nambiar & Yeow, 2011)

In this chapter the advances that have been made in this approach were reviewed, putting special emphasis on the practical aspect and trying to classify the role of CPs in each described device. The topic will be divided into separate sections, each taking into account the analytical characters of this contribution. Thus, we try to provide examples and general reference in the topic for both researchers and postgraduate students.

2. Electrochemical sensor

2.1 Heavy metals control

Heavy metals are common pollutants in the aquatic environment and their hazardous nature is indisputable in our times. Consequently their admissible concentration ranges from zero to a few ppb depending on their toxicity, forms, and the matrix in which they will be determined. Several methods have been developed to control these components with required sensitivity and selectivity. Electrochemical methods have attracted considerable interest in this subject. The most commonly used techniques were based on pre-concentration stripping approaches (either anodic or cathodic), solid phase micro-extraction, and ionic selective electrodes.

Thus, common conducting polymers were used without any modifiers in the pre-concentration / electroanalysis of metal ions, and instead by simple absorption in the heteroatom of CPs, or by its reduction on the surface of the modified electrode and its stripping oxidation control.

Polypyrrole (PPy) micro and macro-electrodes, incorporating chloride ions as the counterion, were grown galvanostatically, and silver ions were pre-concentrated onto the electrode surface for ten minutes from a 1 mol L^{-1} $NaNO_3$ solution. Then the trapped silver was subsequently determined voltammetrically in 1 mol L^{-1} NaCl applying cathodic polarization (Jone & Walace, 1990). A similar approach was optimized by Song and Shiu (Song & Shiu, 2001) achieving detection limit of 0.2 ppm, and using differential pulse cathodic voltammetry, without significant interferences of divalent ions of other heavy metals, especially at low concentration.

However, synthesis of PPy film in the presence of para-sulphonate and its subsequent treatment with 0.5 mol L^{-1} NaOH and 0.5 mol L^{-1} of HNO_3 (base treatment to deprotonation and removal of sulphonate ions and that acidic to return the film to its oxidized state) resulted in the stripping anodic sensor being able to detect 5 ppb of silver ions after its reduction by mercury (Pickup et al., 1998). Modified gold Poly(3-methylthiophene) (P3MeT) was used to detect Hg^{++} using differential pulse voltammetry. The approach was consisted in two steps: first micro-extraction by simple incubating of electro-polymerized P3MeT at open circuit potential for 30 min and then measuring differential pulse voltammetric anodic signal at around pH 2. Low detection limit of 0.1 10^{-9} mol L^{-1} was achieved and the peak of mercury oxidation was not affected by the presence of ions such as Cd^{2+}, Ag^{+}, Fe^{3+}, Cu^{2+}, $Cr_2O_7^{2-}$ and Cr^{3+}(Zejli et al., 2004).

Approaches based on the use of CPs and some molecules (Fig. 1) with notable affinity to heavy metals acting as doping ions were also explored, especially to achieve ionic selective electrodes (ISE).

Electrodes modified by Poly(3,4-ethylenedioxythiophene) (PEDOT) and PPy doped with p-sulfonic calix[4]arene (C4S), p-sulfoniccalix[6]arene (C6S) and p-sulfonic calix[8]arene (C8S) (Fig. 1) were proposed as ISE to silver ions (Mosavi al., 2005). The same strategy was published using PEDOT and several p-methylsulfonated calix[4]resorcarenes (Rn[4]S) with alkyl substitutes of different chain lengths (R1=CH3; R2=C2H5; R3=C6H13) (Fig. 1) was used to detect the same ions (Vázquez et al., 2005a). In these two studies the chemical structure of the backbone of the conjugated polymer seems to play a more important role in the selectivity of potentiometric Ag^{+} sensors based on conducting polymer, than the size of the sulfonated calixarene dopants.

The glassy carbon electrode was polarized in an aqueous solution containing 0.5 mol L^{-1} pyrrole monomer and 0.125 mol L^{-1} Eriochrome Blue-Black B (Fig. 1) as the only added anion, at fixed potential of 0.75 V vs. Ag/AgCl until reaching an optimum electro-polymerization charge of 0.8 mC. This modified electrode was proposed as differential pulse anodic stripping voltammetry and simple potentiometric Ag^+ sensor. For the first method Ag^+ was detected in 0.2 mol L^{-1} KNO_3; pH 2 as electrolyte with low detection limit around 6 10^{-9} mol L^{-1} and the presence of 1000-fold excess of Cd^{2+}, Cu^{2+}, Cr^{3+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Pb^{2+} have been tolerated (Zanganeh & Amini, 2007). Modified glassy carbon electrode with PEDOT doped with hexabromocarborane ($CB_{11}H_6Br_6^-$) (Fig. 1) was also developed by applying a constant charge of 0.014 mA (0.2 mA cm^{-2}) for 714 s to the electrode in the presence of 0.01 mol L^{-1} EDOT and 0.01 mol L^{-1} $AgCB_{11}H_6Br_6$ in acetonitrile, and a resulted modified electrode was used as ISE sensor for Ag^+ (Mosavi et al., 2006). The electrochemical polymerization of PPy was carried out in acetonitrile containing 0.1 mol L^{-1} Tetraphenylborate (Fig. 1) and pyrrole at constant potential of 0.9 V (vs. Ag/AgCl). The film was highly stable and the modified platinum electrode was used as ISE for Zn^{2+} ions (Pandy et al., 2002).

Methods based on electrochemical synthesis and these in turn were based on modifying the electrode with liquid membrane [the membrane consisted of mixture of dissolved poly(3-octylthiophene) (POT) and several other modifiers: potassium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (KTpFPB), [2.2.2]p,p,p-cyclophane (Fig. 1), and silver hexabromocaborane], were described and compared by Vázquez et al. with the aim of making ISEs for silver. Among these sensors the Nernstian response of Ag^+ was observed for those prepared by electrochemical polymerization of POT only; and those based on thick films of POT doped with the immobile and lipophilic anion $CB_{11}H_6Br_6^-$ (Vázques et al., 2005b).

Bi-polymer of undoped polycarbazole and polyindole was also used as modifier to make ISE to Cu^{2+} (Prakash et al., 2002). The approach was based on consecutive constant potential polymerization of each monomers (polycarbazole at 1.3 V and polyindole at 1V vs. Ag/AgCl) and in removing perchlorate dopant by polarizing at -0.2 V. The undoped ISE achieve detection limit of 10 10^{-6} mol L^{-1} with negligible response to other heavy metals.

The conductometric mercury [II] device was achieved, by the absorption of cryptand-222 (Fig. 1) as a receptor on electro-polymerized polyaniline (PANI), with sensitivity segments in the range of 10^{-12}_10^{-8} mol L^{-1}. The best response of the sensor was observed at pH 2, whereas the authors propose a composite of PANI with surfactants poly(styrene sulphonate) [PSS] and sodium dodecyl sulfate (SDS) to work in neutral media and adapt the method to in-situ analysis (Muthukumar el al., 2007).

Modification of CPs with complexing agent EDTA was also used as a strategy to create height-sensitive sensors for heavy metals. EDTA was covalently attached to electro-synthesized poly(diamino-terthiophene) and extraction of Cu^{2+}, Pb^{2+} and Hg^{2+} was achieved. The detection was carried out in another electrolyte by the reduction of ions at -0.9 V (vs. Ag/AgCl) and re-oxidation by potential scanning from -0.9 to +0.7 V (Rahman et al., 2003). Chemical synthesis of *N*,*N*-ethylenebis[*N*-[(3-(pyrrole-1-yl)propyl) carbamoyl) methyl]-glycine] as pyrrole-EDTA like and its electro-polymerization for the simultaneous determination of Cu^{2+}, Pb^{2+} and Cd^{2+} by the combination of extraction and stripping was reported by Heitzmann et al. Surprisingly, a polymer synthesized in the presence of Cd^{2+} shows more selectivity to this ion (Heitzmann et al., 2007)

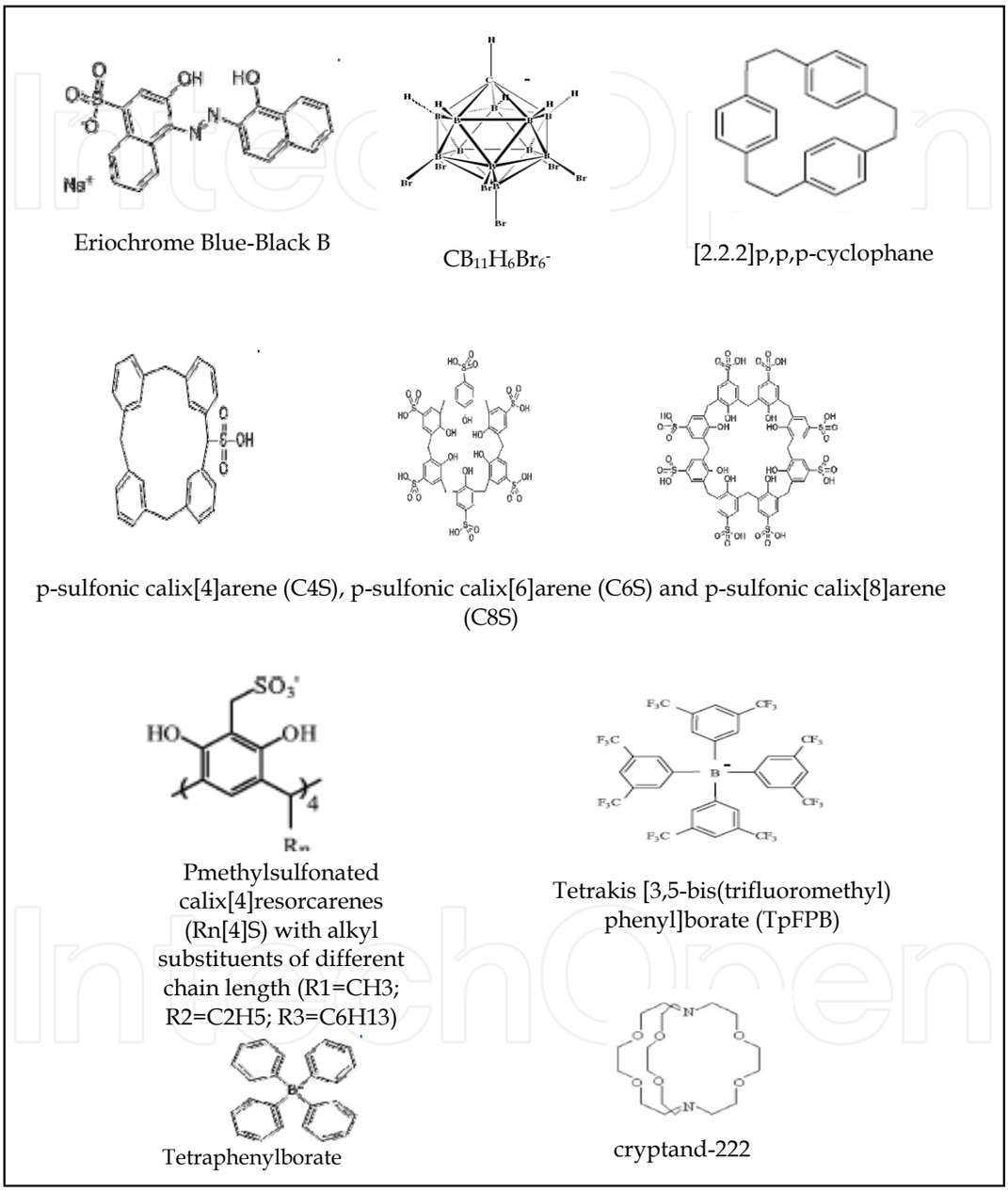


Fig. 1. Structure of some modifier-dopont used for heavy metals ISE based CPs developments (see text for details)

2.2 Neurotransmitters electroanalysis

Electrocatalytic activity of neurotransmitters and derived forms of pyrogallol and catechol at unmodified conventional electrodes has several disadvantages such as low sensitivity, irreversibility, strong absorption, and hence fouling of the electrode by quinones oxidatives product. Catalysis of their electro-oxidation by the use of conventional CPs, were described in several works. It has been found that oxidation responses of modified P3MeT/Pt electrode to catechol, dopamine, and epinephrine were 4-10 time larger as compared to those of unmodified platinum electrode (Atta et al., 1991). This increase was attributed to the fact that electron transfer occurs at the polymer-solution interface (Mark et al., 1995) not at the inert electrode surface after diffusion through the polymer matrix or through pores (Wang et al., 1989).

Between P3MT, Poly-N-Methylpyrrole, PANI, and Polyfuran, modified polythiophene electrode is the most effective for oxidation of the neurotransmitters (Mark et al., 1995; Atta et al., 1996). Therefore, possibly thanks to sulfur heteroatoms that provides a suitable environment for the electron transfer step for this class of molecules (Kelly et al., 2005).

However in spite of the advantage that CPs have in resolving the previously cited drawback of an unmodified electrode, the resolution of the voltammetric signal of the mixture of catecholamine was not resolved. This was enhanced by the use of micro-electrodes (Galal., 1998; Lupu et al., 2003). But using these electrodes it has been concluded that the peak separation decreases with the increase of monomer concentration, deposition time, and voltage. This is opposite to the achievement of admissible film thickness and electro-modification at -20 ^{o}C was proposed as the most acceptable combination of film thickness and electrode properties (Zhang et al., 1997).

Nevertheless, this direct electro-catalysis and current signal magnitudes of CPs modified electrode might be affected by several factors such as electro-synthesis condition, film pre-treatment and the history of the film before its use. The last factor can be considered as inopportune because exposure to some analytes cause a change in film morphology; and the response of this film in proceeding analyses might be totally different to freshly prepared ones.

Electrochemical studding of electro-polymerized P3MeT films by Pd and Pt nanoparticule (NPs) was also explored to achieve stable and selective catalytically CPs based sensors (Atta & El-Kadi, 2009; 2010). It was found that the Pd nanoparticules modified by CPs electrodes (PbNPs, with average diameter of 60 nm , were electro-deposited by cyclic voltammetry method, scanning the potential between −0.25 and +0.65 V (vs Ag/AgCl) at a scan rate of 50 mV^{-1}; 2.5 10^{-3} mol L^{-1} $PbCl_2$ in 0.1 mol L^{-1} $HClO_4$) has the best analytical performance, contrary to that of PtNPs.

Introduction of neutral ɣ-cyclodextrines into P3MeT doped hexafluorophosphate (Bouchta et al., 2005) and β-cyclodextrines into PPy in the presence of perchlorate (Izaoumen et al., 2005) was described. Cyclodextrines (CDs) are cyclic oligosaccharides consisting of six, seven or eight glucose units called α-, β- and ɣ-cyclodextrins respectively. These CDs possess a hydrophobic inner cavity and a hydrophilic outer surface. Their well-known ability to form supramolecular complexes with suitable organic and inorganic, neutral, and ionic substances has resulted in the design of selective electrodes. Films growth by cyclic voltammetry from a mixture of monomers and cyclodextrines (100:1 and 20:1 for P3MeT/ɣ-CD and PPy/β-CD respectively) lead to sensitive surfaces to dopamine, L-dopa and other nuerotransmiters without significant interference of ascorbic acid. A sulfonated β-

cyclodextrin was also used as a unique doping ion for PPy film growing at 0.80 V (vs. SCE) in a 0.20 mol L^{-1} pyrrole and 0.01 mol L^{-1} CDs, to achieve a highly selective electrochemical sensor for dopamine, without interference of ascorbate anion, and a detection limits around 3 10^{-6} mol L^{-1} (Harly et al., 2010).

2.3 Moleculary imprinted conducting polymers

Molecular imprinting technology (MIPs) aims to create artificial recognition sites in synthetic polymers. Classically, the process consists of the co-polymerization of monomer and cross linkers in the presence of a template. Thus, the target molecule's "template" is cross linked in the polymer network and the removal or extraction of this template, by adequate solvent, forms binding sites that are complementary in both size and shape.

Solution polymerization, or synthesis, to give small particles with controlled size and physical properties was largely used as the initial process in producing MIPs. This must then be followed by the immobilization of the recognition spheres in close proximity to the electrode; for example through its incorporation into the carbon paste or into supporting gels, as shown in Fig. 2 (Haupt & Mosbach, 2000). The process can also be performed in situ; inside a surface of the sensor, "or transducer", involving imprinted films or membranes. These can be prepared with the deposition of linear polymers in the presence of a template, or by direct polymerization of monomers, cross linkers if it is required, and templates on sensor surfaces, see Fig. 3.

Naturally, the use of conducting polymers to achieve electrochemical sensors based on molecularly imprinted polymers has been utilized in the two ways sited. However thanks to their outstanding intrinsic characteristics theses polymers have also been used as transducers and immobilization matrices.

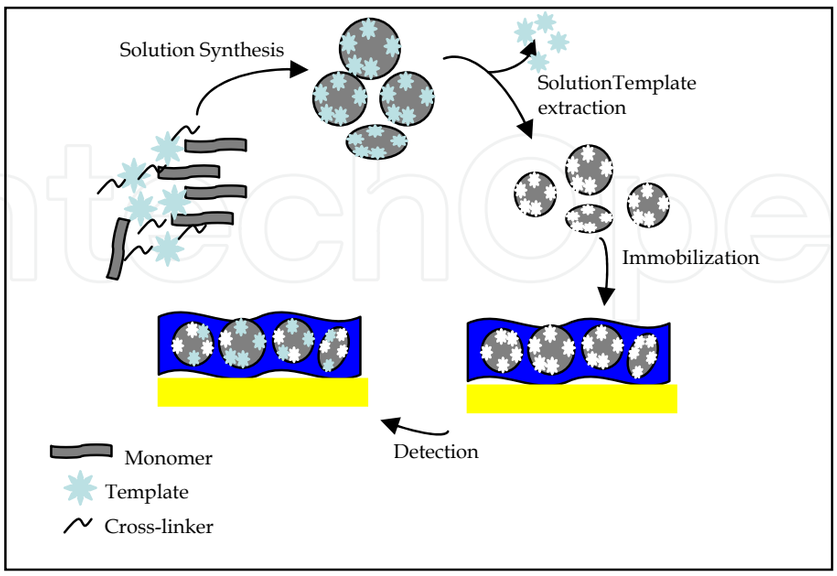


Fig. 2. Solution synthesis of MIPs beads and its immobilization on electrode surface

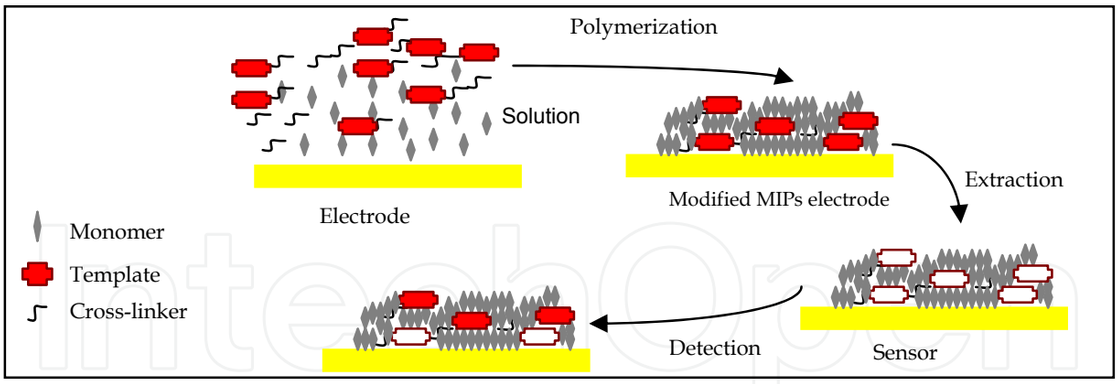


Fig. 3. In-situ synthesis of MIPs

Imprinting polymerization of glucose was performed by the electro-polymerization of poly-o-phenyldiamine (P-*o*-PD) by cyclic voltammetry (20 cycles) in the potential range of 0–0.8 V (vs. Ag/AgCl) at a scan rate 50 mV s^{-1} from a mixture solution of *o*-PD (5 10^{-3} mol L^{-1}) and glucose (0.02 mol L^{-1}) in 0.01 mol L^{-1} acetate buffer (pH 5.18) (Cheng et al., 2001). In this study the authors tried to use this modified electrode as a capacitive sensor, therefore the electrode surface must be well insulated to avoid the penetration of aqueous conducting solution to the layer which in turn increases its capacitance due to the great polarity of water. An insulation process of the electrode consisting in its dipping in 1-dodecanethiol ethanol solution overnight was proposed. The hydrophobic interface was then achieved and the extraction of template process was carried out by simple washing of the electrode. The concentration of glucose was correlated to capacitance (C) values which were calculated directly from the imaginary Z_{im} impedance after recording the current response to an a.c signal with a peak-to-peak amplitude of 10 mV at frequency of 25 Hz. The formula used was:

$$C = 1 / 2\pi f Z_{im} \tag{1}$$

Voltamperometric MIPs sensors for L-glutamate (L-glu) were achieved thanks to over-oxidation of PPy with dopant complementary cavities. The approach consisted of depositing the film of PPy/L-glu galvanostatically (0.5-0.1 mA cm^{-2}) in aqueous solution containing 0.5 mol L^{-1} monomer and 1.0 mol L^{-1} sodium L-glu as dopant and template for 120-90 min. PPy was over-oxidized potentiodynamically over the range of -0.3 to 1.0 V (vs. Ag/AgCl) at a scan rate of 40 mV s^{-1} at pH 6.9 phosphate buffer. Then the template was removed. Recognition was achieved at pH 1.7 (0.2 mol L^{-1} KCl and 0.2 mol L^{-1} HCl) with the appearance of a pair of peaks at formal potential of +0.2 V attributed to the insertion of cationic form of L-glu in cathodic scan and its rejection in the anodic polarization (Deore et al., 1999).

A similar detection approach was recently used to detect small molecule of atrazine pesticide by Pardieu et al. Film of poly(3,4-ethylenedioxythiophene-co-thiophene-acetic acid) printed with atrazine, due to the hydrogen bonding between the acetic acid substituent and nitrogen heteroatom of atrazine, was synthesized electrochemically. Thus after the association of 3-acetic acid thiophene functional monomer 0.03 mol L^{-1} and atrazine 1.5 mol L^{-1} in CH_2Cl_2 and tetrabutylammonium trifluoromethane sulfonate (TBATFMS as electrolyte and doping ions) for 10 min, 3,4-ethylenedioxythiophene 7.5 mol L^{-1} was added and a

constant potential of 1.45 V vs. Pt during 10 min was applied. To remove a template a mixture of protic solvents, methanol/acetic acid solution (0.7:0.3 v/v), was used for 10 min. The sensing process resulted in a cyclic scan of the electrode from -0.5 to 0.4 V, with a scan rate of 25 mV s^{-1}, in CH_2Cl_2 solution containing TBATFMS (0.1 mol L^{-1}). No peak was observed but the author correlated atrazine concentration to relative charge calculated according to:

$$r(Q) = \frac{Q(0M) - Q}{Q(0M)} \quad (2)$$

Where *Q(0M)* is the charge in absence of atrazine and *Q* is in it presence (Pardieu et al., 2009). The mechanism of charge decreasing has not been elucidated by the authors. This could possibly be attributed to a doping/undoping phenomenon, similar to that described by Deore et al. (Diore et al., 1999), but in the inverse sense.

Also, saccharide-imprinted poly(aniline boronic acid) was described involving the selective complexation of boric and boronic acid compounds with saccharides. In this way, the electro-polymerization of derived aniline was achieved at pH (5-7) in the presence of fluoride and the saccharide template was removed by soaking the film in pH 7.4 phosphate buffer solution overnight. A high resolution of potentiometric response of mixture of sacharide such as glucose and fructose was obtained (Deore & Freund, 2003).

On the other hand, micro-spheres with a diameter of 0.5 10^{-6} mol L^{-1} of morphine imprinted polymers were prepared through thermal radical polymerization and the result was immobilized by *PEDOT* conducting polymers into ITO electrode, to be used as amperometric sensors (Ho et al., 2005). Methacrylic acid (MAA) as monomer, and trimethylolpropane trimethacrylate (TRIM) as cross-linker were used. Briefly, 4 ml of MAA and 4 10^{-3} mol L^{-1} of TRIM were mixed in 40 ml of acetonitile, the template morphine was added to be at 57.5 10^{-6} mol L^{-1} and the reaction was initiated by 2,2'-azobisisobutyronitrile. The solution was desoxygenated by sonication for 10 min and then with bubbling nitrogen for 10 min. The breakers were sealed and placed in a water bath at 60 ^{o}C for thermal polymerization. After 9 hours the MIPs particles were collected by filtration and the template was removed with methanol. Modification of the ITO electrode was achieved thanks to the electro-polymerization of EDOT in 0.1 mol L^{-1} lithium perchlorate, and 10 mg of MIPs spheres.

3. Electrochemical biosensors

3.1 Classification

A general definition of a biosensor could be given as a chemical sensor in which the recognition system utilizes a biochemical mechanism. However, biosensors must be distinguished from integrated devices which contain a recognition reservoir, an enzymatic or immunological reactor integrated in a flow system to generate products or consume reactive and identify this variation in a separate detection system. A recommended definition of a biosensor is: an analytical device containing a biological recognition element in intimate contact, or immobilized on a surface of phisico-chemical transducer (Thévenot et al., 1999). Biosensors can be classified depending on the type of recognition process (catalytic or affinity), signal transducer (optical, electrochemical…), or immobilization process (cross linking, covalent linkage, adsorption, retention…).

3.1.1 Biocatalytic recognition based on electrochemical sensor

In this case, the biosensor is based on a recognition catalyze reaction. This can be due to the integration of elements in their original biological environment, or in an advanced manipulation process. Thus, three possible types of biocatalysts entity were envisaged:

a. Whole cells including micro-organisms, such as bacteria, fungi eukaryotic cells or yeast, and cells organelles such as mitochondria and cells walls.
b. Tissue.
c. Enzyme (mono or multi-enzyme) which can be directly isolated and purified from natural microorganisms or engineered to achieve recognition elements with specific characteristic.

The general reaction scheme for biocatalytic process can be expressed as:

$$S + S' \xrightarrow{Biocatalyst} P + P'$$

Where one or more analytes, usually named substrates, S and S', react in the presence of enzyme(s), whole cells or tissue culture and yield one or more products (P and P').

There are three possibilities that use adjacent transducers for monitoring this biocatalysed reaction. These possibilities have been used as chronological classification of the electrochemical biosensors historical evolution, and can be categorized as:

3.1.1.1 First generation biosensors

First generation biosensors rely on the measurement of decreasing initial signal values of co-substrate e.g. oxygen depleted by oxidase, bacteria or yeast reacting layers; or the increase of products such as hydrogen peroxide, quinone, H^+, CO_2, or NH_3, etc. For example if enzyme glucose oxidase is used, glucose concentration can be monitored as a result of either oxygen consumption (the historical Clark and Lyon biosensor was based on this approach), or of peroxide generation following the reaction and corresponding scheme in Fig.4:

$$Glucose + O_2 \xrightarrow{GOX} Glucoonolactone + H_2O_2$$

3.1.1.2 Second generation biosensors

In the last example, hydrogen peroxide was controlled on the surface of the working electrode (a Pt electrode usually functions as the anode at +700 mV). This includes both an oxidation process at high potential and species such as ascorbate, urate, etc., that may electrochemically interfere. Chemically synthesized mediators, usually molecules foreign to the natural catalytic cycle of enzymes, were introduced to the systems in order to react firstly with a product enzymatic reaction - especially peroxide - and then with the enzyme redox centers. Thus the second generation biosensor is defined.

3.1.1.3 Third generation biosensor

In general this class of biosensor is limited to redox proteins and enzymes with one or more electroactive cofactors tightly bound to protein molecules, or integrated in other subunits. Accordingly, this type of biosensor consists of control of direct electronic transfer between these cofactors and the electrode surface. So, following the oxidation or reduction of the substrates, the redox state of active centre of the enzymes is changed, and the electrons are transferred to the electrode or to another acceptor/donor cofactor integrated in subunits of proteins which must ultimately be transferred to the electrode too. Flavin-adenine-dinucleotide (FAD^+), nicotinamide-adenine-dinucleotide (NAD^+), Nicotinamide adenine dinucleotide phosphate ($NADP^+$), pyrrolo-quinoline-quinine (PQQ), heme, and other metallic

derivative centres such as cooper, molybdenum, or iron-sulfur, and the combination of same elements, are the most frequently cofactor units studied in electrochemical biosensors.

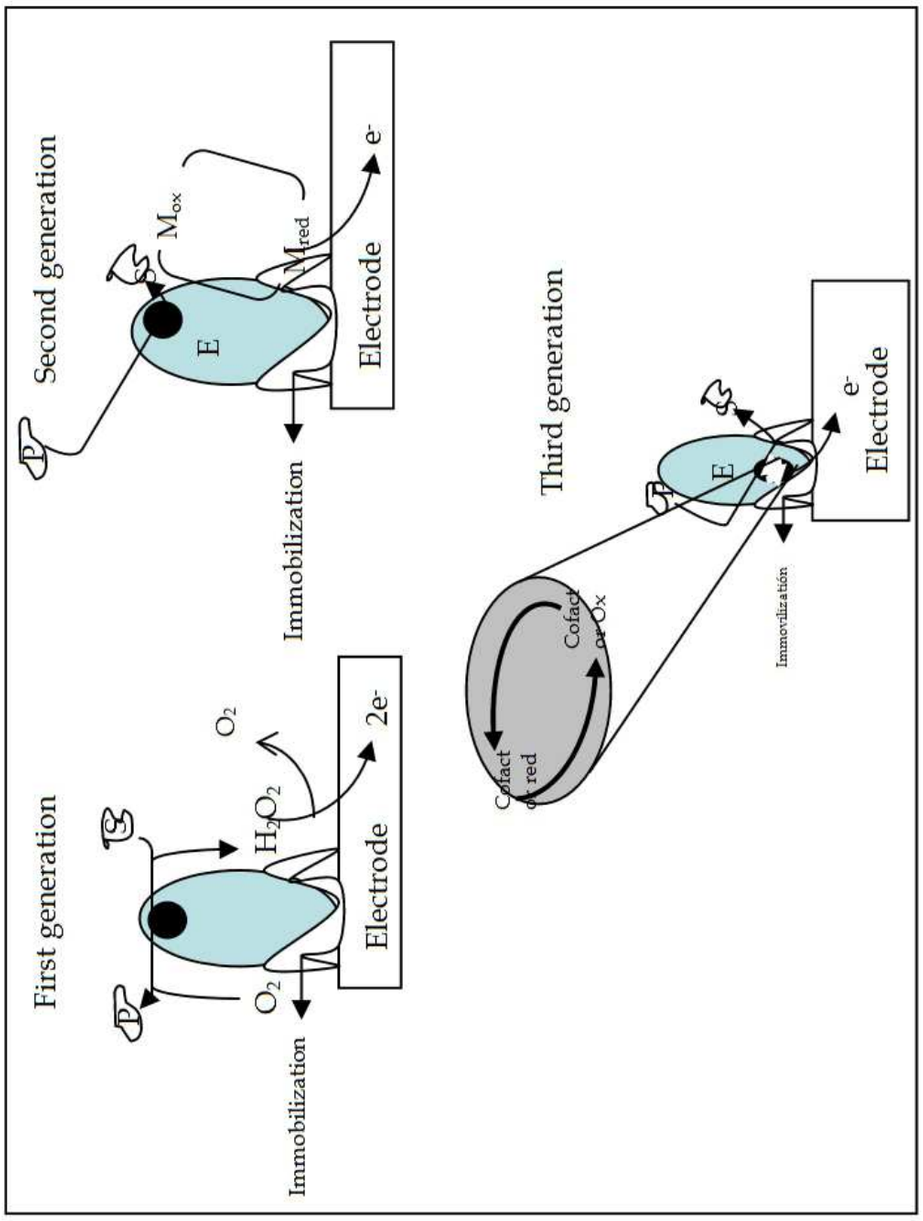


Fig. 4. Three generations of electrochemical biosensors based biological catalyst

3.1.2 Bioaffinity recognition based electrochemical sensor

In this class of biosensor the analyte interacts with a specific site in the biomolecule, or organized molecule assemblies, which have been isolated from the biological environment or synthetically engineered. After this interaction the equilibrium was reached, the analyte is no further consumed by the immobilized recognising agent, but the achieved equilibrium is monitored directly from continuous control of electrochemical surface parameters or

indirectly using a complementary biocatalytic reaction. Steady-state or transient signals are then monitored by the integrated detector. The most commonly developed strategies are based on: immunological specific interaction between an isolated or engineered antibody and analyte antigen (immunoelectrochemical sensor), specific DNA fragments interaction (DNA sensor), and interaction between synthesized oligonucleotide and target analyte (aptasensor).

3.1.2.1 Immunoelectrochemical sensor

Antibodies are proteins produced in animals as an immunological response to the presence of a foreign substance called antigen, and have specific affinity for this antigen. The first categories of immunoelecrochemical sensor was based on the existing schemes of enzymatic immunoassays ELISA (Enzyme Linked Immune Sorbent Assay) which usually need a labelled antibody or antigen, and is classified in two generals subcategories, as shown in Fig. 5. In the non-competitive immunoassay, the antibody is immobilized and, after the addition of the sample which contains the antigen, a conjugated or secondary labelled antibody is added. In competitive assays, the competition can either be between the free antigen (from the sample) and immobilized antigen for limited controlled amount of labelled antibody (complexion of all antigens from the sample and correlation of its concentration with the excess of labelled antibodies); or between the antigen and labelled antigen for limited immobilized amount of antibodies, Fig. 5. Labelling of antigen or antibody is usually achieved using oxido-reductase enzyme, such as alkaline phosphatase, horseradish peroxidase, or glucose oxidase. However the use of direct electro-active chemical compound and metallic nanoparticle was also envisaged.

The second category is label-free electrochemical immunosensor using electrochemical impedance spectroscopy (EIS) and/or volammretry. These have been explored widely due to their high sensitivity and they are based on detecting the electron transfer of foreign redox pairs (usually added to the measure solution), or change in capacitive double layer for non- faradaic processes after antibody-antigen recognition.

For limited example of antibody and antigen couples, label-free immunosensor can be also achieved as a result of the control of current given by electro-active residues in immobilized antibody, thereafter the current changes after the binding of the target antigen with the antibody as well as with the binding of secondary antibody. This process based on protein electro-activity is amplified by the use of carbon nanotube (Vestergaard et al., 2007).

3.1.2.2 DNA hybridization and aptamers based sensors

These sensors rely on the immobilization of a relatively short single standard sequence of natural DNA or synthesized oligonucleotide on the electrode surface. The interaction with a specific complementary region of the target DNA gives rise to a DNA hybridization sensor. The interaction of the oligonucleotide with a target molecule such as a protein drug or environmental effluent gives apatmers biosensors.

Electrochemical signal transduction in these types of biosensors is similar to that of immunosensors. This can be based on; 1) labelling the target or the immobilized oligonuclotide, 2) directly intercalating into the duplex, during the formation of a double-stranded DNA on the probe surface, a molecule that is electroactive, 3) or direct detection of guanine which is electroactive too. Control of the electron transfer of redox pairs, or impedance measurement of non faradaic process by EIS or CVs was also envisaged.

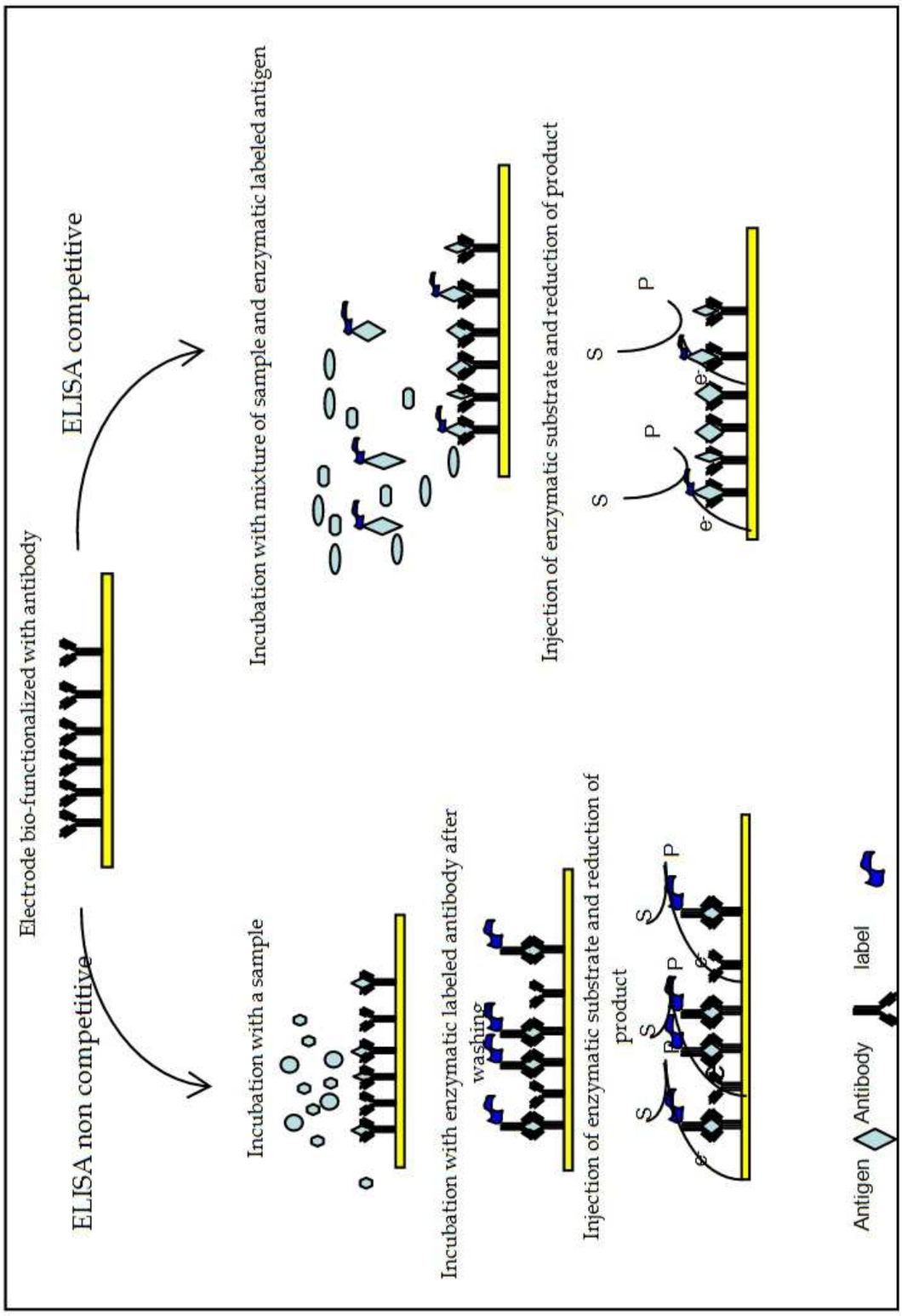


Fig. 5. Configuration of ELISA competitive and non competitive immunosensor

3.2 CPs based electrochemical biosensor

From the definition of a biosensor, development of these devices must undergo three steps: adequate choice of biological recognition element and its modification, its immobilization on an electrode surface, and designs of transduction process. The use of conducting polymers in this area essentially takes place in the two last steps. CPs are therefore used as immobilization matrices especially when their electro-polymerization property is taken into consideration, and as transducers or sensing probes due to their charge donors and acceptors characteristic.

3.2.1 Use of CPs as immobilization matrix

Methods of immobilization of biological recognition element are categorized as physical and chemical. Physical immobilization occurs without any modification of biological element and was based on electrostatic or hydrophobic interaction of these elements and modified electrode, or on simply retention - with membrane - on the top of a metallic electrode. Chemical immobilization was based on covalent binding of the biological element to a modified electrode. The use of CPs as a basis of an immobilization matrix was explored in these two strategies, and also on the legitimised approach to their versatile electropolymerization.

3.2.1.1 Electro-immobilization

Electro-immobilization of biological recognition elements can be achieved in a simple one-step method involving the application of appropriate potential to the working electrode, soaked in a mixture of electro-polymerizable monomer and bimolecules. The mechanism of such immobilization was reviewed by Bartlett and Cooper (Bartlett & Cooper, 1993), and it has been found on the charge complementary between the CPs and the enzyme. Accordingly, since the CPs is deposited as a polycation, electrostatic interaction between the polymers and the biomolecule occurs and the enzyme is then retained in the three dimensional growth film. This mechanism was supported by three pieces of evidence: First, simple adsorption of the bio-molecule onto performed CPs film controlling the net charge of each component (CPs are positively charged in their oxidized form and biomolecules are negatively charged at pH above their isoelectric point). Secondly, the amount of incorporated enzyme and the enzymatic activity of the biofilm decrease, as the concentration of other small anions increases. And thirdly, that it is difficult to incorporate positively charged biomelecules into CPs. However this mechanism has been rebuffed by Schumann who proposes that the entrapment of an enzyme is only due to a statistical enclosure of the enzyme present in the vicinity of the electrode surface (Schumann et al., 1993).

In any case this method has several disadvantages, including the difficulty of co-immobilization of enzyme and mediator, due to their competition during the charge compensation process; the high isoelectric point of some biomolecules which can be incompatible with polymer pH polymerization; and the requirement of high enzyme concentration.

This approach was enhanced by Cosnier and Innocent, using synthesized amphiphilic pyrrole monomers (Cosnier & Innocent, 1992). A monomer and enzyme-saving technique for biosensor preparation has been developed based on initial absorption of the enzyme together with the amphiphilic pyrrole derivative on the electrode surface, followed by solvent evaporation and subsequent electrochemical polymerization in aqueous electrolyte

solution. Enzyme pre-absorption onto the electrode surface prior to the initiation of electro-polymerization process can be also an enhancement of electro-immobilization. Accordingly, if the polymer/enzyme film which is subsequently grown is relatively thin, this will increase the overall concentration of the enzyme and may enhance the sensitivity of the resulting biosensor.

3.2.1.2 Covalent binding

This type of binding includes two steps, activation of a solid matrix and binding of the biomolecule or its derivative forms. Modification of electrode surface with deposited conducting polymers introduces the apparent possibility for activation of these electrodes.
Already activated amine or carboxylic monomer can be polymerized or copolymerized with an unsubstituted parent to give a directly activated surface, which can be covalently biomodified using carbodiimide. Various N- and 3-substituted pyrrole monomers have been synthesized in this order. The synthesized pyrrole derivatives were tested by Schalkhammer et al., for their ability to form polymers as well as to retain the GOX enzyme activity and permeability of peroxide product to electrode surface. The authors recommend the use of co-polymerization of substituted and unsubstitued pyrrole as an efficient way to achieve aqueous stability and reactive polymer films. The most promising substituted pyrrole was 2-(1-pyrrole)-acetylglycine which forms only thin water-soluble polymer films, but can be co-polymerized with pyrrole to obtain porous films with an optimal enzyme load. Also COOH- and NO_2-groups (NO_2 group was reduced after electro-polymerization of corresponding monomer, using a solution of 15% TiC13 or 1% SnCl, in l% HCl for 30 min to form a film with pending NH_2 for enzyme linking), which are stable against oxidation under polymerizing conditions have proved to be optimal for obtaining a modified PPy layer which can be used for covalent coupling of enzymes (Schalkhammer et al., 1991).
Unsubstituted polymer film can be derived from a heterogeneous reaction on the electrode surface. The first example was reported by Schuhmann et al., it is based on nitration of electrogrown PPy film and covalent bending of glucose oxidase. Thus, after electrochemical polymerization of PPy in water and oxygen free 0.1 mol L^{-1} solution of pyrrole in acetonetrile containing 0.1 mol L^{-1} Tetrabutylammonium p-toluenesufonate (TBAPTS). The polymer was nitrated by keeping the modified electrode in a solution of 700 mg $Cu(NO_3)_2.3H_2O$ in 20 mL acetic anhydride for 5 min at 20° C in an Ar atmosphere. The nitro groups were then reduced electrochemically in CH_3CN/TBAPTS, cycling the potential between 500 and -250 mV vs. SCE. Carboxylic side chains of the GOX enzyme were activated with water soluble cabodiimide and the result was covalently linked to such in-situ synthesized poly(3-aminopyrrole) (Schuhmann et al., 1990).
A comparable possible covalent approach could be the binding of a monomer to the enzyme. There are no differences found in the oxidation potential of a simple pyrrole and that of a glucose oxidase-bound pyrrole. Consequently, subsequent polymerization of the protein-functionalized monomer with a non-functionalized gives film with controlled enzyme concentrations, and provides greater sensitivity; possibly arising from the greater porosity of the film. As an example pyrrole-modified glucose oxidase with pyrrole units can be obtained via amide bonds or secondary amines. A stable modified enzyme-pyrrole with an average of 30 pyrrole units was obtained thanks to the formation of amide bonds between caboiimide-derivated N-(2-caboxyethyl)pyrrole derivatives and lysyl residues at the surface of protein (Wolwacz et al., 1992). Similarly, N-(3-aminopropyl)pyrrole has been bound to carbodiimide-activated carboxylic residue of the enzyme (Yon-Hin et., al 1993). A

similarly obtained glucose-functionalized pyrrole monomer was also polymerized with bithiophene to achieve bio-polymers with higher redox potential compared to that of pyrrole; this assures film with more stability toward degradation effect of peroxide product of GOX (Hiller et al., 1996).

The electrochemical polymerization of monomers functionalized by easy leaving groups such as N-hydroxysuccinimide or N-hydroxyphthalimide has also been described as simple post-polymerization functionalization of CPs. The precursor copolymer of poly[(3-acetic acid pyrrole)/3-N-hydroxyphthalimide pyrrole] was electro-copolymerized at 0.9 V (vs. SCE) onto a platinum electrode in acetonitrile and an amino-substituted oligonucleotide was then grafted onto it by direct chemical substitution of the leaving group N-hydroxyphthalimide, in dimethylformamide containing 10% acetate buffer at pH 6.8 during 3 h incubation time (Korri-Youssoufi et al., 1997). The conversion of (5-bromopentyl)-substituted mono, bi and tri-thiophene, to carboxylic acid was accomplished in two steps; first with KCN, H_2O/EtOH and second in KOH, H_2O/MeOH. The carboxylic acid was further converted to the acid chloride by oxalyc chloride and directly reacted with N-hydroxysuccimide (NHS) and triethylamine to give the NHS-ester. A comparison of the electrochemical polymerization process of these thiophenes substituted with activated NHS-ester resulted in an oxidative potential more positive for monothiophene, compared to bi or tri-thiopene which both show similar potential (Bäurele et al., 1996).

Another means of obtaining functionalized CPs films is through electro-immobilization of conventional carboxylic or amine polymers with a CPs monomer. For example, electro-generation of polyaniline/poly(acrylic acid (PANI/PAA) films were polymerized on Boron Doped Diamond electrode (BDD) in 0.1 mol L^{-1} H_2SO_4/0.5 mol L^{-1} Na_2SO_4 solution containing 0.2 mol L^{-1} aniline monomer and 25 mg mL^{-1} poly(acrylic acid) (MW=2000) by potential cycling one time from -0.2 V to +1.2 V (vs. Ag/AgCl) at various scan rates. The carboxylic group density of this film was quantified using the Toluidine Blue O (TBO) method, which consisted of soaking the modified electrode in an aqueous solution of 0.5 10^{-3} mol L^{-1} TBO, adjusted to pH 10 with NaOH. The formation of ionic complexes between the carboxylic acid groups of the PANI/PAA film and cationic dye was allowed to proceed for 5 h at room temperature. After washing the electrode with the same alkaline water solution to remove uncomplexed TBO, the dye was desorbed by 50wt % acetic solution and the amount of carboxylic group was calculated from the optical density of the desorbed dye at 633 nm assuming that 1 mole of TBO had combined with 1 mole of carboxylic group of the PAA. In this study it was noted that such an increase in scan rate during electropolymerisation of PANI/PAA films had a largely negative effect on the carboxylic density of the film; therefore the optimum condition was found to be at a scan rate of 10 mV s^{-1} to attain ultrathin film with the highest carboxylic functionalities (Gu et al., 2005).

3.2.1.3 Affinity interaction with functionalized CPs films

The sensitivity of the biosensor was greatly dependent on the orientation of immobilized biological recognition elements. Chemical binding of a bio-molecule does not fulfill this criterion and therefore cannot ensure the biological activity preservation. However, affinity interaction between functionalized CPs films and bio-molecules, which induces regioselectives grafting was regarded as a convincing method to achieve high oriented bio-surface with required sensitivity.

Specific and high-affinity interaction between biotin and avidin (association constant K_a= 10^{15} mol L^{-1}) leads to strong associations similar to the formation of covalent bonding. This

high and specific linkage was largely valued for binding biological species to the surfaces. Using conducting polymers, this strategy involves the electropolymerization of conducting monomer-modified biotin (Cosnier et al., 1999) or electro-immobilization of streptavidin (Xiao et al., 2007), the resulting conducting polymer is subsequently made operational by avidin (for the first case) and finally by biotinylated bio-molecules. This strategy comes with some distinct advantages, such as availability of several biotinylated bio-molecules - especially antigen and antibody-, a surface modified with avidin provides a passivated interface that prevents further non-specific adsorption of proteins, and also the high accessibility of the resulting immobilized molecule.
Another strategy is based on the formation of transition metal complexes between histidine tagged proteins, where the surface is made functional by immobilized nitrilotriacetic or iminodiacetic (Haddour et al., 2005).

3.2.2 Use of CPs as transducer

In spite of providing several easy means of actually immobilizing bio-molecules, CPs are a good energy transducer thank to their redox activity and efficient protectors of the electrodes against interfering materials. CPs are able to transduce directly the electrochemical signal generated by some redox enzymes; sense DNA hybridization; and detect directly antibody-antigen interaction. They are also capable of making credible third generation, DNA, and immunological biosensors, respectively. The following examples illustrate some representative reports in these areas.
Glucose oxidase considered as a typical flavin enzyme with FAD/FADH as redox prosthetic group was largely studied to contrast the ability of using some material to be the base of third generation biosensor development. In this trial, the nano-structured or thin film of CPs polyaniline and poly(3,4-ethylenedioxythiophene), was seen to promote the direct electron transfer of immobilized GOX.
In their comparative study Thomson et al. show that contrary to GOX electro-immobilized with poly(3,4-ethylenedioxythiophene), the enzyme absorbed onto a vapor phase polymerized PEDOT shows strong peaks attributable to FAD redox activity with average potential at -0.505 mV vs. Ag/AgCl. Vapor deposition of PEDOT was achieved by simple introduction of gold electrode modified with a thin layer of 40% iron(III) para-toluene sulfonate in butanol and 2.3% pyridine in a sealed EDOT monomer chamber at 70 ^{o}C for 30 min (Thompson et al., 2010).
Zhao et al. optimized the synthesis of polyaniline nanofibers and relate its application to direct electron transfer of glucose oxidase. The synthesis of polyaniline nanofibers was carried out at an interface of toluene which contains 0.1 mol L^{-1} aniline and aqueous 1 mol L^{-1} sulfuric acid containing 0.05 mol L^{-1} of ammonium peroxydisulfate. The resulting two-phase system was left undisturbed at room temperature. After 30 s polyaniline appeared at the interface and initiated its migration into the aqueous phase. At this point the phase becomes dark-green and stops changing for 6 h, which was considered as sufficient reaction time. Finally the polyaniline nanofibers were collected by filtering and washing them with water and ethanol and subsequently vacuum-dried. For manufacture of the modified electrode the nano-fibres were suspended in aqueous solution, and then deposited onto the surface of a glassy carbon electrode. After the evaporation of the solvent, the electrode was covalently bio-modified with GOX using EDC-NHS mixture. Contrary to the electrode without GOX, the cyclic voltammograms of these manufactured biosensors showed redox

peaks with formal potential at -418 mV (vs. Ag/AgCl) and a catalytic response to glucose at -0.350 mV (Zhao et al., 2009). This was characteristic of direct electron transfer of FAD/FADH as a redox center in the enzyme. Similar values were obtained by Wang et al. when GOX was electro-immobilized onto the inner wall of highly ordered polyaniline nano-tubes, which were then synthesized using anodic aluminium oxide membrane as a template (Wang et al., 2009).

The use of a template to achieve nano-structured PEDOT was also explored by Arter et al. A nickel nano-trench was lithographically patterned to create devices with several hundred linear arrays on glass. This device was used as the working electrode in the three electrode cells for simultaneous electro-deposition of virus-M 13 and PEDOT after initiation by a pure PEDOT "primer" layer. The resulting device was used as a resistive biosensor for positive antibody (p-Ab). The electrochemical resistivity of the device increases with increasing concentration of p-Ab and a charge-gating was postulated as the mechanism of this increase after p-Ab/M 13 virus affinity binding (Arter et al., 2010).

Conductimetric immuno-biosensors were also reported by Mohammed-Tahir and Alocilja. Their approach can be expressed as 'sandwich' methodology: using a secondary antibody conjugated by polyaniline to achieve highly sensitive *E-coli* and *Salmonella* biosensors. In this manner, the primary antibody was immobilized by a simple glutraldehyde method, the antigen was incubated with this functional surface and a secondary antibody was used to close the electrical circuit between two lateral inert electrodes (Mohammed-Tahir & Alocilja, 2003)

Label-free affinity biosensors using CPs as a transducer were characterized by Sadik et al. The modification of polypyrrole with anti-human serum albumin (anti-HSA) was achieved by simple-step electrochemical immobilization, and the interaction of antigen (HSA) and antibody-PPy electrode was monitored using cyclic voltammetry and impedance spectroscopy at different potentials, corresponding to reduced, doped and overoxidized states of PPy. Impedance measurement showed that large values of double layer capacitance were observed at higher positive potentials with a transition from kinetic control to diffusion control (Sargent et al., 1999). A similar result was observed when anti-bovine serum albumin was electro-immobilized with PPy film and detectable antibody-antigen interaction was measured by the control of the Faradaic behavior of the electrode (Grant et al., 2005).

Change of the doping/undoping redox reaction of CPs to control hybridization of DNA was reported by Mohan et al. Accordingly, chemical immobilization of ssDNS onto electrochemically synthesized poly 5-carboxilic indol, to achieve sensitive biosensor for breast cancer was reported by the plot of change in charge resistance or capacitance with log concentration of target DNA (Mohan et al., 2010).

4. Conclusion and perspective

In this chapter we have summarized the advantageous use of conducting polymers for electrochemical sensing and bio-sensing. These advantages lie predominantly in surface modification controls and electrochemical redox activity which make these materials an useful transducer. Some examples of nano-structured CPs and their use in promoting direct electron transfer, as well as third generation biosensor, were also outlined. The points covered could well be the future focal point of this topic; as well as that of the characterization of signal transduction in label-free bio-affinity biosensors based conducting polymers.

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