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Chemically Modified Electrodes in Biosensing

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Abstract

The development of chemical sensors has received a great deal of scientific interest in the last decades. Not only the chemical industry may benefit from these sensors but also the food industry, bio-industry, medicine, environmental control because of their capability to give continuously and reversibly a selective and fast response to the presence of a specific compound in a complex mixture of components, without perturbing the system. Biosensors combine the power of analytical detection techniques with the specificity of biological recognition system and therefore they are the most promising devices today about this selectivity. Furthermore, biosensors possess many unique features such as compact size, simplicity of use, one-step reagentless analysis, absence of radioactivity, etc., that make them very attractive alternatives to conventional bioanalytical techniques. The present short review highlights some modern aspects of Chemically Modified Electrodes (CMEs) based on redox enzymes used in amperometric biosensing, a detection method which has already found a large number of applications in health care, food industry and environmental analysis. Some relevant applications of amperometric biosensors based on CMEs to real sample analysis are also presented and some possible future trends highlighted.

Keywords: Electrodes; Sensors; Electrochemical methods

Introduction

Electrochemical methods traditionally have found important applications in sample analysis, and organic and inorganic synthesis. The electrode surface itself can be a powerful tool. By controlling the potential, the electrode can be used as a variable free energy source (or sink) of electrons. In addition, electrons crossing the electrode-solution interface can be determined with great sensitivity by measuring current [1].

Chemical sensors consist of a transduction element covered with a biological or chemical recognition layer. The remarkable specificity of biological recognition processes has led to the development of highly selective biosensing devices. Electrochemical sensors, the most rapidly growing class of chemical sensors, hold a leading position among the systems presently available and have already proven themselves in use. Electrochemical sensors are inherently sensitive and selective towards electroactive species, fast and accurate, compact, and portable. Due to its simplicity, electrochemical transduction constitutes a successful route to create low cost biosensors when coupled with enzymes [2].

In biosensing the measurement of electrical properties for extracting information from biological systems is normally electrochemical in nature, whereby a bioelectrochemical component serves as the main transduction element. Although biosensing devices employ a variety of recognition elements, electrochemical detection techniques use predominantly enzymes. This is mostly due to their specific binding capabilities and biocatalytic activity [3]. Typically, the reaction under investigation would either generate a measurable current (amperometric), a measurable potential or charge accumulation (potentiometric) or measurably alter conductive properties of a medium between electrodes (conductometric) [4]. References are also made to other types of electrochemical detection techniques, such as impedimetric, which measures impedance (both resistance and reactance) [5], and field-effect, which uses transistor technology to measure current as a result of a potentiometric effect at a gate electrode [6].

The biosensor can usually be considered a subset of chemical sensors because the transduction methods, sometimes referred to as the sensor platforms, are the same as those for chemical sensors. Biosensors are special chemical sensors in which the recognition system utilizes a biochemical mechanism. The biological recognition system, usually a receptor protein, antibody, enzyme, translates the information from the biochemical domain into a chemical or physical output signal, with a defined sensitivity. Due to the intrinsic, highly selective properties of the biomolecular species in comparison to the inorganic catalysts, enzymes based biosensors are the most selective one.

Biosensors are usually classified into various basic groups, according to the signal transduction and to the biorecognition principles. The transducer is component of biosensor, which has important role in the signal detection process. On the basis of the transducing element, biosensors can be categorized as electrochemical, optical, piezoelectric, and thermal sensors. Depending upon the nature of electrochemical changes in detection during a biorecognition event, electrochemical biosensors fall into one of four categories: amperometric, potentiometric, impedimetric and conductometric; field effect transistors may be also used as transduction devices [6].

Amperometric sensors are based on the detection of electroactive species involved in the chemical or biological recognition process. In the amperometric detection, the current signal is generated as a function of the reduction or oxidation of an electro-active product on the surface of a working electrode. The applied potential between the working electrode and the reference electrode serves as the driving force for the electron transfer reaction of the electroactive species. As certain molecules are oxidized or reduced (redox reactions) at the working electrode (eg. Pt, Au, carbon, etc.), electrons are transferred from the analyte to the working electrode or to the analyte from the electrode. The direction of flow of electrons depends upon the properties of the analyte which can be controlled by the electric potential applied to the working electrode. If the working electrode is driven to a positive potential an oxidation reaction occurs, and the current flow depends on the concentration of the electroactive species (analyte) diffusing to the surface of the working electrode. Similarly, if the working electrode is driven to a negative potential then a reduction reaction occurs. The resulting current is a direct measure of the rate of the electron transfer reaction. It is thus reflecting the rate of the

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recognition event, and is proportional to the concentration of the target analyte [7]. Amperometric sensors utilize also for their chemical reaction mediators, i.e. molecules which are able to transfer electrons. They can participate in the redox reaction with the target analyte and help in the faster electron transfer. As a result it is possible to work with low potentials, thus the influence of different interferants on response decrease.

In potentiometric sensors, the analytical information is obtained by converting the recognition process into a potential signal, which is proportional (in a logarithmic fashion) to the concentration (activity) of species generated or consumed in the recognition event. Potentiometric devices measure the accumulation of a charge potential at the working electrode compared to the reference electrode in an electrochemical cell when zero or no significant current flows between them [3]. The reference electrode is required to provide a constant half-cell potential. The indicator electrode develops a variable potential depending on the activity or concentration of a specific analyte in solution. Potentiometry provides information about the ion activity in an electrochemical reaction [8]. For potentiometric measurements, the relationship between the concentration and the potential is governed by the Nernst equation [9]. The transducer employed in the potentiometric technique is usually a gas-sensing electrode or an ion-selective electrode. Potentiometric sensors are very attractive for field operations because of their high selectivity, simplicity and low cost. They are, however, less sensitive and often slower than their amperometric counterparts.

In conductometry the analytical information is obtained by measuring of electrolyte conductivity, which varies with the changes in the ionic species concentration. In other words, conductometric based transduction provides information about the ability of an electrolyte solutions to conduct an electric current between electrodes. Most reactions involve a change in the ionic species concentration and this can lead to a change in the solution electrical conductivity or current flow [10]. Normally an AC (alternating current) voltage is applied across the electrodes, which causes a current flow to be sustained between two metal electrodes (usually platinum or silver) which are separated by certain distance. When ions or electrons are produced during the course of a (bio) chemical reaction, the ionic composition changes, and the overall conductivity or resistivity of the solution is changing. Electrical conductance/resistance of the solution represents the parameter which is measured when using this transducer; an Ohmmeter (or multimeter) is used to measure the change in conductance between the metal electrodes. The conductivity is a linear function of the ion concentration; therefore, it can be used for sensor applications. However, it is nonspecific for a given ion type. Unfortunately, one of the major issues with this technique is that the sensitivity is generally inferior compared to other electrochemical methods [10].

Electrochemical Impedance Spectroscopy (EIS) is a sensitive indicator of a wide variety of chemical and physical properties. EIS based transduction method is not a typically used electrochemical detection method; however this technique has only recently become popular tools for bioreceptor transduction [7,11-13]. Through the application of a small sinusoidally varying potential (between 5-10 mV), one measures the resulting current response which depends on various processes [13]. By varying the excitation frequency of the applied potential over a range of frequencies (between 10 kHz and 10 MHz), one can calculate the complex impedance, sum of the real and imaginary impedance components, of the system as a function of the frequency (i.e. angular frequency) [14]. The electrical impedance resulting from the sample is calculated as the ratio of voltage over current. The resulting electrical impedance measurement has both a magnitude and a phase, a complex number. For any time-varying voltage applied, the resulting current can be in phase with the applied voltage (resistive behavior), or out of phase with it (capacitive behavior). Typically, a conventional three-electrode system (i.e., counter, reference and the working electrode) is used to monitor the current variations, noting that a potentiostat/galvanostat and a frequency response analyzer are used in the detection process. EIS is a useful tool in the development and analysis of materials for biosensor transduction. The EIS type measurement is suitable for real time monitoring since it is able to provide a label free or reagentless detection [15].

The purpose of a biosensor is to provide rapid, real-time, accurate and reliable information about the analyte of interest. Ideally, it is a device that is capable of responding continuously, reversibly, and does not perturb the sample. Biosensors have been envisioned to play a significant analytical role in medicine, agriculture, food safety, homeland security, bioprocessing, environmental and industrial monitoring [16].

Given the impressive progress in the electrochemical sensors and biosensors area, and their growing impact on analytical chemistry, it would be impossible in the context of this review to mention all the advances in research. Other review articles on electrochemical sensors and biosensors are found in the literature [17-28].

his short review article describes different CMEs electrode modifications with a brief introduction given to explain several phenomena occurring at chemically modified electrodes and the mechanism of detection, followed by some interesting applications of Chemically Modified Electrodes (CMEs) in biosensing of some important biomolecules. The concept of bio-recognition, which is at the heart of biosensor technology, is also described. Some relevant applications of amperometric biosensors based on CMEs to real sample analysis are also reviewed and some possible future trends highlighted

Chemically Modified Electrodes (CMEs)

Some facts reduce the applicability of the electrodes in analysis. First many compounds that are important biologically and environmentally show no response within a potential window at solid electrodes or necessitate an overpotential. Direct electrochemical detection (EC) usually requires high potential for such compounds. This can produce large background current, resulting in inferior detection limits. Also, passivation and/or deactivation of the electrode surface, due to the adsorption of macromolecules (e.g. proteins and surfactants) or of reaction products, greatly affect the stability of electrode response. More, coexisting components, which may be present in concentrations much larger than the analytes, may severely interfere with the determination of trace analytes. Complicated sample pretreatments are often employed to eliminate or separate interfering components [17].

The phenomena mentioned above often can be controlled by manipulating the chemical nature of the electrode surface and a promising route for overcome this problems is based on tailoring of the electrode surface – the application of chemically modified electrodes – for improving quantitative measurements. CMEs have attracted considerable interest over the past decades as researchers attempted to exert more direct control over the chemical nature of an electrode surface. By deliberately attaching chemical reagents to it, one hoped that the electrode surface would take on the chemical

properties of the attached reagents. If the proper reagents were chosen, desirable properties such as reagent-based control of the rates and selectivities of electrochemical reactions (i.e. electrocatalysis), freedom from adsorptive and coating effects, and special optic or excited state features might be obtained [17]. Thus, either the physical properties of the electrode material are adapted for specific uses or functional groups are immobilized on the surface to improve the performance of the electrode. [1,27].

What we define as CME is a deliberate control of the molecular structure at the surface, aimed to tailor the electrode to meet specific applications [28]. This appealing concept of rational molecular design of electrode surfaces has enjoyed considerable success and stimulated much research.

These electrodes, which are made by incorporating specific chemical groupings or "microstructures" on conventional electrode surfaces, are of interest because their responses have two completely separate components: the usual electrochemical component determined by the potential at which the electrode is maintained instrumentally and an additional chemical component determined by the reactivity of the attached group. Consequently, CMEs offer not only easily variable redox characteristics but also the possibility of adjustable physical and chemical properties (such as charge, polarity, chirality, permeability) [17]. The ability to manipulate the molecular architecture of the bulk matrix of an electrode and its surface in particular has led to a wide range of analytical applications of CMEs and created powerful opportunities for electroanalysis.

The terminology, definitions, preparation methods and analytical aspects of CMEs have been described in some IUPAC reports [29-31].

Electrocatalysis by bound mediators

An important motivation for modifying electrode surface is electrocatalysis of the electrode reaction of an analytically desired substrate, being one of the most important topics of research on CMEs. Chemically modified electrodes employing immobilized redox mediators can facilitate the electron transfer of such analytes [32].

Almost all catalytic CMEs have relied on the immobilization of redox center on the electrode surface. The immobilized redox center acts as a fast electron transfer mediator for substrate species, which is oxidized or reduced slowly (or not at all) at the naked electrode. The basic principle involved in CMEs electrocatalysis by a surface confined electron-transfer mediator is illustrated in Figure 1, for a generalized oxidation process. In this sequence, the analyte diffuses from the bulk solution to the electrode surface, where it is oxidized in a purely chemical reaction with the oxidized form of the mediator (M_{Ox}). The potential of the electrode is maintained at a value sufficiently positive for M_{Ox} to be stable state of the mediator and its reduced form (M_{Red}) to be rapidly re-oxidized to the catalytically active form. Thus the heterogeneous electron transfer takes place between electrode and mediator and not directly between the electrode and analyte. In essence, then, the mediator can be considered to function simply as an electron shuttle between the electrode and the analyte.

There are three important characteristics of mediated electrocatalysis. With an electrocatalytic CME, the oxidation (or reduction) of the analyte is made to take place at the redox potential of the mediator catalyst couple unless a catalyst-substrate adduct is formed, in which case reaction occurs at the potential for the adduct. Second, the mediator catalyst and substrate formal potentials should be similar. Finally, a successfully catalyzed reaction of S occurs at less

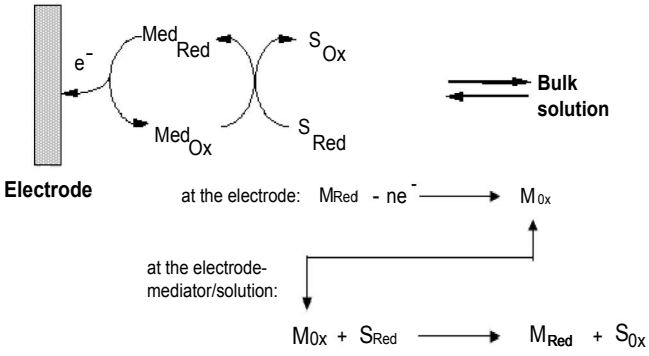

Figure 1: The model of electrocatalytic reaction on CME, where SOx/SRed and MOx/MRed are the oxidized and reduced forms of analyte to be detected and of mediator respectively.

negative or positive potential for reduction or oxidation, respectively, than the naked electrode reaction of S would require.

Bioelectroanalytical studies based on CMEs have spread in several directions. One of them is concerning with the utilization of CMEs in bioelectrochemical studies for a better understanding of the electron transfer reactions in biological systems which implies redox enzyme and protein carriers and proceed at very high rates. By using CMEs it was possible to study biochemical compounds which show very slow heterogeneous electron transfer at conventional electrodes and consequently exhibit irreversible electrochemical behavior. It was demonstrated that CMEs are a viable alternative to homogenous electron transfer mediator in solution and also that they can be used for quantitative determination of proteins and enzymes. Another area of interest for electroanalytical studies based on CMEs is the construction and characterization of immobilized enzyme chemically modified electrodes (IECMEs), which combine the specificity, selectivity and catalytic power of an enzyme for its natural substrate with the advantages of electrochemical detection. So, in addition to the basic characterization of CMEs, such surfaces have been used widely in electroanalysis and of the disciplines directly affected by these developments is analytical chemistry [32]. All the important analytical properties of electrodes – sensitivity, selectivity, reproducibility and even applicability – have been shown to be capable of enhancement by the judicious use of chemical modification and all of this could offer many practical applications.

Chemically modified electrodes based on enzymes

The simplification of an analytical methodology to a level where practical, fast, routine measurement of a test analyte becomes possible, preferably with no sample pretreatment and a minimum demand upon operator skills, is still a great challenge for the modern analytical chemistry. To this purpose highly selective analytical methods based, e.g. on the use of selective reagents need to be developed.

Extraordinarily selective and versatile reagents are provided by Mother Nature in the form of e.g. enzymes, antibodies, receptors, etc., which can be integrated within a physico-chemical transducer to produce an analytical device i.e. a biosensor (Figure 2). In a simple approach the biosensor is a detector which converts biochemical signal in measurable ones and capable of providing either qualitative or quantitative results.

An electrochemical biosensor is considered a chemically modified

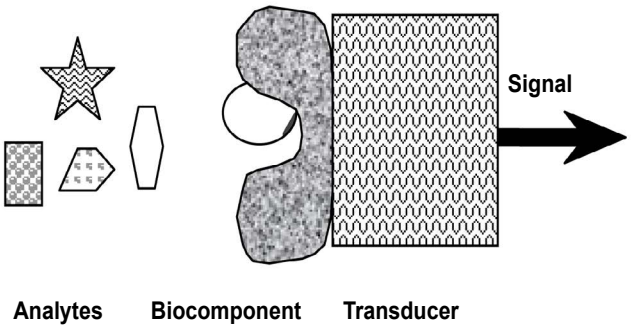

Figure 2: Schematic representation of a biosensor.

electrode [29] since electronic conducting, semi-conducting or ionic conducting material is coated with a biochemical film [33]. A typical biosensor construct has three features – a recognition element, a signal transducing structure and an amplification/processing element. As the electrochemical biosensor is a self-contained integrated device the biochemical receptor should be retained in direct spatial contact with an electrochemical transduction element. Biosensors usually yield a digital electronic signal, which is proportional to the concentration of a specific analyte or group of analytes [34]. Although in theory many different types of biosensors can be constructed, by far the most frequently used type is the enzymatic amperometric biosensor, which uses amperometric detection to allow exploitation of oxidoreductase enzymes.

In principle, an enzymatic amperometric biosensor is a device which consists of an electrochemical sensor in contact with a layer of immobilized enzyme. Enzymatic amperometric biosensors are based on the measurements of the current resulting from the oxidation or reduction of an electroactive species, by keeping a constant potential at the working electrode with respect to a reference electrode. The resulting current is correlated to the bulk concentration of the electroactive substance or its reaction within the adjacent bio-catalytic layer. The function of enzymatic amperometric biosensors is related to Electron-Transfer (ET) processes between the active site of an (immobilized) enzyme and an electrode surface which is poised to an appropriate working potential. In most employed enzymatic amperometric biosensors, the enzyme does not communicate directly with the electrode. Instead, a conventional electrode such as oxygen, Pt, Au, carbon, etc., is situated in close proximity to the enzyme and detects the substrate or products of enzymatic reactions.

Enzyme-based biosensors most frequently use an enzyme of the class of oxidoreductases. In particular, oxygen-dependent oxidases are applied, but NAD^+-dependent dehydrogenases, PQQ-dependent dehydrogenases, peroxidases and multi-cofactor enzymes are also used. The substrate of interest is oxidized by these enzymes and in turn the prosthetic group of the enzyme (e.g. FAD, NAD^+, FMN, PQQ, heme, transition metals) is reduced. Next, the prosthetic group needs to be re-oxidized in order to regenerate the enzyme and to prepare it for further substrate recognition and conversion reactions [33].

The biosensor has to participate in this process in order to mediate a fast electron transfer between enzyme and the electrode surface. However, often the redox enzymes are designed by nature to protect the integrated cofactor against unwanted redox processes. This implies that an efficient regeneration of the active site of the enzyme is of key importance and that a proper functioning of the biosensor depends largely on the kinetics of this ET process [35,36]. This ET process is influenced to a great extent by the specific properties of the used enzyme, e.g. the physical chemical properties of the prosthetic group, the accessibility and distance of the prosthetic group from the protein surface, the nature of the redox cofactor, the intrinsic protein stability and the possibility of immobilizing the enzyme to the electrode surface.

Since efficient coupling of the enzyme and sensor is critical to the overall performance of the biosensor, it will be desirable to employ an enzyme that could transfer electrons directly or by means of a mediator to an electrode [37]. The easiest ET mechanism would be the electrochemical recycling of the enzyme's prosthetic group directly at the electrode surface involving an electron tunneling mechanism (referred to as "third generation type of biosensors" which is not discussed here).

Normally, redox enzymes do not exchange electrons with simple metal electrodes due to the fact that the active site is located deep inside an insulating protein shell and, even in the closest approach configuration, the distance between the redox centre and the electrode surface is still too large to permit an efficient electron transfer [35,36]. This implies that the ET transfer between the enzyme's prosthetic group and the electrode surface has to be arranged otherwise, which has resulted in the development of many different types of biosensors. Problems and specific features of architectures for amperometric biosensors using different electron-transfer pathways such as mediated electron transfer, electron-hopping in redox polymers, electron transfer using mediator-modified enzymes and carbon-paste electrodes, direct electron transfer by means of self-assembled monolayers or via conducting-polymer chains are discussed in literature [35].

Actually, amperometric enzyme based biosensors may be classified into 3 generations; it is possible to distinguish amperometric biosensors of first, second and third generations, according to the different mechanism of the electronic transport [37,38]. Next, a brief summary of the first two generations of biosensors will be given.

First generation of amperometric biosensors

The first generation of amperometric biosensors works by means of the direct detection, at a suitable electrochemical transducer, of electroactive species that are enzymatically produced or consumed. Oxidase enzymes incorporated into amperometric biosensing devices use molecular oxygen as the "natural" electron acceptor; the prosthetic group of the enzyme is recycled by freely diffusing oxygen (O_2), and either the decrease of O_2 or the increase of the reaction product, i.e. hydrogen peroxide (H_2O_2), can be amperometrically monitored.

The first biosensors [39,40] belong to this class; Glucose Oxidase (GOD) was immobilized on top of a Clark oxygen electrode (near the electrode surface). The biosensor was proposed to oxidize glucose to gluconic acid using an electrode to amperometrically detect the consumption of oxygen, since its consumption is proportional to glucose concentration. Glucose oxidation actually uses a prosthetic group (flavin adenine dinucleotide - FAD) to transfer the electrons from the substrate. After substrate oxidation, $FADH_2$ returns to FAD in the presence of oxygen; the consumption of O_2 was detected by monitoring changes in the reduction of O_2. The traditional glucose sensor, can detect also the hydrogen peroxide produced in the oxidation process catalyzed by a glucose oxidase enzyme. During the catalytic cycle the enzyme is first reduced and then regenerated by oxidation with the molecular oxygen in the sample solution:

$$\text{Glucose} + \text{GOD-FAD} \rightarrow \text{Gluconolactone} + \text{GOD-FADH}_2$$

$$\text{GOD-FADH}_2 + O_2 \rightarrow \text{GOD-FAD} + H_2O_2$$

Although the architecture of the first generation type of biosensor is relatively simple, it does have certain disadvantages. The most important drawbacks of this sensor related to the fact that the response can become limited by the oxygen concentration (in particular when substrates are monitored at high concentrations, for example glucose or lactate), the high reduction or oxidation applied potential required to respectively reduce O_2 or oxidize H_2O_2 which implies that several electroactive species can be also oxidized, and the denaturation of some enzymes (e.g. GOD) by H_2O_2 at high concentration.

Second generation of amperometric biosensors

To overcome or at least to minimize these drawbacks, in case of second-generation of biosensors several attempts have been made [41] to replace oxygen with diffusing artificial mediators; in this case the mediator (M_{ox} - electron acceptor) is reduced to M_{red} by the enzymatic cofactor and then oxidized back to M_{ox} when the mediator is in contact with an electrode polarized at the appropriate potential (Figure 3). Replacement of oxygen with mediators is not a simple task since the artificial mediator should compete efficiently with O_2, should display stable oxidized and reduced forms, should exhibit fast reaction rate with both the enzyme and the electrode surface, and the possibility of a tight attachment into the architecture of the biosensor, should be nontoxic, and finally should display a redox potential sufficiently removed from that of other electroactive species to avoid interferences. Both mediator and enzymatic substrate must be in the analytical solution.

In the simplest configuration, artificial mediators -mainly soluble low-molecular-weight metal complexes with reversible electron transfer properties (e.g. ferrocene derivatives, $K_4[Fe(CN)_6]$, ruthenium or Os-complexes) are added to the sample and used as freely-diffusing ET shuttles. Some other materials are also used, e.g. quinones, Tetrathialfulvalene (TTF), tetracyanoquinodimethane (TCNQ), conducting salts (e.g. TTF-TCNQ, N-methylphenazin (NMP)-TCNQ, etc) and organic dyes (e.g. methylene blue, prussian blue, phenazines, methyl violet, Alizarin yellow, thionin, toluidine blue, azure A and C, etc.) [35]. Apart from the use of soluble mediators other approaches have been attempted: co-entrapment of mediator and enzyme [42,43].

In the case of e.g. flavin-oxidases or PQQ-dehydrogenases, a mediator is required to shuttle electrons between the electrode surface and the enzyme redox couple. In this process the mediator is cycled between its oxidized and reduced state producing the analytical signal (current) while the enzyme reacts with the substrate giving the product. Nicotinamide adenine dinucleotide (NAD) dependent dehydrogenases behave differently from other enzymes since during the catalytic cycle it is the coenzyme, not the enzyme itself, which is reduced (or oxidized), according to a specific mechanism [41]. Reoxidation of the reduced form of the coenzyme (that provides the analytical signal) requires, at a clean Pt or Au electrode surface, potential values as high as + 1.0 V vs. Ag/AgCl. Modified electrodes or efficient mediators are required to lower the working potential [41]. The mediator shuttles the electrons involved in the redox process from the active center of the enzyme to the electrode or vice versa [44-53].

Practical Applications

The primary purpose behind modifying an electrode is to improve its analytical performance either by, increasing its sensitivity and selectivity or by protecting the surface from unwanted reactions.

Pesticides

Pesticides (herbicides, fungicides, insecticides) are widely used throughout the world, and millions of tons are used each year in agriculture, medicine, industry and related activities [54]. Because similar compounds have been produced as possible nerve poisons, a further area of application is in the military [55]. Many of them are highly toxic, and their accumulation in living organisms can be cause of serious diseases. Even if they present a low persistence, their acute toxicity creates a need for fast responding detection systems in order to protect human health during manufacturing and application processes and subsequently sensitive systems for reliable control of food products and environment pollution [56].

The mode of action of these pesticides is based on irreversible inhibition (non-competitive) of acetylcholinesterase [57], and the same principle is utilized for analysis. Free [58] or immobilized [59] enzyme can be used. With acetyl- or butyryl-choline as the substrate, bienzyme electrodes coupling a cholinesterase (acetyl- or butyryl-cholinesterase) with choline oxidase (ChOx) have been reported, with measurement based on the detection of either oxygen [60,61] or hydrogen peroxide[62,63], this latter being more sensitive.

The design and optimization of biosensor towards pesticides can be simplified using mono-enzymatic systems based on the hydrolysis

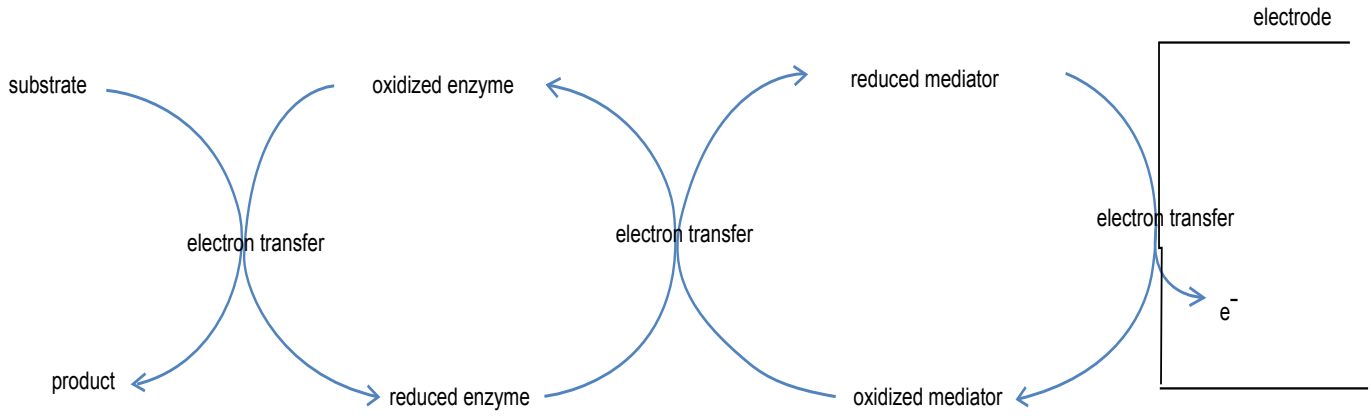

Figure 3: Mediated electron transfer path between a redox enzyme and an electrode.

of acetyl- or butyryl-thiocholine by a selected cholinesterase. In this case, detection is based on the oxidation of thiocholine produced at a platinum electrode. Using such an approach, the applied potential is lower than that used for the previously described oxidation of hydrogen peroxide [64].

By using different electronic mediators such as cobalt phthalocyanine [55,56,65-67], tetracyano p-quinodimethane, tetrathiafulvalene and 1,1'-dimethyl- ferrocene [68-70] it was possible to decrease the applied potential. The interference due to oxidizable substances possibly present in real samples was dramatically decreased in this way. The FePC chemically modified carbon paste electrodes exhibited an electrocatalytic response for the reduction of peroxides [71]. In our work [72], we chose to examine acetylcholine/choline system at carbon paste ferrophthalocyanine (FePC) chemically modified electrodes by using an acetylcholinesterase (AChE)/choline oxidase (ChOx) bienzymatic system, and subsequently to evaluate the suitability of these electrodes for pesticide detection. We developed a sensitive and effective bienzymatic sensor for pesticide detection based on the following reactions sequence:

$$H_3C\text{-}COO\text{-}(CH_2)_2N^+(CH_3)_3 + H_2O \xrightarrow{AChE} HO\text{-}(CH_2)_2\text{-}N^+(CH_3)_3 + CH_3\text{-}COOH$$

Acetylcholine (AChO) *Choline (ChO)*

$$(CH_3)_3N^+\text{-}(CH_2)_2\text{-}OH + 2O_2 + H_2O \xrightarrow{ChOx} (CH_3)_3N^+\text{-}CH_2\text{-}COOH + 2H_2O_2$$

Betaine

$$H_2O_2 \xrightarrow[350\ mV\ vs.\ Ag/AgCl]{Cathodic\ reduction} \text{peroxide reduction product}$$

When a kinetically controlled bienzyme sensor with a low activity of acetylcholinesterase is used, a diminished sensitivity is obtained for acetylcholine with an increased sensitivity towards inhibitors such as pesticides. Inhibition of AChE, proportional to the pesticide concentration, was measured as a decrease of the formation rate of hydrogen peroxide, which was correlated with the corresponding decrease in the chatodic current generated by the biosensor. In this paper the results obtained with co-immobilized enzymes are presented. By this approach it was possible to detect down to 10^{-10} M paraoxon and carbofuran.

DNA

The detection of DNA has a particular interest in genetics, pathology, criminology, pharmacogenetics, food safety and many other fields [73]. Studies on the electrochemical behavior of DNA and its bases in aqueous solution have been reported previously at different electrodes [73-77]. These nucleic acid bases show well-defined oxidation peaks at +0.9 and +1.2 V vs. Ag/AgCl, respectively [77]. Guanine and adenine are the only nucleic acid bases which can be oxidized at carbon paste electrodes [78]. Wang and coworkers [79] described a CNT modified glassy carbon electrode, which improved detection of DNA hybridization event and greatly enhanced the guanine oxidation signal. Previous reports on the solution electrochemistry of uncatalyzed guanine oxidation have shown that the electron transfer is slow at most electrode surfaces [80-82]. Electrodes possessing specific chemical functionalities intentionally linked to their surface have been demonstrated to possess distinct advantages over conventional electrode substrates; in principle, such Chemically Modified Electrodes (CMEs) should be able to provide enhanced performance in the area of electroanalysis. Ruthenium complex, $[Ru(bpy)_3]^{2+}$, was used as catalyst for guanine oxidation [75,76,80-83]. The electrochemical behavior of guanine at cobalt(II) phthalocyanine carbon paste electrode (CoPc-CPE) was studied by Abbaspour and coworkers, who observed the oxidation potential of guanine at about +0.9 V vs. Ag/AgCl in acetate buffer medium [84]. Zhu and coworkers [85] developed a novel label-free hybridization assay based on poly (amidoamine) dendrimer/MWCNT-CoPc/glassy carbon electrode for the electrochemical detection of DNA sequence related to Avian Influenza Virus genotype. The modified electrode presents excellent amplification of the guanine oxidation response. Screen printed carbon electrodes modified with CoPc were also used for guanine detection, whose oxidation signal was enhanced with ca. 15% [86]. We develop a novel assay for the electrochemical detection of guanine based on carbon nanotubes paste electrodes (CNTPEs) modified with cobalt phthalocyanine (CoPc) [87]. The results indicated that the modification of a CNTPE with this compound results in amplification of the guanine oxidation response in contrast to that on the unmodified CNTPE. The electrochemical behavior of the modified electrode and the mechanism of the oxidation of guanine were investigated using Cyclic Voltammetry (CV) and differential pulse voltammetry (DPV). The methods parameters were optimized. A detection limit of $1.3.10^{-7}$mol.L^{-1} was obtained for guanine using the electrocatalytic oxidation signal corresponding to the Co(II)/Co(III) redox process. This modified electrode was further applied for determination of single-stranded DNA by differential pulse voltammetry with a detection limit of $9.86.10^{-8}$mol.L^{-1}. The advantages of convenient fabrication, low-cost detection, short analysis time and combination with nanotechnology for increasing the sensitivity make the modified electrodes worthy of special emphasis in the nonlabeled detection of DNA hybridization reaction and in the development of DNA based biosensors for toxic chemicals, toxins and pathogens determination.

With respect to potentialities of Deoxyribonucleic Acid (DNA) to recognize some ranges of analytes, the DNA-based electrochemical probes were reviewed [88-90] and compared with enzymatic and immunochemical biosensors [91]. Considerable efforts have been made at the electrochemical detection of a sequence-specific DNA hybridization process. Formation of the DNA duplex from an immobilized single stranded (ss) DNA probe and a complementary target single strand can be detected using electroactive DNA intercalators and minor-groove binding indicators [92]. Short DNA sequences related to human HIV [93], Mycobacterium tuberculosis DNA [94], E. coli DNA [95], and Hepatitis B virus [96] were determined. Nevertheless, known redox indicators do not satisfactorily fulfill requirements for high sensitivity and specificity [90].

Labuda and coworkers [97] applied DNA surface and bulk-phase modified carbon paste electrodes to the accumulation/voltammetric determination of electrochemically active phenothiazine and azepine type drugs [98] as well as catechin and acridine derivatives [99].

Biotechnology related to DNA immobilization on electroactive surfaces was used for the determination of a wide range of biomolecules such as norepinephrine [100], dopamine, uric acid [101], cytochrome *C* [102], adenine, guanine and thymine [103]. In a recent paper [104], the influence of double-stranded DNA (dsDNA) physical immobilization on the electrocatalytic behaviour of different carbon matrices was studied using three neurotransmitters: dopamine, epinephrine and norepinephrine as model analytes. The carbon matrices selected

for the study were: fullerenes C_{60}, Multi-Walled Carbon Nanotubes (MWCNTs), and diamond paste. Neurotransmitters: dopamine, epinephrine, norepinephrine were used as model analytes. The study proved that the arrangement of atoms in the matrix – Bucky balls, multi-walled carbon nanotubes, and diamond is having a high influence on the behavior of the modified dsDNA biosensors. The response characteristics, selectivity and recoveries of model analytes were compared between unmodified and modified matrices. As a result, for C_{60} and carbon nanotubes matrices there were improvements only when dopamine was assayed; no significant improvement was recorded for the assay of epinephrine, and a decrease in sensitivity was recorded for the assay of norepinephrine. While no response for the assay of domanine, epinephrine and norepinephrine was recorded with diamond paste based sensor, modification with dsDNA of diamond paste made possible the assay of epinephrine and norepinephrine at very low concentration, and decreased limits of quantification and detection for the same analytes, although the sensitivity of this biosensor was lower than 100 nA/nmol/L (value recorded for e.g., MWCNT and dsDNA based biosensor). In terms of selectivity, utilization of dsDNA has not improved the performance of the sensors based on plane pastes. This behavior was also reflected in the recovery tests (when the interference occur), when one can make a selection of the applications of the sensors for pharmaceutical or clinical field. These studies helped to understand the importance of correlating the arrangement of carbon atoms in the molecules used as matrices with the behavior of the sensors, and also that addition of molecules such as dsDNA will not always improve the selectivity and sensitivity of the sensors. Although the DNA has a strong affinity towards CNTs and fullerenes, not the same effect can be recorded for diamond material.

Catecolamines

Monoamines, Dopamine (DA), Norepinephrine (NE), serotonin (5-hydroxytryptamine) and their oxidative metabolites are involved in propagation, or modulation of the propagation of neural information in the neural system of animals. Low levels of DA have been found in caudate of patients with Parkinson's disease [105]. Unusual levels of monoamines have been reported in patients suffering from other diseases such as schizophrenia, HIV infection etc [106]. Serotonin (5-HT) is an important catecholamine neurotransmitter in biological systems which regulates mood and sleep and is a major target for pharmaceutical treatments of depression [107].

Both DA and 5-HT are readily oxidized, hence, electrochemical techniques have been explored for their analysis [108-112]. In order to increase the rate of electrode reaction, that is to enhance the voltammetric signal, working electrodes modified with electrocatalytic layers have been employed. Among them catalytic layers formed by electric pretreatments [113], or by addition of mediator species like Prussian Blue [114], different polymers [115] etc, are the most often selected ones. Simultaneous determination of 5-HT and DA is important, since both occur together in biological systems. There are reports on the simultaneous detection of DA and 5-HT on graphite electrodes reinforced by carbon [116] and on iron tetrasulfophthalocyanine ($[Fe^{II}TSPc]^{4-}$) modified carbon paste electrode [117].

Voltammetric response of DA suffers from the interference of Ascorbic Acid (AA) which exists in vivo as anions in high concentrations and possesses an oxidation potential very close to that of DA on unmodified carbon electrodes and in weak alkaline media. In order to increase selectivity perm selective membranes with ion exchange character like Nafion [118-120] were applied in DA determination, which is known to repel AA at physiological pH. However, the negatively charged polymers suffer from drawbacks such as non-uniform thickness and poor reproducibility.

Metallophthalocyanines are a possible choice for preparing voltammetric modified sensors due to their catalytic activity for a wide range of redox processes [121,122]. The sensitivity and the selectivity of the (bio)sensors can be greatly improved as a result of the electrocatalysis by metallophthalocyanines [123,124]. For example, Oni and Nyokong [117] studied the electrocatalytic activity of iron(II) phthalocyanine complexes to DA at its modified carbon paste electrode, and also pointed out that the bulk cobalt and nickel phthalocyanine complexes did not have electrocatalytic activity to DA. The electrocatalytic behavior of these complexes towards oxidation of species is believed to be mediated by the Fe^{III}/Fe^{II} couple [125]. Tetraaminophthalocyanine nickel ($Ni^{II}TAPc$) electropolymerised on a glassy carbon electrode has been studied as an electrocatalyst for the determination of DA [115]. A cation surfactant Cetyltrimethylammonium Bromide (CTAB) and iron(II) octanitro phthalocyanine modified carbon paste electrode was fabricated and applied to simultaneous determination of ascorbic acid, dopamine and uric acid [126]. The anodic peak potentials of DA and AA were separated with good sensitivity in the presence of CTAB. Moreover, 100-fold AA did not interfere in the determination of DA [127]. Recently, immobilization of phthalocyanines and metallophthalocyanines at the surface of carbon nanotubes has been tested [128,129]. Carbon Nanotubes (CNTs) working electrodes have been proved beneficial helping the electron exchange reaction in redox processes of different species like DA, epinephrine, and 5-HT among other compounds [130,131]. We develop a novel assay for the enhanced electrochemical detection of low dopamine concentrations in the presence of serotonin in deproteinized serum samples based on the electrocatalytic action of Carbon Nanotubes Paste Electrodes (CNTPEs) modified with iron(II) phthalocyanine (FePc) has been investigated [132]. The developed FePc-MWCNTP electrodes were able to detect dopamine with a detection limit of $2.05 \cdot x\ 10^{-7}$ M in the presence of 5-HT, comparable with data found in the literature or even better [117,127]. There was no electrochemical response for Ascorbic Acid (AA) added in the sample and electroactive species like uric acid and paracetamol did not interfere in dopamine determination due to a good peak separation at the developed sensor. Thus, the strengths of the chemically modified electrode approach include its sensitivity and selectivity. The monoamine neurotransmitter measuring method has been tested in analyzing deproteinized serum clinical samples with very good results.

Conclusions

It is clear that the development and popularization of practical CMEs has created a variety of new and potentially powerful detection opportunities electroanalysis. As our ability to manipulate precisely and imaginatively the chemical nature of the electrode/solution interface continues to grow, it can be expected that applications in the areas considered above will expand and completely new applications be developed.

The most intriguing possibilities for new work are the detection of analytes not readily sensed at conventional electrodes and the detection of conventional analyte with increased selectivity. Many of the CMEs studies above had these objectives as their driving force and the continued use of biologically active molecules as electrode modifiers will undoubtedly have increased selectivity as a principal focus. As CME systems continue to develop, completely new kinds of applications can be expected.

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