[Frontiers in Bioscience E3, 663-689, January 1, 2011]

Recent advances in nano-based electrochemical biosensors: application in diagnosis and monitoring of diseases

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1. ABSTRACT

Based on biological molecules combined with nanostructured components, the news generations of electrochemical biosensors can employ different transducers (potentiometric, amperometric and impedimetric) converting the chemical information into a measurable amperometric signal. Following this contemporary theme, our main focus in this review is to discuss different methodologies for application in biosensing, whose signal transduction is based on electrochemical principles. We apply a discussion on recent trends involving different nanostructured materials, but without daring to contemplate all nanomaterials incessantly cited in literature, which leads us to believe that this moment is an unprecedented revolution in the preparation of electrochemical biodevices. Besides, some structures of bio-nano interface and different electrochemical biosensors involved in diagnosis systems are also discussed. We outline in several parts of the report how nanoscience technologies are emerging in diagnostic medicine, as well as convergence of electrochemistry and bio-nanoscience. Our hopes for this review are that it can help different categories of researchers to understand the broad application area of electrochemistry and bioelectrochemistry, in order to detecting several types of diseases and biological phenomena.

2. INTRODUCTION

In the last decade, the scientific literature on biosensors has focused heavily on preparation of biodevices using nanostructured materials. Also, there are still a number of objectives to be addressed, such as the establishment of appropriated processes for immobilization of biological molecules in combination with nanomaterials at electrodes surfaces. It's well-known that the science involving biosensors range from medicine to molecular engineering and thereby approaches different themes for the application of electrochemical biosensors.

Just to try a definition, biomolecules or microorganisms (e.g. enzymes, antibodies, antigen, DNA, cell receptors, fungi, bacterial, tissue, biomimic component among others) when immobilized over a transducer surface are called biosensors. In the electrochemical biosensor system the specificity of the biologic compound is aggregated to the selectivity and sensitivity of the matrix used. Intimate contact with a suitable transducer device converts the biochemical signal into quantifiable electric signals. The electronic signals produced are proportional to the concentration of specific analyte. A range of these devices configurations give great versatility in the biosensors development, which can have several

applications depending of the biological compound and electrochemical transducer properties.

The electrochemical detection systems present several advantages over the other systems, e.g the wide diversity of transducer modification with chemical or electrochemical procedures for better probe incorporation. Recently, some studies have pointed that electrochemical techniques are the most suitable for hybridization events (1-3). Biosensors can be classified basically in two classes: the catalytic and affinity ones, which use immobilized enzymes and antibodies, DNA or peptides as bio-recognition molecules, respectively. The enzyme specific activity over a determined compound (substrate) or a determined class of compounds, in general way is responsible for the numerous enzymatic biosensors proposed in the literature for determination of a wide class of compounds using several matrices (4-5). Also, using electrochemistry approach may be advantageous because distinct experimental procedures can be used, including amperometric, voltammetric and impedimetric imeasurements.

One of the main challenges in the use of biomolecules and/or nanostructured materials containing biomolecules onto electrode surface is the preservation of the bioactivity, particularly because biomolecules components are outside of their natural environment. One may mention that tens of proteins, enzymes, DNA fragments, antibody or antigen has been successfully immobilized by different research groups (2, 6-8). Interest in the development of nanostructured biosensors in recent decades is attributed due to their capacity to detecting and monitoring biomolecules with specificity and selectivity. Modified nanostructured electrodes can provide a high performance in electrochemical biosensors that depend depends of the appropriate combination of supramolecular architectures and specific bioprobes. The use of hybrid nanomaterials (9,10) has provided different strategies to preserve catalytic properties of biomolecules an enhance the signal response in electrochemical devices (11-18).

Thus, our main focus in this review is give a general overview of the possible application of biosensors and as well discuss different methodologies for application in biosensing, whose signal transduction is based on electrochemical principles. We will apply a recent discussion on trends involving different nanostructured materials. Some structures of bio-nano interface are discussed here, as well as different electrochemical biosensors that can really act in diagnosis systems. In the following paragraphs we shall describe several electrochemical biosensors made with different nanomaterials. Also, the main topics covered here are: nanostructured electrochemical biosensors and nanosensors; electrochemical DNA biosensors; conventional methods for gene analysis; genosensors and diagnosis of diseases; immunoassays and immunosensors for clinical analysis; potentiometric immunosensors applied for diagnosis of diseases; amperometric immunosensors and enzyme-based nanostructured biosensors. Also, here we shall concentrate on the immobilization of biological molecules in combination with nanostructured materials, where the molecular architecture can be controlled accurately. Contemplating the final part of this review, we make an allusion to novel models for diagnosis and monitoring of diseases, since from nanostructured biosensors to body fluids measurements.

3. NANOMATERIALS AND ELECTROCHEMICAL BIOSENSORS: DIFFERENT PATHS TO THE SAME GOAL

Nanostructured materials has been exploited in last years as an important tool for amperometric biodevices development and several works have been reported to detect endogenous biomolecules at real time where the biocompatibility is one of the most important steps for *in vivo* diagnosis, in which continuous signal measurement by amperometric enzyme biosensor has been desired specially in a large range of analyte concentration. For example, Jeong and co-workers (11) have developed a calibration method for subcutaneous amperometric glucose sensor in which a thick film of enzyme is used as a transducer on direct current measurement in blood samples, an interesting approach for diabete diagnosis. However, the biocompatibility in most materials for implantable sensors was evaluated by Abel and co-workers (19) as materials that are more suitable for the diffusion of analyte through polymer membranes. Interesting molecular arrangements by using electrochemical systems on medicine has been made extensively because of low cost and to detecting small concentration of biological molecules (20-23). In this case, electrochemical sensors were studied by Griveau (20) to detecting NO in rat tumor cells, when method miniaturized electrochemical sensor with a stable hexacyanoferrate probe to detecting NO *in vivo* tumor cells in order of nM concentration, an interesting tool to investigate the efficiency of anticancer drugs.

In another way of research, the use of biocompatible matrix in electrochemical sensors has been reported in several works to detect neurotransmiters (24-26) and control possible severe depletion of them in organism. Dopamine is one of the neurotransmitters most investigated in order to avoid and desenvolve a pre-treatment to Parkinson's disease. Njagi and coworkers (24) developed an implantable biosensor based on chitosan and oxide nanoparticles for dopamine detection. In this system an optimal and sensitive electrode demonstrated detection in order of 10nmol L^{-1} to 220 μmol L^{-1} of dopamine. In summary, the main challenge today is to fabricate biodevices based on modified electrodes to monitoring continuously biological systems with high reproducibility and selectivity, an important step for medical applications.

In the case of DNA biosensors, the probe immobilization is the major step, which insures the performance and operation of the sensor. Specific oligonucleotide sequences or even DNA fragments can be immobilized by adsorption, complexes involving biotin-avidin interaction, covalent bindings and immobilization over composite electrode surfaces. Bonnani and co-workers (25) reported and discussed the major properties of each one of these immobilization techniques. DNA is considered

a functional material which can interact specifically or selectively with several chemical substances. Are many kinds of interactions between chemical molecules with DNA, which enables its indirect detection. The bound of compounds to DNA, for indirect detection, can be classified into three major ways: intercalation, minor groove binding and covalent binding (26,27). Beyond these, Minasyan and co-workers (28) suggested that the interaction between low weight ligands to DNA may also occur through non-covalent bonds and electrostatic interaction. This can alter the macromolecules conformations in different ways. The formation of many DNA complexes after ligand interaction may lead to changes in its structure and functional activity. In this kind of interactions, it is known that due to the planar structure of aromatic compounds, they can intercalate into the DNA base pairs (29). In this way, the hybridization can be detected directly by DNA intercalators (30-36) or by electroactive species which interacts with the guanine base from the DNA sequences (37, 38). Due to the electroactive properties of these compounds, the hybridization event may be detected indirectly by the intercalation effect. Among the many intercalators presented in the literature, the most cited ones are methylene blue (MB), and ethidium bromide (EB). The interactions types and kinetics between MB and ssDNA (single-stranded DNA) or dsDNA (double-stranded DNA) have been intensively studied (39-49). Other interactions as the MB bound with dsDNA grooves, electrostatic interactions, dependence on ionic strength, MB/DNA ratios and sequence of DNA base pairs received considerable interest as electroactive probe in the DNA hybridization detection (50-54).

MB interacts electrostatically (50), directly by dsDNA intercalation (55) or through the free guanine base present in ssDNA (56). The interaction between DNA and EB has been studied by several methods, among them, absorption, fluorescence and NMR (57-59). Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide), a well-known DNA fluorescent intercalator, is able to bind with the nitrogenous bases of DNA and has been used to monitor the DNA hybridization reaction because a double-stranded DNA (ds-DNA) has a much different affinity for EB compared with a single-stranded DNA (ss-DNA). Recently the use of electrochemical methods to study DNA intercalation has had an emerging progression (60-62). The electrochemical insights of small molecules to DNA can provide a complement to the spectrophotometric methods for non absortive species (60). Experimental results for complex formation with ss-polynucleotides are relevant to the conditions not excluding the presence of ds-sites on DNA (60). The electrochemical methods differ in its sensitivity, simplicity and versatility providing information about the binding mechanisms. Berg and Eckardt (63, 64) performed the first studies about the molecules interaction with DNA, interacted with DNA based on its different electrochemical activities, free or bound to DNA.

As previously noted, DNA is a very interesting molecule that has intrigued many scientific segments regarding to the development of biosensors, and it will be discussed in the following topic.

4. ELECTROCHEMICAL DNA BIOSENSORS

4.1. DNA Structure

Deoxyribonucleic acids (DNA) were firstly isolated by Friedrich Miescher in the 19th century (65), and your structure and function were described by Watson and Crick (66) in 1953. DNA is composed of four repeating nucleotides formed of a phosphate-deoxyribose backbone and a nitrogenated base. The bases are of two types, the purines (adenine and guanine), formed by two fused rings and the pyrimidines (thymine and cytosine) consisting of only one ring. The chain is coiled to form a double helix composed of two antiparallel strands (double-stranded DNA, or dsDNA) kept together by hydrogen bonds. dsDNA can be broken by heat or high pH (denaturation), but on removal of the heat source or pH extreme, the DNA molecule will re-form (reanneal) into the double stranded configuration (hybridization).

DNA has very important role in life, responsible for the storage and transfer of genetic information. The complementarity of two single stranded DNA (ssDNA), based in interaction of hydrogen bonds only with specific complementary bases: adenine pairs with thymine, and cytosine pairs with guanine, and the ability to hybridization of the ssDNA, form the basis of DNA molecular diagnostics for detection of specific gene.

4.2. Conventional methods for gene analysis

Conventional methods for analysis of specific gene sequences are based on direct sequencing or on DNA hybridization and amplification methods, more commonly used in diagnostics because of its simplicity (67). The development of these methods was possible after the 1970s, with the sequencing technology created by Maxam (68) and Gilbert and Sanger (69), which allowed the amplification by PCR (Polymerase Reaction Chain), and with the implantation of solid-supported hybridization for DNA analysis, using membrane-based blots (70,71).

DNA amplification, PCR and Real-time PCR technology techniques are powerful tools for pathogen detection and DNA diagnosis. However, several factors like nucleic acid contamination of reagents or laboratory materials, minimum laboratory requirements to keep its performance, great influence of the environment, and high costs have limited the use of these technologies. Also, PCR analysis uses carcinogenic agents, such as ethidium bromide in gels with UV detection. Analysis can be more complicated when the southern blot or dot blot methodology is considered, which is highly affected by the concentration of both probes and samples, type of membrane, labeling procedure (radioactivity), and hybridization conditions.

4.3. New electrochemical approaches in DNA biosensors

DNA biosensors, also called genosensors, have received increasing interest for specific sequence detection (72-78) and they can be used as an alternative to PCR, gel

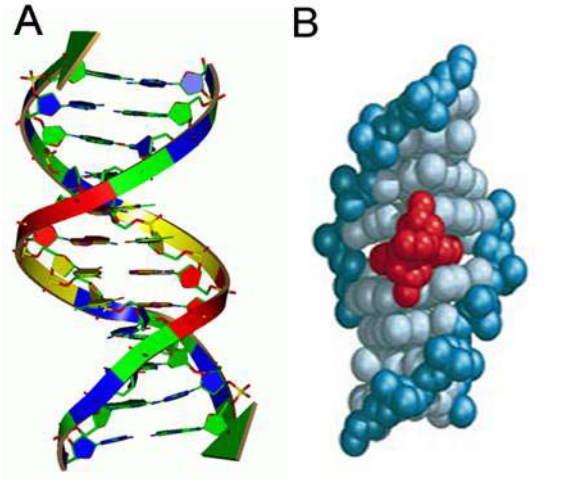

Figure 1. (A) DNA structure. (B) View of the crystal structure of intercalator bound to double strand DNA. The intercalator is depicted in red, and the DNA double helix is in blue. Reproduced with permission from (72).

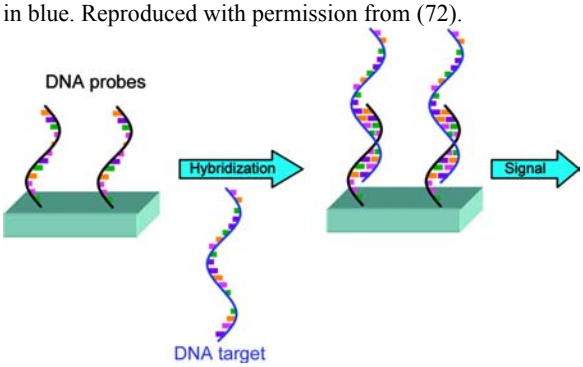

Figure 2. Steps involved in the detection of a DNA sequence. Reproduced with permission from (89).

electrophoresis, and marking with radioactive. DNA biosensors are formed by immobilized ssDNA sequences (probe) on the sensor surface, as biological recognition agent, conferring selectivity, and a transducer that provides sensitivity, and converts the recognition of the sequence-specific hybridization into a measurable electronic signal (Figure 1).

DNA biosensors portable, battery-operated instrument, easy-to-use, with low cost, low sample, rapid analysis, multiplexing capability of the system, selective, highly sensitive to detection of specific DNA sequences and compatible with microfabrication technology and mass production have potential application in several areas, including agricultural, bioterrorism agents detection, clinical, environmental, food industry and veterinary (79). Electrochemical detection of nucleic acids has attracted interest because of the high sensitivity allowed by electrochemical methods, and use of relatively inexpensive instruments (80). The probe (single-stranded DNA) can be immobilized onto a surface by adsorption, crosslinking, encapsulation, avidin–biotin complexation and covalent attachment (81). DNA probes, immobilized on a surface, should be accessible to the DNA target, being this factor important to the biosensor performance.

Approaches for development of biosensors have been suggested to detect sequence DNA hybridization, including optical (82, 83), piezoelectric (84, 85) and electrochemical (86) transduction techniques. The first paper dealing with electrochemical analysis of nucleic acids was published by Berg (87), in 1957 and the first electrochemical DNA biosensor was reported by Millan and Mikkelsen (88) in 1993, based on hybridization indicators of cobalt complexes. Since this time, the electrochemical DNA biosensors have attracted considerable attention.

Electrochemical detection of DNA hybridization can be performed by label-free systems (89) divided into: (a) direct oxidation of DNA bases, based on the intrinsic electroactivity of the DNA bases upon hybridization or (b) change in interfacial properties, and labeled systems based on specific binding event of hybridization labels with the DNA (90) (Figure 2).

The combination of electrodes with functionalized polymers is a promising strategy for immobilization of DNA probes in the development of DNA biosensors. Studies of electropolymerization have indicated that monomers containing aromatic groups directly bonded to oxygen are easier to polymerize, presenting reproducibility and mechanical resistance of the film, allowing higher stability of the modified electrode (91,92). Madurro and co-workers (93) conducted studies showing that graphite electrodes modified with electrodes modified with polymer films are efficient to immobilize nucleotides. All four bases (adenine, guanine, cytosine and thymine) can be determined simultaneously by differential pulse voltammetry onto graphite electrodes modified with poly-aminophenols (94-96) and poly(4-hydroxyphenylacetic acid). Hybridization studies of oligonucleotides (97,98), based on direct oxidation of DNA bases onto graphite electrodes modified with polymer films showed that the oxidation currents of purine bases decrease after hybridization, indicating that hydrogen bonds formed between complementary sequences leading to a duplex difficult the oxidation of the bases, decreasing the oxidation peak current of the guanosine and adenosine. Also, the single stranded presents higher proximity onto the electrode surface, due to its higher conformational flexibility that, facilitate the charge transfer between the nucleotides and the electrode (98, 99).

Besides direct oxidation of purine bases and electrocatalytic oxidation by redox indicators, e. g. ruthenium (100) and osmium complexes (101) have been showed. The oxidized mediator removes electrons from guanine in dsDNA, and after, the reduced mediator is oxidized at the electrode, returning to the oxidized state. The oxidation current is proportional to the amount of guanine in the DNA hybridized on the electrode surface (102).

Changes in properties of the DNA, such as impedance, capacitance or conductivity, can be used to detect the

hybridization between complementary ssDNA (103,104). Electrochemical impedance spectroscopy (EIS) is a non-destructive steady-state technique, often used to study interfacial properties, analysing changes on resistive and capacitive properties of materials that occur at the electrode–electrolyte interface.

In labeled methods, the transduction of the DNA hybridization is based on interaction of a redox compound and the DNA. Some hybridization indicators (e.g. acridine orange (105), daunomycin (106) and ethidium bromide (107) can bind at the DNA through intercalation between base pairs, having higher affinity with dsDNA than with single-stranded probe, leading to the accumulation of redox indicator at the hybrid formed onto electrode surface, increasing the current signal, after hybridization.

Others indicators, e.g. Hoechst 33258 (108), cobalt complexes (109), methylene blue (110) presents higher electrostatic interaction with ssDNA, in well-defined binding sites. The effect is a differential accumulation of the indicator in the DNA layer near the surface of the electrode when ssDNA or dsDNA is attached, which correlates with different voltammetric peak currents, occurring a decrease in peak current after hybridization occurrence.

Molecular diagnostic to microbial and viral pathogens detection of clinical interest and genetic diseases, based on the analysis of specific DNA sequences, using nucleic acids as biorecognition element, has stimulated the development of DNA biosensors based on nucleic acid as biorecognition element, being excellent candidates for rapid and inexpensive diagnosis for point-of-care.

4.4. Genosensors and diagnosis of diseases

In recent years, research in DNA biosensors is being focused on the development of procedures aiming the direct application of genosensors in diagnosis and monitoring of diseases. In sequence are shown examples of these applications.

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) leading to opportunistic infections. HIV infection in humans is pandemic, already have occurred millions of deaths from this infection. Niu and co-workers (111) developed a human immunodeficiency virus DNA biosensor by immobilizing covalently single-stranded HIV DNA fragments to modified glassy carbon electrode and monitoring the hybridization using a cobalt complex as electrochemical indicator, with a detection limit of 27 pmol L^{-1} and a linear range from 1.6 x 10^{-10} to 6.2 x 10^{-9} mol L^{-1}.

Recently, a multi-electrode DNA biosensor as developed for simultaneous detection of the human immunodeficiency virus (HIV) oligonucleotide sequences, HIV-1 and HIV-2 (112). The electrochemical array was fabricated by self-assembling each of two kinds of thiolated hairpin-DNA probes onto the surfaces of the corresponding three working electrodes. The hybridization was monitored by squarewave voltammetry using methylene blue as redox indicator. The linear range was from 20 to 100 nmol L^{-1} for HIV-1 and HIV-2, with the same detection limits of 0.1 nmol L^{-1}.

Breast cancer is the second most common type of cancer and the second-leading cause of cancer death in women. An electrochemical breast cancer biosensor based on a chitosan-co-polyaniline copolymer coated onto indium tin oxide was developed by Tiwari and Gonga (113) , immobilizing the complementary DNA probe (42 pb) associated with the breast cancer, monitoring the peak oxidation current of $[Fe(CN)_6]^{3-}$ ion. The bioelectrode exhibited linear range of 0.05–25 fmol L^{-1} of the ssDNA target, sensitivity of 2.104 μA $fmol^{-1}$ and shelf life of about six months, stored at room temperature.

Dengue virus is one of the most significant causes of arthropod-borne diseases on Earth, transmitted among humans by the Aedes aegypti mosquito. Dengue is recognized in over 100 countries and the worldwide annual infection rate is estimated to be between 50 and 100 million infections per year. An electrochemical microfluidic biosensor integrated with a minipotentiostat was developed by Baeumner and co-workers (114). The probe was coupled to liposomes entrapping couple potassium ferri/ferrohexacyanide. The capture probes were coupled to magnetic beads that were isolated on a magnet. The liposomes were lysed to release the electrochemical markers that were detected on an interdigitated ultramicroelectrode array in the biosensor. The system was tested to detection of Dengue virus and the peaks measured were 7–10 times higher than those recorded in the absence of target.

A method for electrochemical detection of a synthetic 20-bp oligonucleotide sequence related with dengue virus genome was described by De Lima-Filho and co-workers (115). Electrochemical detection of hybridization was performed by cyclic voltammetry, using ferrocene (Fc^{+}) as hybridization label. Leprosy is a chronic granulomatous infection, mainly affecting the skin and peripheral nerves, caused by the obligate intracellular organism *Mycobacterium leprae*, also known as *Hansen's bacillus*. *Mycobacterium tuberculosis* is a pathogenic bacterium that infects the lungs, causing tuberculosis and leads to millions of deaths annually. Ozsoz and co-workers (116) presented a method for detection of multiple point mutations in the Mycobacterium tuberculosis rpoB gene using an electrochemical genosensor. The device contained five different capture probes, which are designed to hybridize with several sequence segments within the bacterial rpoB gene hotspot region. Point mutations were detected by monitoring the guanine oxidation with differential pulse voltammetry after hybridization between PCR amplicons and inosine modified capture probes at graphite surface.

It is a very worrying health problem worldwide, most prevalent in Asia, Africa and Latin America with approximately 249.000 new cases being detected in 2008. Brito-Madurro and co-workers (117) developed a bioelectrode for gene detection of *Mycobacterium leprae*,

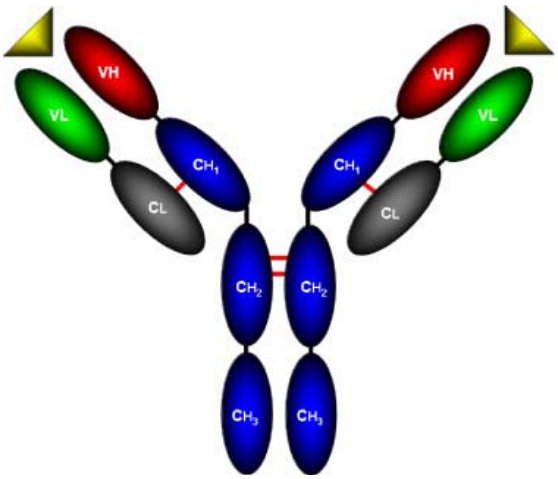

Figure 3. Basic structure of an IgG molecule. Reproduced with permission from (125). .

also known as *Hansen's bacillus***,** immobilizing a single stranded DNA (ssDNA) with 78 bases long (specific gene related to *Mycobacterium leprae*) on graphite electrode modified with poly(4-aminophenol). Hybridization between probe and target was monitored by voltammetry, using ferrocenecarboxyaldehyde as electrochemical DNA hybridization indicator. The hybridization of nucleic acid probe with the DNA target resulted in significant decrease in the oxidation peak current of ferrocenecarboxyaldehyde, indicating greater affinity of this compound for ssDNA than for dsDNA. The linear range of detection for the DNA target was found to be 0.35 – 35 ng μL^{-1} ssDNA.

Hepatitis C virus infection is a major public health threat. Frequently, this infection progresses to chronic diseases, which can lead to liver cirrhosis and hepatocellular carcinoma. According to the World Health Organization, this infection currently affects approximately 170 million people worldwide. There is no comprehensively effective therapy for chronic HCV infection and in most cases current treatments call for major improvements. Hejazi and co-workers (118) developed an electrochemical DNA biosensor, using a gold electrode modified with a probe related to the hepatitis C virus genotype 3a, and methylene blue as indicator. The detection limit was 5.7 x 10^{-11} mol L^{-1}.

Helicobacter pylori is a bacterium that lives in the stomach. It causes more than 90 percent of ulcers, which are sores in the lining of the stomach or the duodenum. A biosensor has been constructed by immobilization of a thiolated capture probe sequence from *Helicobacter pylori* onto gold electrodes, employing as electrochemical indicators to detect hybridization a molecule containing ortho quinone functional group [N,N'-Bis(3,4-dihydroxybenzylidene)-1,2-diaminobenzene (3,4-DHS)] (120). After hybridization, 3,4-DHS was accumulated and monitored by differential pulse voltammetry. The linear range of complementary target sequences of H. pylori was 8.9–22.2 μmol L^{-1}, with a detection limit of 8.3±0.4 μmol L^{-1}.

Sexually transmitted diseases constitute the most common infectious diseases around the world. An assay for electrical biosensing of syphilis DNA was proposed by Zhang and co-workers (119), using target-guided formation of polyaniline (PANI) based on an enzymatically catalyzed method. The current response of PANI was linearly related to target DNA concentration between 1.0 pmol L^{-1} -1.0 nmol L^{-1} , and detection limit of 0.5 pmol L^{-1}.

Avian Influenza virus infections are a major cause of disease in poultry, and infections can occur in humans. A DNA electrochemical biosensor for determination of DNA sequence of Avian Influenza Virus genotype was showed by Zhu and co-workers (120). A multi-walled carbon nanotubes–cobalt phthalocyanine and poly(amidoamine) were modified on the glassy carbon electrode. DNA probes were immobilized on the modified electrode, and the hybridization was monitored by differential pulse voltammetry, based on the oxidation signal of guanine. The biosensor presented linear range was from 0.01 to 500 ng mL^{-1}, and detection limit of 1.0 pg mL^{-1}.

5. IMMUNOASSAYS AND IMMUNOSENSORS FOR CLINICAL ANALYSIS

5.1. General Aspects

Among the methods described for analysis of proteins and peptides, many are based on a combination of physical separation and sensitive detection. Immunoassay methods are capable of direct and specific detection in 'real' samples, like serum or plasma. However, these traditional methods are time-consuming and may have sensitivity problems (121-124). The property of highly specific molecular recognition of antigens by antibodies leads to high selectivity of assays based on immune principles for immunoassays and are in wide use as analytical tools in clinical and pharmaceutical sciences. Antibodies are structurally very similar. Of the five classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM), which differ in, e.g. glycosylation and number and positions of the disulfide bridges, mainly IgG (150 kDa) is used for immunoassays. An IgG consists of two heavy and two light chains, which are interconnected by disulfide bridges (see Figure 3) (125). All chains have a variable and a constant region. The variable regions of the heavy and light chain combine in one interaction site for the antigen, which is called the antigenic site. Thus, an IgG molecule has two identical binding locations for the antigen.

The most common type of enzyme immunoassay in clinical analysis is known as enzyme-linked immunosorbent assay, or ELISA. There are different schemes of enzyme immunoassay, and in clinical laboratory practice the most popular are the 'sandwich' method (Figure 4) (126).

Traditional immunoassays are performed by microtitration and are usually slow (several hours) and labor-intensive (127,128). Currently, most immunoassays are performed with 96-well microtiter plates in which samples can be processed simultaneously. However this assay needs of laboratories and requires technically trained

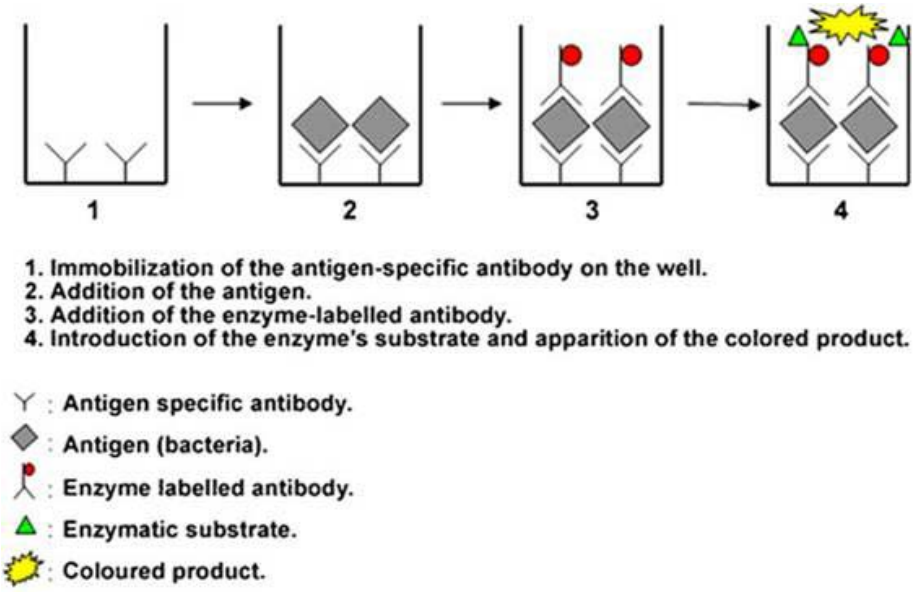

Figure 4. Schematic representation of the sandwich-ELISA protocol. Reproduced with permission from (126).

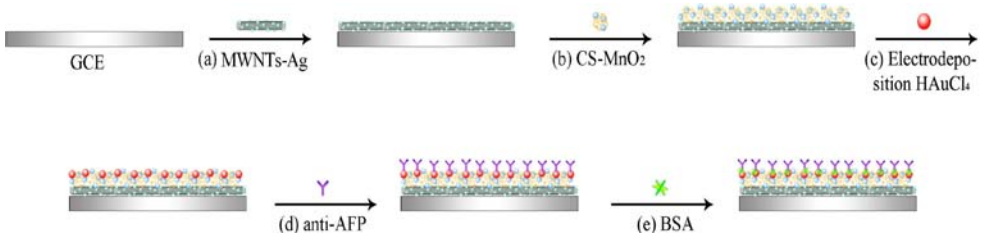

Figure 5. Schematic illustration of stepwise process of an immunosensor. BSA: bovine serum albumin solution for to block possible remaining active sites. Reproduced with permission from (133).

personnel beyond being a process in multistage process, resulting in a general complexity, the automation of the multistage measurement and conventional cannot be conducted under non-laboratory (129). For a formal definition, but not specific, immunosensors are biosensors based on the immunologic reaction of an antigen (A_g) with the antibody (A_b) forming the corresponding Ag-Ab complex (130). In these cases, when Ab or Ag is immobilized over the electrode surface, depending of the kind of response from the transducer, several devices can be obtained (131,132). A fact that should be considered is not just the selectivity but also sensitivity generated by the sensor. Two great classes in the development of these devices are the amperometric immunosensors in which enzymes are employed as markers and the impedimetric immunosensors in wich direct measurements are performed through the electric properties of the surface.

Different species and subclass of immunoglobins are used in development of immunosensors, which combine antibody antigen interaction (Figure 5) (133). In these immunoassays, the interaction between an antibody (Ab) and electrochemical immunosensors capable of direct and specific measurement of very low protein concentrations have been developed (134).

Correlation between antibody affinity and disease status has been proposed and the affinity of serum antiviral antibodies against rubella, cytomegalovirus and human herpes virus-6 has been used to distinguish primary infections from secondary or chronic infections. Accurate measurement of protein biomarkers in human serum and tissue is important for early disease detection and diagnosis, signaling pathway studies, and drug development (135).

Immobilized creatine kinase M (CK-M) antibody is used for detection of the cardiospecific CK-MB isoenzyme. Goat antihuman CKM Ig G was immobilized on an electrode, can be used for several assays and is regenerable (136). Such type of sensors has excellent selectivity because of high antibody–antigen specificity. Enzymes are extremely useful as labels in immunoassays as their catalytic properties allow the detection and quantitation of low levels of immune reactants. The enzymes most commonly used are alkaline phosphatase, horseradish peroxidase, glucose oxidase and βeta-galactosidase.

Electrocatalytic properties of redox enzymes permit their application as labels for potentiometric

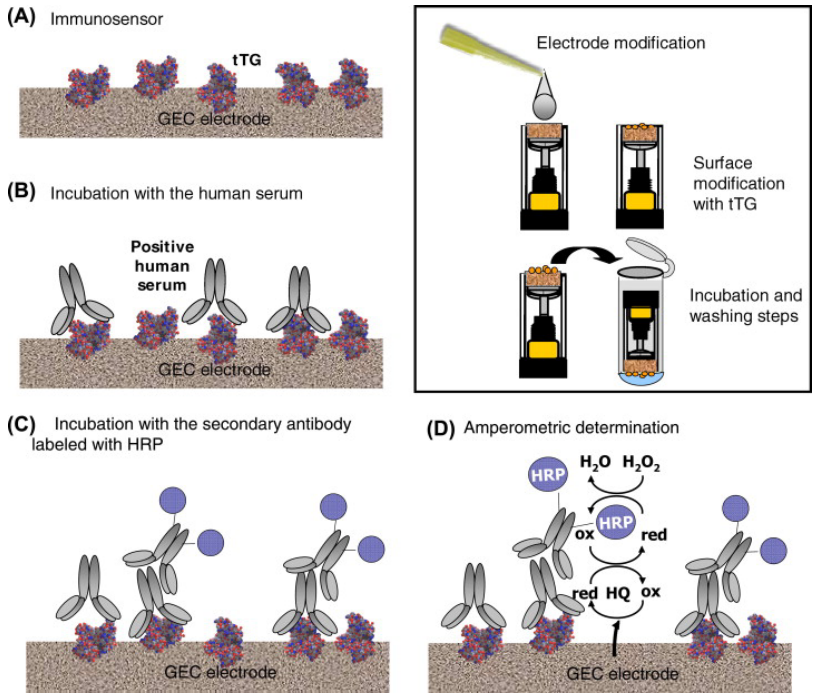

Figure 6. Electrochemical immunosensor by indirect detection for the diagnosis of celiac disease. Schematic representation of graphite–epoxy composite (GEC) material showing the adsorbed transglutaminase (tTG) (A), immunological reaction with the human serum (B), immunological reaction with the secondary antibody (C), and amperometric determination (D). Reproduced with permission from (138).

immunosensors. Antigen immobilized onto electrode surface interacts with enzyme-labeled antibody, resulting in attachment of the enzyme to the electrode surface. Therefore, the formation of antigen-labeled antibody complex on the electrode surface is accompanied by an electrode potential shift. The presence of free antigen in the solution leads to competition in the binding of labeled antibodies (conjugate) with free and immobilized antigen. The competition results in a decrease of the rate of potential shift. The decrease in rate of the potential shift, in this case, is proportional to free antigen concentration in the solution (137).

To be able to detect the interaction, one of the immunoagents has to be labeled. Various labels have been applied, of which radioisotopes were among the first, because of their inherent sensitivity. Other frequently used labels are chemiluminescent compounds, and enzymes (e.g., alkaline phosphatase and horseradish peroxidase), convert an enzyme into measurable product (Figure 6) (138). Immunoassays performed with immobilized antibodies have resulted in sensitivities in the nanomolar range for complementary antigens and a reported dynamic concentration range of 10^{-3}–10^{-12} mol L^{-1} (139). For macromolecular antigens, such as IgG, direct measurement is possible using immobilized IgG antibodies. However, for lower molecular weight analytes, such as theophylline, a sandwich assay using a second antibody is needed.

Immunosensors combining antibody antigen interaction with electrochemical measurements have been developed using different techniques (e.g., potentiometric, amperometric and impedimetric).

5.2. Potentiometric immunosensors applied for diseases

The strategy of potentiometric immunosensor is based on the change of the potentiometric response before and after antigen-antibody reaction. Either antibodies or antigens in aqueous solution have a net electrical charge polarity, which is correlated with the isoelectric points of the species and the ionic composition of the solution (140).

Electrocatalytic properties of redox enzymes permit their application as labels for potentiometric immunosensors. In presence of the substrate, the attachment of the electrocatalytic active enzyme on the electrode surface initiates an electrocatalytic reaction resulting in a potential shift. Antigen immobilized on the electrode surface interacts with enzyme-labeled antibody, resulting in attachment of the enzyme to the electrode surface. Therefore, the formation of antigen-labeled antibody complex on the electrode surface is accompanied by potential shift. The presence of free antigen in the solution leads to competition in the binding of labeled antibodies (conjugate) with free and immobilized antigen. This competition results in a decrease of the rate of potential shift proportional to free antigen concentration in the solution (141).

Liang and co-workers (142) developed a potentiometric immunosensor for determination of breast cancer antigen (CA15-3) utilizing glutaraldehyde to link

CA15-3 antibody on a functional sol-gel film. This immunosensor showed a low LOD (5 U ml^{-1}) and satisfactory stability in a storage period of 30 days. However, the application of the potentiometric immunosensors is limited by non-specific binding between heterogeneous antigen and antibody and the influence of the high background signal.

Tang and co-workers (143) developed an immunosensor for detection of hepatitis B. In this study, surface antigen has been developed by means of self-assembly to immobilize hepatitis B surface antibody on a platinum disk electrode based on gold nanoparticles, nafion and gelatin as matrices. The detection was based on the change in the electric potential before and after the antigen–antibody reaction. These strategy, in contrast to the commonly applied methods (e.g., the glutaraldehyde crosslinking procedure), could allow for antibodies immobilized with a higher loading amount and better retained immunoactivity, as demonstrated by the potentiometric measurements. A dynamic concentration range of 4–800 ng mL^{-1} and a detection limit of 1.3 ng mL^{-1} were observed.

Tang and co-workers (144) described an immunosensor based on immobilization of antibody specific for hepatitis B onto platinum electrode modified with silver colloids and polyvinyl butyral. Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus. It is a major global health problem and the most serious type of viral hepatitis. It can cause chronic liver disease and puts people at high risk of death from cirrhosis of the liver and liver cancer. Worldwide, an estimated two billion people have been infected with the hepatitis B virus (HBV), and more than 350 million have chronic (long-term) liver infections. The resulting immunosensor exhibited a sigmoid curve with log antigen of the Hepatitis-B-Virus concentration, wide linear range from 16.0 to 800 ng mL^{-1} with a detection limit of 3.6 ng mL^{-1}, fast potentiometric response < 3 min and stability > 4 months.

A potentiometric immunosensor for diphtheria has been developed by means of self-assembling compound nanoparticles to a thiol-containing sol-gel network. Diphtheria is a bacterial infection that spreads easily and occurs quickly. It mainly affects the nose and throat. Children under 5 and adults over 60 years old are particularly at risk for contracting the infection. A cleaned gold electrode was first immersed in a 3-mercaptopropyltrimethoxysilane sol-gel solution to assemble a silica sol- gel. A direct potentiometric response to diphtheria antigen was obtained from the immobilized diphtheria antibody. The potentiometric response of the resulting immunosensor was rapid and the linear range was from 22 to 800 ng mL^{-1} with a detection limit of 3.7 ng mL^{-1}.

Qu and co-workers (145) reported a micro-potentiometric hemoglobin (Hb) immunosensor based on electrochemically synthesized polypyrrole-gold nanoparticles composite. Specific antibody for HbA1c was immobilized onto the electrode surface. The HbA1c level reflects the blood glucose concentration of the previous 2–3 months and has potential to become an inexpensive and portable device for monitoring of diabetes. HbA1c is measured as a relative content of total Hb with the clinical range 5–20%, and 4–6% is estimated as the normal value for a healthy adult. The sensor response was linear over the concentration range 4-18 μg mL^{-1} HbA1c.

5.3. Amperometric immunosensors applied for diagnosis of diseases

Amperometric immunosensors, based on the surface charge or potential change upon immunoreaction on the interface of the detection device, usually use a nonlabeled technique (146). The first amperometric immunosensor for tumor markers, for the determination of human chorionic gonadotropin (hCG), was reported in 1979 (147). Monoclonal anti-hCG was immobilized on an amperometric oxygen electrode. Catalase-labeled-hCG and hCG in the sample competed for binding sites of immobilized anti-hCG on the electrode surface. After a washing step to remove nonspecifically bound hCG, the sensor was then reacted with the substrates. Membrane-bound catalase generated oxygen that was sensed by the oxygen electrode. The detection limit of the sensor was 20 IU L^{-1}.

Kutyreva and co-workers (148) developed an amperometric immunosensor for diagnose of *Candida albicans* (CA), wich is a fungus normally present on the skin and in mucous membranes such as the vagina, mouth, or rectum. The fungus also can travel through the blood stream and affect the throat, intestines, and heart valves. The method for determination of CA based on combination immunochemical reactions and voltammetric indication of analytical signal was developed. Dilutions different of antibody (Ab) against antigen (Ag) of CA immobilizing in common with choline esterase (CE) were studied. The dynamic range of concentrations determined of Ag depends on degree of dilution of Ab used for manufactory biosensitivity part of sensor. The data indicate that the [Ab-Ag] immune complexes are stable.

Ju and co-workers (149) proposed a method for determination α-1-fetoprotein (AFP) in human serum by using a horseradish peroxidase (HRP) label in an enzyme-linked immunosorbent assay (ELISA). AFP is measured in pregnant women, using maternal blood or amniotic fluid, as a screening test for a subset developmental abnormalities: increased in open neural tube defects and omphalocoele and decreased in Down syndrome (150). α-1-Fetoprotein is an important tumor marker for the diagnosis, and even early detection of original liver carcinoma. Its concentration in healthy human serum (in the age range of 18-40 years old) is as low as the average value of 3.4 ng mL^{-1} (151). AFP concentration rises to about 400 ng mL^{-1} in the serum of a severe liver cancer patient. The peak current produced by differential pulse voltammetry is proportional to the concentration of AFP in the range of 0.5-400 ng mL^{-1} (R =0.9993) under optimum conditions.

Based on the direct electrochemistry of HRP-labeled to immunoreagent, a reagentless and mediatorless

immunosensor for the detection of CA125 (cancer antigen 125 or carbohydrate antigen 125) has been firstly proposed (152). CA-125 has found application as a tumor marker or biomarker that may be elevated in the blood of some patients with specific types of cancers. This marker is most consistently elevated in epithelial ovarian cancer, but can be expressed in a number of gynecologic (endometrial, fallopian tube) and non-gynecologic (pancreatic, breast, colon and lung) cancers (153). The immunosensor was prepared by immobilizing CA125 in titania sol–gel formed by vapor deposition method on a glassy carbon electrode, where the current decrease was proportional to CA 125 concentration ranging from 2 to 14 units mL^{-1} with a detection limit of 1.29 units mL^{-1}.

Yamanaka and co-workers (154) described the antigen immobilization process in order to develop a biosensor for detection of Chagas disease antibodies in serum samples, using as comparison the spectrophotometric indirect immunoenzymatic assay, ELISA. Chagas disease is a chronic and incapacitating illness, caused by the protozoan parasite *T. cruzi* when trypomastigotes invade host cells (155). It was possible the detection of antibodies for Chagas disease The response time to allow antigen–antibody and antibody–peroxidase-labeled IgG interactions was 20 min with a reactivity threshold at −0.104μA.

Zhang and co-workers (156) developed an amperometric immunosensor for carcinoembryonic antigen (CEA) based on colloidal gold nanoparticles modified chitosan membrane on the surface of an indium-tin oxide electrode. Lately it was found that CEA has been expressed in many malignancies. The concentration of serum CEA is related to colon cancer (153) lung cancer (157) ovarian carcinoma (158), breast cancer (159) and cystadenocarcinoma (160). Carcinoembryonic antigen could be determined in the linear range from 2.0 to 20 ng mL^{-1} with a detection limit of 1.0 ng mL^{-1}, showing good stability and reproducibility for batch fabrication.

Nassef and co-workers (161) developed an electrochemical gliadin amperometric immunosensor specific for celiac disease based on the spontaneous self-assembly of antigliadin Fab fragments (CDC5-Fab) on Au surfaces. The analytical performance of this immunosensor is compared with a similar containing whole CDC5 antibodies previously modified with thiol groups (CDC5-SH) as the recognition element. The amperometric immunosensor based on Fab fragments showed a highly sensitive response with an LOD of 3.29 ng mL^{-1}.

Kim and co-workers (162) developed amperometric immunosensors for diagnose of lung cancer through the detection of Annexin II and MUC5AC. Overexpression of Annexin II, which has been reported in various carcinomas, is thought to be associated with cell proliferation, differentiation and cell-cell adhesion in the pathogenesis of carcinoma (163). To fabricate the sensor probe, a conducting polymer (poly-terthiophene carboxylic acid) (TTCA) was electropolymerized onto a gold nanoparticle/glassy carbon electrode and a dendrimer was covalently bonded to the poly-TTCA through amide bond formation, where AuNPs were doped onto the dendrimer. The final sensor probe was examined before and after interaction with Annexin II and MUC5AC. The performance of the immunosensor for the Annexin II was evaluated for the apical surface fluid labeled with GOx by the standard addition method and presents detection limit of the 0.051 ng mL^{-1}.The Annexin II concentration in the secretions collected from squamous metaplastic cells was determined to be 280±8.0 pg mL^{-1}.

5.4. Impedimetric immunosensor applied for diseases

In the impedimetric immunosensors, the biological signal transduction is performed through the Electrochemical Impedance Spectroscopy (EIS) (164). The impedance of electrode before-after immunoreaction changes indicates the amount of the antibody or antigen in the analyte (165). One significant advantage of impedance detection for biosensing is that antibody-antigen binding can be directly detected, allowing the development of immunosensors. This immunosensors are based in the changes of the electrical properties by recognition of antigen/antibody layer immobilized on electrodes. The major drawback of impedance methods to biosensors is the need of an interfacial engineering to reduce non-specific adsorptions (166-168).

Many groups demonstrated the potential use of this technique to direct immunoreactions (169-178). According Prodromidis (179), based on the measurement signal nature, the impedimetric immunosensor can be classified into two classes: capacitive (where the electrode surface is completely covered by a dielectric layer and all the electrode behaves as an insulating) and faradaic or faradaic impedimetric (where the electrode surface, which is partially or completely covered by a non-insulating layer is able to catalyze a redox probe, which does not exists in the solution.

Ma and co-workers (180) developed an electrochemical impedance immunosensor (EIS) for human mammary tumor-associated glycoprotein. Antibody proteins were immobilized by spontaneous adsorption of antibody on gold. (EIS) measurements of a gold electrode coated with the antibody showed changes in charge–transfer resistance after the addition of the specific anti-A capacitance immunosensor for transferrin, a potential marker for prostatic carcinoma, based on an oligomer layer on gold has been developed (181) . This immunosensor was prepared by electrochemical polymerization of *o*-aminobenzenethiol self-assembled layer on polycrystalline gold surface and then cross-linking transferring antiserum to the oligomer of *o*-ABT with glutaraldehyde as a coupler. When the immunosensor was immersed in a solution that contained the transferrin, the interaction of antibody with the antigen led to the increase of the dielectric layer and induced a capacitance decrease, which was directly related to the amount of antigen in a linear range of 1.25–80.0 ng mL^{-1} with a detection limit of 0.12 ng mL^{-1}.

Balkenhohl and co-workers (182) developed an immunosensor for electrochemical detection of anti-

transglutaminase antibodies in human sera onto screen-printed gold electrodes which were covered with a polyelectrolyte layer of poly (sodium-4-styrensulfonic acid).The concentration of anti-transglutaminase antibodies in human sera is an important analytical marker for the diagnosis of the autoimmune disorder celiac disease. Celiac disease makes it difficult for the body to properly absorb nutrients from foods. Symptoms include various intestinal difficulties, recurring abdominal bloating and pain, nausea, anemia, among others. Changes in the interfacial properties of the sensor electrode were determined by electrochemical impedance spectroscopy. Incubation of these disposable immunosensor chips with various anti-transglutaminase antibody concentrations resulted in changes in their charge transfer resistance.

Chen and co-workers (183) described an impedimetric immunosensor for human interleukm 5 (IL-5) using an electropolymerized nanocomposite film containing polypyrrole, porypyrrolepropylic acid, and Au nanoparticles. Immunosensor for human interleukm 5 is an important sensor for disease pathology study, clinic diagnosis, and pharmaceutical research. This indicator can induce exclusive migration of eosinophils. Eosinophils in the tumor tissue represent a positive prognostic indicator. Using the optimal fabrication parameters, the detection limit for IL-5 was 10 fg mL^{-1} in phosphate buffered saline and 1 pg mL^{-1} in 1% human serum with good specificity and a dynamic range of 3 orders of magnitude.

An immunosensor based on non-faradaic process for the diagnosis of dengue infection using antigen–antibody conjugation method was developed. Dengue virus exists as four antigenically distinct serotypes (Dengue 1-4) and is transmitted among humans by the *Aedes aegypti* mosquito (184). As a proof of concept, pre-inactivated dengue virus was firstly immobilized indirectly onto the immunosensor surface, pre-coated with sol–gel derived barium strontium titanate thin film and modified with organic self-assembled monolayer formed by 3-aminopropyltriethoxysilane and a cross-linker glutaraldehyde over the interdigitated electrodes. The modified sensor surface served as selective sensing probe to capture/conjugate the dengue antibody molecules present in patient's serum (185). By monitoring the impedance or current change, the antibody molecules in the patient's serum could be positively detected.

A immunosensor for leishmaniasis was development using the Leishmania kinesin antigen, rK39, found in *L. chagasi* and immobilized onto graphite electrode modified with poly*(*4-hydroxyphenylacetic acid (186). Antibodies that recognize the rK39 antigen have been detected by enzyme-linked immunosorbent assay in nearly 98% of the investigated sera from patients with visceral leishmaniasis. *Leishmania* are obligate intercellular parasites of the macrophages and cause a number of important human disease ranging from self-healing cutaneous lesions (Cutaneous leishmaniasis - CL) to diffuse cutaneous and mucosal manifestations or disseminated and often fatal visceral leishmaniasis (VL) (187). Specific, positive IgG binding was clearly demonstrated by comparing the Nyquist curve with the nonspecific IgG binding. The results obtained evidenced that specific antibody was successfully assembled onto the electrode surface (188).

6. ENZYME-BASED NANOSTRUCTURED BIOSENSORS

Regarding to enzyme-based nanostructured biosensors, nanomaterials bring new possibilities and different architectures for biosensors development at the same time to improve and amplify the electrical signal origin in biocatalytic system. The electron transfer between enzyme and electrode surface is controlled by organization energy, potential differences and enzyme orientation on the electrode and distances between enzyme and electrode. In practice, the use of polyelectrolytes, proteins and other organic molecules introduces a kinetic barrier between enzyme and electrode surface for electron transfer. However, several approaches have been reported, based on the use of artificial electron-transfers that actuates in order to improve the electrical signal in biodevices. The electron transfer in biological systems has been extensively studied due to their implications on improvement of the biosensors.

Several studies have reported the use of metal nanoparticles in modified electrodes to provide a high sensitive analytical detection (189-202) due to their capability to improve the charge transfer through the modified electrode film and their biocompatibility with biomolecules. In recent studies, the use of gold nanoparticles in modified Pb nanowires has provided good sensibility (189) at the same time exhibit excellent electrocatalytic activity and good response for glucose detection. Indeed, the response time is less than 5 s with a linear range of 5 to 2200μmol L^{-1}. On the other hand, a good approach have reported by Kumar and co-workers (190), where cooper nanoparticles and zinc oxide was electrodeposited onto electrode producing an electroactive surface for glucose detection in human urine with high sensibility.

Carbon nanotubes have also been subject of intense research in biosensors development due to their electronic and redox properties. Recent studies show that carbon nanotubes can be used as a transducer and improve the electron transfer in biological systems (203-229). As an example, Lin and co-workers (203) proposed a novel biodevice based carbon nanotubes modified with silver nanoparticles in chitosan for glucose biosensor. This system shows that glucose was detected in a linear range of 0.5 to 50 μmol L^{-1} with high sensitivity (135.9μA mmol L^{-1}), suggesting that carbon nanotube act as an electron transducer for glucose detection. However, several studies has been reported about the electroactivity of carbon nanotubes in which the electrical signal can be improved in biocatalytic reactions (230-232), in which the metal impurities may cause electron transfer in catalytic reactions. Besides carbon nanotubes, other types of 1D structure can be used in the manufacture of chemical sensors and biosensors (233-235). A full review can be found on reference 233, where Liu has showed the recent advances

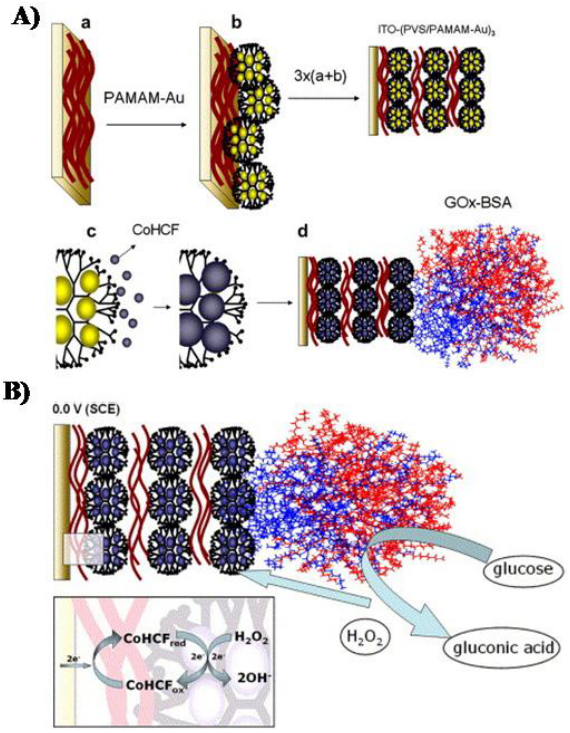

Figure 7. A) Schematic fabrication of LbL films comprising PVS and PAMAM-Au. The sequential deposition of LbL multilayers was carried out by immersing the substrates alternately into (a) PVS (a) and PAMAM-Au (b) solutions for 5 min per step. After deposition of 3 layers, an ITO-(PVS/PAMAM-Au)$_3$@CoHCF electrode was prepared by potential cycling (c). The enzyme immobilization to produce ITO-(PVS/PAMAM-Au)$_3$@CoHCF-GOx (d) was carried out in a solution containing BSA, glutaraldehyde and GOx. B) Schematic representation of reaction of glucose at ITO-(PVS/PAMAM-Au)$_3$@CoHCF-GOx electrode. Reproduced with permission from (236).

in the development of sensors including gas sensors, humidity sensors, and immunosensors using semiconducting metal-oxide-based nanowires or nanotubes (231).

An interesting approach is to explore the capability of Au nanoparticles to enhance the charge transport in nanostructured films producing electroactive nanostructured membranes (ENM) (236). In this context, PAMAM dendrimer containing metallic nanoparticles has been assembled in LbL films onto conducting substrates and used as modified electrodes (Figure 7). The strategies has involved indium tin oxide (ITO) substrates on glass, with polyvinylsulfonate (PVS) as negatively charged polyelectrolyte alternating with the positively charged PAMAM in the LbL film structure. New systems are described based on ENM membranes of ITO-PVS/PAMAM-Au LbL electrodes, with a redox mediator (Me) electrodeposited around Au nanoparticles.

The concept of ENM can be generalized to a wider variety of redox mediators (237). All electrodes modified with hexacyanoferrates showed electrocatalytic activity towards hydrogen peroxide, which is promising for the preparation of novel biosensors and nanodevices requiring redox mediators. Indeed, a new approach is described to produce nanostructured electrocatalytic membranes using a combination of three methods; LbL technique, PAMAM dendrimers with cobalt hexacyanoferrates-modified gold nanoparticles (alternated with poly (vinylsulfonic acid) layers on ITO and immobilization of enzyme in the presence of bovine serum albumin and glutaraldehyde as cross-linker. By using glucose oxidase as model, the modified electrode was successfully applied as a enzymatic biosensor for the amperometric measurement of glucose at 0.0 V vs. SCE.

In recent works from Caseli and coworkers (238) , Langmuir–Blodgett (LB) technique have been used in order to obtain controlled architectures of enzyme (such as urease) immobilized in solid supports, whose physicochemical properties are investigated in detail. For example, urease molecules were adsorbed at the air–water interface and incorporated into Langmuir monolayers of the phospholipid dipalmitoyl phosphatidyl glycerol (DPPG). The authors concluded that the incorporation of urease made DPPG monolayers causes a more flexible and dynamic elasticity of the film onto a solid substrate. Also, urease and DPPG–urease mixed monolayers can be transferred onto solid substrates, forming LB films with a close packing arrangement of urease, especially in the mixed LB films. Theses results were inferred by using nanogravimetry and electrochemistry measurements. Interstingly, the blocking effect of the LB films deposited onto ITO electrodes, as well as the electrochemical properties of the LB films pointed to a charge transport controlled by the lipid architecture.

Nanoscaled 1D materials (nanotubes and nanowires) have been employed in conjunction with biomolecules in electrochemical devices, taking advantage of the high specific area and fast charge transport exhibited by these systems . The challenges regarding the manipulation of nanotubes and nanowires for modified electrodes is the positioning of the materials perpendicularly to the electrode surface, in a way that both enzyme activity and fast electron transfer can be achieved. In a recent study, it was describe a simple and efficient strategy to fabricate enzymatic devices based on the deposition of glucose oxidase on aligned and highly oriented CoNiMo metallic nanowires (239). CoNiMo nanowires with an average diameter of 200 nm and length of 50 μm were electrodeposited on Au-covered alumina substrates via electrodeposition, using alumina membranes as templates (Figure 8). Enzyme-modified electrodes were fabricated via enzyme immobilization using a cross-linker. To minimize nonspecific reactions in the presence of interfering agents, a permselective membrane composed of poly(vinylsulfonic acid) and polyamidoamine dendrimer was deposited via electrostatic interaction. The formation of hydrogen peroxide as a product of the enzymatic reaction was monitored at low overpotential, 0.0 V (vs Ag/AgCl). The detection limit was estimated at 22 μmol L^{-1} under an applied potential of 0.0 V. The apparent

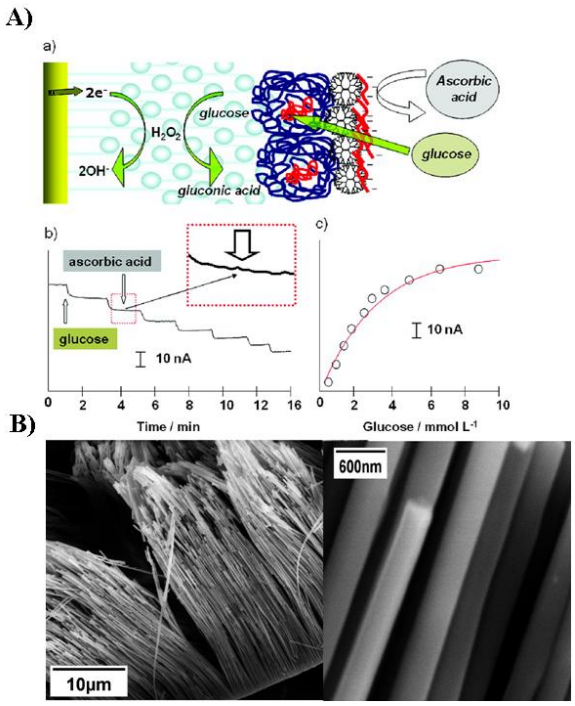

Figure 8. A) a) Schematic illustration of the glucose oxidation at CoNiMo-NWs/GOx electrode after the PAMAM/PVS membrane deposition. b) Cronoamperometry of the CoNiMo-NWs/GOx electrode after the PAMAM/PVS membrane deposition in 0.1 mol L^1 phosphate buffer (pH 7.0) with addition of 0.1 mM glucose. Note that the enzyme electrode containing a PAMAM/PVS layer was subject to the glucose biocatalysis in the presence of ascorbic acid (0.5 mmol L^1) and no current from ascorbic acid addition was observed. c) Michaelis-Menten response curves of GOx using the same system described in (b). Applied potential: 0.0 V (Ag/AgCl). B) a) SEM images showing the aligned CoNiMo NWs deposited on Au-covered alumina substrates. Reproduced with permission from (239).

Michaelis-Menten constant determined from the Lineweaver-Burke plot was 2 mmol L^{-1}.

Some important points should be considerate in order to understand the operational capability of as enzyme-based biosensor. For example, for direct amperometric biosensors, enzyme is immobilized over a conducting electrode and the electronic transference is detected during the redox reaction biologically mediated. However, the active site must be closer to the electrode surface and easily accessible to the analyte in solution. In many cases, the electronic transference occurs much far from the electrode surface and the electronic transference rate drops exponentially with the distance (240). This problem can be reduced through redox mediators but the detection become limited by the mediator mass transference (241). Indirect amperometric biosensors detect the product of a reaction catalyzed biologically, as example, hydrogen peroxide. However, the analyte normally contains additional species, (e.g ascorbate ureate) which can also be electrochemically oxidized or reduced.

Other simple strategy to apply nanomaterials in order to obtain an efficient enzyme bio-electrochemical device based on urease-modified ITO electrodes is by using an ENM comprising polyaniline and silver nanoparticles stabilized in polyvinyl alcohol (242). The suitable environment near the ITO/PAni/PVA–AgNP/urease electrode surface promoted efficient catalytic conversion of urea into ammonium and bicarbonate ions. It was observed that after enzyme conversion, fast diffusion of ammonium ions occurred into the PVA–AgNP layer, which is highly permeable. In this case, the electrodes showed an efficient response toward urea detection, in which two methods were used to calculate the apparent Michaelis–Menten constant (K_M) and both led to similar values, ca. 2.7 mmolL^{-1}. This low constant suggests that the modified electrode architecture employed here is suitable for new enzymatic devices with high bioactivity preservation.

Recently, a novel class of nanosensors has been developed in order to create biochips for single devices and single molecules detection (243). As example, it was reported the bioelectrochemical study using an individual indium tin oxide (ITO) nanowire (ITO-NW) electrode modified with glucose oxidase enzyme (GOx), in which the enzymatic activity and the biocatalytic activity was evaluated (Figure 9). In the latter, it was demonstrated the possibility of immobilizing an ITO-NW electrode on gold contacts deposited on top of a microchip (oxidized Si wafer), in which a protective polymer layer containing an aperture over the sample area was photolithographically deposited over the microchip to isolate the metallic contacts. This approach looking for new tools for studying redox enzymes at the single-molecule level, and can be used as bioelectrochemical devices, such as nanobiosensors.

7. NOVEL MODELS FOR DIAGNOSIS AND MONITORING OF DISEASES: FROM NANOSTRUCTURED BIOSENSORS TO BODY FLUIDS MEASUREMENTS

The recent advances in use of nanostructured bioelectrochemical field have enabled for reliable early and accurate diagnosis (244) and detection diseases (245-249). Particularly, the nanodiagnosis has provided an excellent tool to detect the mechanisms of disease, because improves the sensitivity with both fast responses and low cost, which has extended the present limits of contemporary molecular diagnostic. Also, nanostructured electrochemical sensors have showed important devices for the diagnosis and control of diseases, resulting in significantly technological gains to society and human health. For this purpose, the NPs such as metal nanoparticles, semiconductor quantum dots, and even composite nanoparticles are excellent candidates because offer as suitable models due large surface area, excellent electroactivity and biocompatibility allowing an increase of the signal from single analyte target molecules.

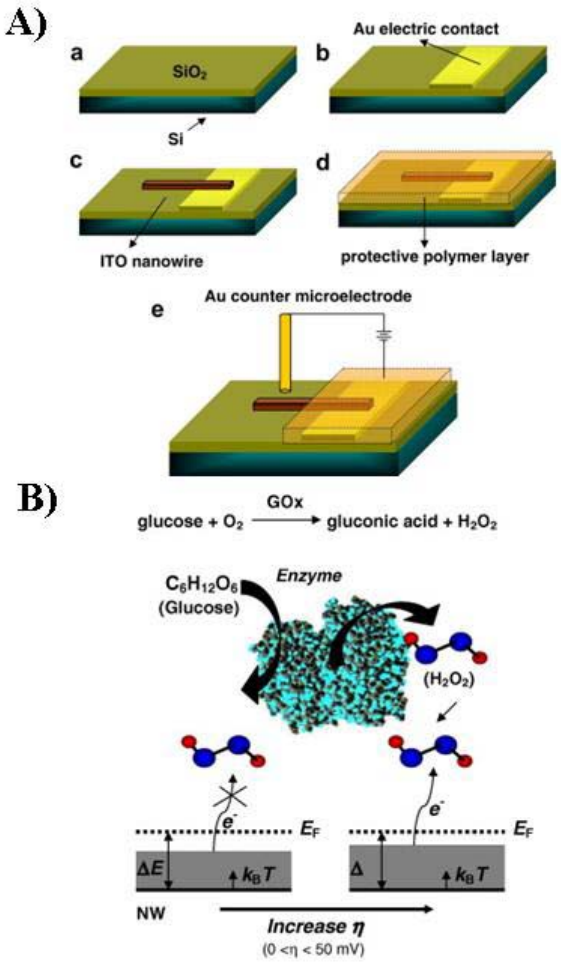

Figure 9. A) Illustrative representation of the process used for the fabrication of microfabricated metal electrodes. Firstly, a gold contact was deposited atop of the microchip. A polymer protect layer containing an aperture over the electrode area was photolithographically deposited over the microchip, in order to isolate the metallic contacts. B) Schematic illustration of the glucose oxidation at ITO-NW electrode with cathodic profile (peroxide reduction) for electron-rich electrode surface (0-50 mV). Reproduced with permission from (243).

Regarding to applications involving immunosensors have the studies by Lin and co-workers (250) that presented a nanoparticle (NP) label/immunochromatographic electrochemical biosensor (IEB) for rapid and sensitive detection of prostate-specific antigen (PSA) in human serum. This IEB integrates the immunochromatographic strip with the electrochemical detector for transducing quantitative signals. It is found that th biosensor is very sensitive with the detection limit of 0.02 ng mL^{-1} PSA and is quite reproducible (with a relative standard deviation (R.S.D.) of 6.4%). This method is rapid, clinically practical, and less expensive than other diagnostic tools for PSA; therefore, this IEB coupled with a portable electrochemical analyzer shows great promise for simple,

Jiang and co-workers (251) observed that gold nanoparticles with size ranging from 2 to 100 nm affect directly signalling process essential for basic cell functions due to binding and activation of membrane receptors and subsequent protein expression. These finding must help on the design of new intelligent nanodevices for diagnosis and therapy where an adequate nanoparticle size control is required to better understanding of interaction between particular nanoparticles and biological system.

In recent years much attention has been focused on detection of DNA hybridization in both homogeneous solutions and at the liquid solid interface (252-256), since gene expression patterns in human cancer were related to the detection of pathogens. Particularly, an interesting strategy adopted was based on electrochemical DNA (E-DNA) sensor that employs a modified electrode containing specific molecular beacon. For example, utilizing the E-DNA approach Lubin *et al* (257) were able to understand the influence of probe length, probe geometry, and redox–tag placement on the E-DNA signalling based on both stem-loop and linear probes, as illustrated on (Figure 10). From this study, the choice of an optimal E-DNA platform must involve both DNA sequence specificity and signalling. Likewise, Ricci and co-workers (258) developed a new platform for anti-DNA antibody detection using screen printed electrode modified with thiol-gold self assembled monolayer. In fact, E-DNA-like sensor exhibited sensitivity performance better that conventional electrochemical enzyme-linked immunosorbent assay (ELISA) assay been able to detect and discriminate between the anti-DNA antibodies and non-specific proteins at low nanomolar concentrations. The E-DNA approach has brought great benefits for sensors designs, which open news perspectives for diagnosis and monitoring of diseases.

Also, Liu and co-workers (259) exploited the covalent supramolecular (Figure 11) approach to construct electrochemical sensor based on cooperatively of two proximate poly-T oligonucleotides in coordination with mercury (II). This work revealed that this sensor have a good sensitive mercury (II) response with detection limit of 0.5×10^{-9} mol L^{-1} (nM) using minimal reagents, an excellent potential for application in mercury detection.

Drouvalakis and co-workers (260) demonstrated a label-free peptide-coated carbon nanotube-based immunosensor for the direct assay of human serum. A rheumatoid arthritis (RA)-specific (cyclic citrulline-containing peptide), was immobilized to functionalized single-walled carbon nanotubes deposited on a quartz crystal microbalance (QCM) sensing crystal. Serum from RA patients was used to probe these nanotube-based sensors, and antibody binding was detected by QCM sensing. The sensitivity of the nanotube-based sensor (detection in the femtomol range) was higher than that of the established ELISA and recently described microarray assay systems, detecting 34.4 and 37.5% more RA patients with anti-citrullinated peptide antibodies than those found by ELISA and microarray, respectively. There was also an 18.4 and 19.6% greater chance of a negative test being a true indicator of a patient which does not present RA, either by ELISA or microarray, respectively.

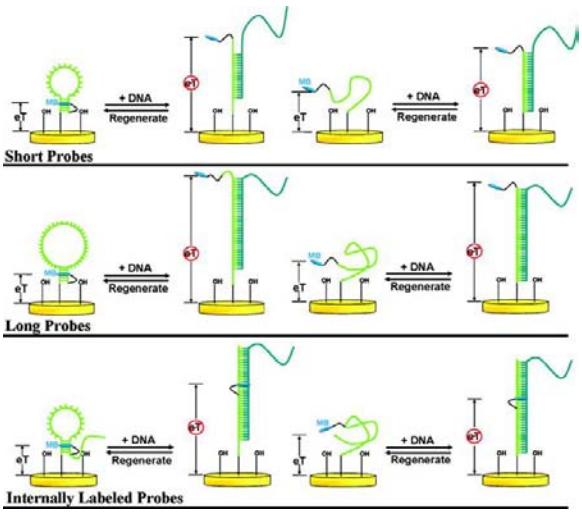

Figure 10. Design and electrochemical response from six distinct E-DNA sensors with different probe DNA sequence containing three probe geometries: one short (17 bases) and two longer (34 bases) recognition elements. Reproduced with permission from (257).

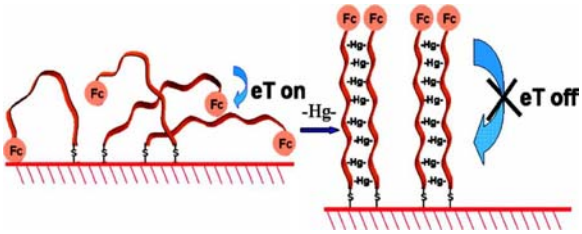

Figure 11. Supramolecular covalent strategy for Hg^{2+} detection. In the absence of Hg^{2+} ions, the poly-T adopt a random-coil conformation that allow the Fc-tag comes close of electrode and consequently an effective electron transfer (left), however after coordination of the probes to the Hg^{2+} centers the Fc tags away from electrode and electron transfer reaction is blocked (right). Reproduced with permission from (259). Copyright©2009 American Chemical Society.

Qureshi and co-workers (261) shown a highly sensitive and label-free multianalyte capacitive immunosensor that was developed based on gold interdigitated electrodes (GID) capacitor arrays to detect a panel of disease biomarkers. C-reactive protein (CRP), TNFα, and IL6 have strong and consistent relationships between markers of inflammation and future cardiovascular risk (CVR) events. Early detection of a panel of biomarkers for a disease could enable accurate prediction of a disease risk. The detection of protein biomarkers was based on relative change in capacitive/dielectric properties. Two different lab-on-a-chip formats were employed for multiple biomarker detection on GID-capacitors. In format I, capacitor arrays were immobilized with pure forms of anti-CRP, -TNFα, and -IL6 antibodies in which each capacitor array contained a different immobilized antibody. Here, the CRP and IL6 were detected in the range 25 pg mL^{-1} to 25 ng mL^{-1} and 25 pg mL^{-1} to 1 ng mL^{-1} for TNFα in format I. Sensitive detection was achieved with chips co-immobilized (diluted) with equimolar mixtures of anti-CRP, -IL6, and -TNFα antibodies (format II) in which all capacitors in an array were identical and tested for biomarkers with sequential incubation. The resulting response to CRP, IL6, and TNFα in format II for all biomarkers was found to be within the range of 25 pg mL^{-1} to 25 ng mL^{-1}. The capacitive biosensor for panels of inflammation and CVR markers show significant clinical value and provide great potential for detection of biomarker panel in suspected subjects for early diagnosis.

Assays based on nanomaterials, for example, are now beginning the transition from laboratory to the clinic. But the potential for such assays to become part of routine medical testing depends on many scientific factors, including sensitivity, selectivity and versatility, as well as technological, financial and policy factors (262). Recently, Roy and Gao (263) reported developments in the application of nanostructured-based biosensors with an electrical transduction mechanism, in ultrasensitive detection of biological entities, especially nucleic acids and proteins. Sepúlveda and co-workers (264) describe the basis behind Localized Surface Plasmon Resonance (LSPR) sensing and summarize the latest progress regarding nanostructure fabrication techniques and biosensing applications. The authors finally discuss the challenges that LSPR biosensors should face in order to be used in the near-future as commercial devices. However, Goulart and co-workers (265) presented comparisons between conventional methods of detection and biosensors obtained from synthetic peptides immobilized over the surface of graphite electrodes modified with polymeric films. The potential application was verified for three diseases as model (dengue, leprosy and leishmaniasis). Some of the more recent applications of the detection by biosensors of disease related analytes in body fluids have recently appeared in the literature including enzymes and antibody/antigen.

For applications involving enzymatic biosensors have the studies by Rahman and co-workers (266) that described the development of a biosensor for bilirubin determination in a human serum sample. The fabrication of the amperometric bilirubin biosensor begins complexing the Mn(II) ion with a conducting polymer and covering the surface with a thin polyethyleneimine (PEI) film containing ascorbate oxidase (AsOx). The biosensor specifically detect bilirubin through the mediated electron transfer by the Mn(II) ion. A linear calibration plot for bilirubin was obtained between 0.1 µmol L^{-1} and 50 µmol L^{-1} with the detection limit of 40 ± 3.8 nM. The bilirubin sensor exhibited good stability and fast response time (<5 s). Han and co-workers (267) showed the fabrication of a glucose biosensor based on enzyme reaction of glucose oxidase. The symptomatic therapy of diabetes mellitus requires reliable assessment of blood glucose level at frequent intervals. Tomato skin membranes have been successfully employed to entrap glucose oxidase for fabrication of glucose biosensor. The response of the biosensor showed a linear relationship with a concentration range of 1.0-30.0 mmol L^{-1} glucose. The limit of detection was

0.20 mmol L^{-1}. The recovery was 95.0-110.0% for 30 serum samples analysis. Hooda and co-workers (268) developed a system for measurement of serum total cholesterol. Inner bottom surface of PVC beaker was chemically modified for covalent immobilization of cholestryl esterase and cholesterol oxidase using glutaraldehyde as a coupling agent. HRP incorporated carbon paste working electrode was fabricated for amperometric measurement. The sensor showed optimum response within 20 s at pH 7.0 with incubation temperature of 45 °C. K_m and V_{max} for cholesteryl acetate were 760 mg dL^{-1} and 0.9 mA s^{-1}, respectively. The reaction cell lost 50% of its initial activity during its regular use for 200 times over a period of 100 days when stored in 0.1 mol L^{-1} sodium phosphate buffer, pH 7.0 at 4 °C. No metabolite interference was observed.

In other avenue of research, Jamal and co-workers (269) showed an electrochemical method to determine alanine aminotransferase (ALT) activity over its normal and elevated physiological range. The biosensor was developed based upon detection of L-glutamate at a glutamate oxidase-modified platinum electrode. Measurements were carried out in the presence of ALT co-substrates L-alanine and α-ketoglutarate and current response from either the oxidation of hydrogen peroxide or the re-oxidation of the mediator ferrocene carboxylic acid was employed. The sensitivity of the device was found to be 0.845 nA U^{-1} L ALT with t_{90} = 180 s, linear range 10–1000 U L^{-1} and LOD of 3.29 U L^{-1}. The enzyme electrode was tested over a 6-month period and found to retain 79% of its original activity towards ALT detection with >200 measurements performed over this time. Also, Patil and co-workers (270) investigated a biosensor for determination of Cytokeratin-7 (CK-7), a protein expressed in epithelial tissue, used to differentiate between types of cancers. Functionalized Au nanowires were used to enhance the sensitivity and selectivity of the cancer biomarkers detection. An enzymatic reaction between alkaline phosphatase enzymes with the *p*-nitrophenyl phosphate substrate resulted in an electroactive *p*-nitrophenol and redox active intermediate hydroquinone that has been detected electrochemically. A strong dependence of the anodic peak current with the concentrations of CK-7 resulted in detection down to 10 ng mL^{-1} concentration. Additionally, the cross-validation was assured using quantum dots-655 fluorescent markers. Hegnerová and co-workers (271) describes direct label-free detection of 17beta-hydroxysteroid dehydrogenase type 10 (17β-HSD10) using a Surface Plasmon Resonance (SPR) biosensor. This multifunctional mitochondrial enzyme is involved in pathogenesis of Alzheimer disease (AD) and represents a potential target for AD diagnostics. A multichannel SPR sensor with spectral modulation is functionalized with amyloid beta or polyclonal antibody against 17β-HSD10 peptide using a self-assembled monolayer of alkylthiolates and amino coupling chemistry. Detection of a synthetic peptide of 14 amino acids from the 17β-HSD10 molecule (residues 133–146 aa) and whole enzyme 17β-HSD10 is performed. Detection of 17β-HSD10 enzyme in artificial cerebrospinal fluid (ACSF) buffer is also carried out. It is demonstrated that the reported SPR biosensor is capable of detecting 17β-HSD10 enzyme at ng/ml levels.

8. SUMMARY AND PERSPECTIVE

As can be seen throughout this review, different types of electrochemical biosensors can be used to detect various diseases as well as the implementation of various systems of diagnosis. Also, we covered here different pathways in order to obtain nanostructured electrochemical biosensors and nanosensors; electrochemical DNA biosensors, electrochemical methods for gene analysis and molecular biology, immunoassays and immunosensors for clinical analysis (potentiometric immunosensors applied for diagnosis of disease and amperometric immunosensors) and enzyme-based nanostructured biosensors. As concluding remarks, we make an allusion of novel models for diagnosis and monitoring of diseases, since from nanostructured biosensors to body fluids measurements. Although remarkable versatility of electrochemistry applied to systems of diagnosis, it is believed that the next step in implementing these biosensors is linked to the development of more robust experimental apparatus and cheaper devices. Based on several papers in the literature, it's showed that the electrochemical biosensors are becoming increasingly attractive in the reproductive and diversity of molecular recognition systems. Combined with recent advances in methods for the preparation of nanomaterials and their use in electrochemical interfaces, it is foreseeable that in the near future new methods based on microchips and *in situ* and *in vivo* diagnosis becomes a reality. These are the great efforts that involve the development of electrochemical biosensing.

9. ACKNOWLEDGMENTS

The authors kindly thank the Brazilian agencies for financial support in their research activities, which were FAPESP, FAPEPI, FAPEMIG, CNPq, INEO and CAPES (Rede NanoBioMed-Brazil).

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Abbreviations: Deoxyribonucleic acids (DNA), methylene blue (MB), ethidium bromide (EB), ssDNA (single-stranded DNA), dsDNA (double-stranded DNA), single-stranded DNA (ss-DNA), PCR (Polymerase Reaction Chain), Electrochemical impedance spectroscopy (EIS), Human immunodeficiency virus (HIV), acquired immunodeficiency syndrome (AIDS), polyaniline (PANI), immunoglobulins (IgA, IgD, IgE, IgG, IgM), antigen (A_g), antibody (A_b), creatine kinase M (CK-M), breast cancer antigen (CA15-3), hepatitis B virus (HBV), hemoglobin (Hb), human chorionic gonadotropin (hCG), Candida albicans (CA), choline esterase (CE), α-1-fetoprotein (AFP), horseradish peroxidase (HRP), immunosorbent assay (ELISA), cancer antigen 125 (CA 125), poly-terthiophene carboxylic acid (TTCA), human interleukm 5 (IL-5), electroactive nanostructured membranes (ENM), indium tin oxide (ITO), polyvinylsulfonate (PVS), redox mediator (Me), Langmuir–Blodgett (LB), dipalmitoyl phosphatidyl glycerol (DPPG), glucose oxidase enzyme (GOx), indium tin oxide nanowire (ITO-NW), nanoparticle (NP), immunochromatographic electrochemical biosensor (IEB), prostate-specific antigen (PSA), rheumatoid arthritis (RA), quartz crystal microbalance (QCM), gold interdigitated electrodes (GID), C-reactive protein (CRP), cardiovascular risk (CVR), Localized Surface Plasmon Resonance (LSPR), polyethyleneimine (PEI), ascorbate oxidase (AsOx), alanine aminotransferase (ALT), Cytokeratin-7 (CK-7), 17beta-hydroxysteroid dehydrogenase type 10 (17β-HSD10), Alzheimer disease (AD), artificial cerebrospinal fluid (ACSF).

Key Words: Biosensors, Nanomedicine, Nanoscience, Electrochemistry, Review

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