Pure Appl. Chem., Vol. 76, No. 4, pp. 861–878, 2004.
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Trends and challenges in biochemical sensors for clinical and environmental monitoring*

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Abstract: Biochemical sensors have emerged as a dynamic technique for qualitative and quantitative analysis of different analytes in clinical diagnosis, environmental monitoring, and food and process control. The need for a low-cost, reliable, ultra-sensitive, and rapid sensor continues to grow as the complexity of application areas increases. New biosensing techniques are emerging due to the need for shorter sample preparation protocols. Such novel biosensor designs make field and bed-site clinical testing simpler with substantial decrease in costs per sample throughputs. In this paper, we will review the recent trends and challenges in clinical and environmental biosensors. The review will focus on immunological, nucleic acid, and cell-based clinical and biological sensors. Special emphasis will be placed on the approaches used for immobilization or biological reagents and low-cost electrochemical biosensors. The promising biosensors for rapid diagnosis of cancer or HIV are also discussed.

INTRODUCTION

As a result of the need for cheap, fast, and easy to use analytical tools during the last decades, biochemical sensors have emerged as a dynamic technique for qualitative and quantitative determination of different analytes for environmental, clinical, agricultural, food, or military applications. In a recent report, Thevenot et al. reviewed numerous definitions and classifications of electrochemical biosensors under the International Union of Pure and Applied Chemistry (IUPAC) nomenclature recommendations [1]. These definitions could be extended to other types of biosensors. A biosensor is defined as a self-contained quantitative or semi-quantitative analytical device, which is capable of providing quantitative or semi-quantitative analytical information using a biological recognition element either integrated within or intimately associated with a physicochemical transducer [1,2]. The review also affirmed that "a biosensor should be clearly distinguished from a bioanalytical system which requires additional processing steps, such as the addition of reagents". Patel et al. defined the term "sensor" as a device or system that responds to a physical or chemical quantity to produce a measurable output of that quantity [3]. This device also includes control and processing components: a biological receptor or recognition element as well as a physicochemical transducer.

The biological recognition component provides the specificity of the sensors, and this may be an enzyme, antibody (Ab), cell, receptor, or nucleic acid. Biosensors can be divided into two main groups based on the nature of the bioactive elements. These include bioaffinity sensors, which utilize the selective binding of the analyte of interest to a surface that has been modified by a specific receptor such

*Plenary lecture presented at the Southern and Eastern Africa Network of Analytical Chemists (SEANAC), Gaborone, Botswana, 7–10 July 2003. Other presentations are published in this issue, pp. 697–888.

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as antibodies or nucleic acids. The second group uses an immobilized biocatalyst such as a single or a multiple enzyme, cell, or an organism, which recognizes, binds, and subsequently transforms the target analyte (substrate) [1,3–7]. The reaction can be facilitated by the presence of electrochemical mediators, which also have an important role in removing interferences [5,8]. In enzymatic sensors, both the catalytic conversion of the substrate and the enzyme inhibition can be used to monitor the target analyte. Enzyme sensors are the most extensively studied and can be used for the detection of a large variety of analytes that are important for many practical applications. The first commercial success of biosensor was recorded as an enzyme sensor, currently known as a "glucose pen" used by diabetic patients for daily determination of glycemia in the blood [4]. The most developed bioaffinity sensors are immunoreceptors, which are based on antigen-antibody (Ag–Ab) interactions. The transducer is used to convert biological information into a quantifiable signal and could be optical [4,5,7,9,10], thermal [5,11], piezoelectric [3–5], or electrochemical [1,5,8]. Of these different transducers, electrochemical (amperometric or potentiometric) biosensors have been the most successful and the most widely used.

Biosensors should meet or exceed certain requirements so as to make them comparable or even better than the traditional analytical systems. They must be simple to handle, small, cheap, and able to provide reliable information in real-time. They also need to be sensitive and selective for the analyte of interest, and suitable for in situ monitoring. The exceptional combination of a biological element in straight contact with a physical transducer makes it possible to fulfill all of these requirements. When designing biosensors, it is important to understand the multiple factors that influence the performance of the sensing system. It is also important to consider the limitations of the biosensor, especially when the final goal is for application in real-sample monitoring. Despite extensive research in biosensors, few biosensors are routinely used in real applications [12–14]. In this paper, we present a review of the trends and challenges in biochemical sensors with specific focus on the most recent and promising applications in clinical and environmental monitoring.

IMMOBILIZATION OF BIOLOGICAL REAGENTS

The successful attachment of a biological receptor onto an electrode surface is considered the most critical step in the development of biosensors [1,4,7]. This is not surprising since the analytical performance of a biosensor is strongly affected by the chemistry of the immobilization process. Consequently, the success of the immobilization steps is strongly linked to biosensors' improved operational and storage stability, fast response time, wide dynamic range, as well as good sensitivity and reproducibility [15–18]. At present, it appears that there is no ideal immobilization technique available for all bioactive elements.

Depending on the nature of the electrode surface and physical transducer, several immobilization schemes have been used for attaching biologically active elements. The simplest procedure is physical adsorption, but it suffers from poor stability because of its weak bonds resulting from van der Waals interactions [4,7]. In addition, the adsorbed biomaterials are exposed to changes in the pH, temperature, and ionic strength. The most widely used method is the covalent binding with bifunctional reagents (such as glutaraldehyde or carbodiimide) [1,4,19–23] that allow a stable attachment and a quick response time. On the other hand, poor reproducibility and significant loss of activities are common with this method. The method is mainly used for enzyme immobilization [4,21] and could also be extended to other biological elements (such as antibody or nucleic acids) [20,22–25].

Covalent attachment of amino-modified oligonucleotide probe has been used to develop a DNA microarray onto glass and silicon surfaces using 1,4-phenylene diisothiocyanate (PDITC) as bifunctional linker [24]. Also, we recently reported a novel approach for the covalent attachment of dsDNA molecules inside a glass capillary tube using a bifunctional succinimidyl cross-linker that was anchored to the substrate via mercaptosilane. The resulting glass-modified capillary was subsequently used to develop a fluorescence biosensor [20]. The same procedure could be used to immobilize Ab onto the surface of identical capillary [23]. In order to develop an integrating waveguide, avidin was covalently at-

tached to the interior surface of the capillary and was treated with appropriate biotin-conjugated captured antibodies. In another work, antibodies were successfully immobilized onto the surface of a glass sensor chip modified with dextran (coupled either covalently via silane or via biotin/avidin). This was subsequently used to bind to the corresponding Cy5-labeled antigen used in a fluorescence sensor assay [26].

A particularly attractive method to immobilize biomolecules can be achieved via entrapment in the electrogenerated conducting polymers (such as polypyrrole, polyaniline, or polythiophene), thus resulting an extremely simple, rapid single- or multiple-step procedure [26–29]. Electrodeposited polymers could be developed in the presence or absence of biomolecules. The ideal polymer for immobilization of biomolecules should possess functional groups to facilitate covalent binding with the bioreagent. Due to low oxidation potential necessary for its synthesis, polypyrrole (PPy) has been the most widely used polymer. In addition, its electropolymerization can occur in aqueous solutions, and this feature is compatible with most biological molecules. For instance, following a galvanostatic electropolymerization of PPy, we have successfully entrapped anti-IgG onto a Pt surface [25]. The immobilization of Ab was also achieved by electrostatic binding between the terminal cyano group of the *N*-substituted PPy-bearing CN and the hydroxyl groups on the heavy chains of the Ab [29]. Electropolymerization of pyrrole-modified biotin allows successive attachment of avidin and biotin-labeled glucose oxidase, thus resulting in an efficient glucose biosensor [28,30]. We have demonstrated that under applied pulsed potential, Ab–Ag binding can occur in a reversible manner [31]. By immobilizing polyclonal anti-PCB antibody into the conducting PPy membrane, we obtained the highest activity compared to similar immunosensors prepared through physical entrapment or simple adsorption [30,31].

The use of electropolymerized PPy also allows DNA immobilization on a variety of surfaces. Basically, the procedure involves the formation of biotinylated PPy subsequently used as anchoring points for the immobilization of avidin units due to the high affinity of the biotin/avidin interaction. The attachment of the biomolecules to biotinylated polymers through avidin/biotin affinity interactions was fully discussed by Cosnier et al. [27]. These grafting units can be regenerated by breaking the biotin/avidin bridge, thus rendering the matrix reusable [30]. This method allows the control of polymer/biomolecule layer, but could also lead to the denaturation of the active element during the immobilization process, while the amount of immobilized biomolecules is restricted to a monolayer at the polymer-solution interface [27]. The method is suitable for the fabrication of small surface area biosensors, thus opening a new way for the development of microbiosensors. Commercially available biotinylated reagents and avidin-conjugated biomolecules offer a great promise in the future. Preservation of bioactivity during immobilization can be achieved by using physical entrapment in a polymer matrix. This method is preferred due to its simple procedure [16]. However, it suffers from several limitations, such as possible diffusion barriers, imposed by the presence of the polymer layer. Recently, low-temperature sol-gels have been used as immobilization matrix on different supports. So far, these have mostly been applied for the stable attachment of enzymes [16,32].

In addition to those already discussed, other techniques are now available for the immobilization of biological elements. These include Langmuir–Blodgett films [3], self- assembled monolayers [21,33,34], or surfaces modified with metal chelate such as nickel–nitrilotriacetic acid (NTA) [17,18]. These methods are especially attractive because they ensure a controlled and oriented immobilization, thus improving the sensitivity and the detection limit of the biosensor. For instance, Storri et al. have investigated the use of SAM with further covalent binding of proteins onto the gold piezoelectric quartz crystal [35]. The analytical characteristics of the resulting immunosensor were compared with simple adsorption and avidin/biotin binding procedures. These sensors were used to study the reaction of the immobilized BSA and anti-human IgG with their specific antibodies. The last two methods have been also used to attach synthetic single-stranded oligonucleotides onto other surfaces such as graphite screen-printed electrode [36]. More recently, Tombelli et al. presented an interesting comparison between four different methods based on SAM and their application for oligonucleotide immobilization

on gold-coated piezoelectric crystals [37]. The resulting DNA sensors were tested and characterized with respect to the activity of immobilized probe, measurement of the hybridization reaction, possibility of regeneration, and nonspecific adsorption. Three of the four immobilization procedures involved biotin–streptavidin interaction, which is widely used for the immobilization of biotinylated DNA. In this case, regeneration of the single-stranded probe was obtained using HCl after each hybridization cycle.

An interesting approach for the immobilization of dsDNA on screen-printed modified electrode was recently reported by Wang et al. [38]. The attachment was realized through 5-ft phosphate groups of dsDNA by the formation of phosphoramidate bond with the amino groups of a self-assembled cystamine monolayer. We reported a new immobilization chemistry by means of enzyme-modulated cleavage of dsDNA. This new immobilization chemistry was designed using the specificity of cytosine residues at ss vs. ds DNA loci on metal substrates after enzyme cleavage. Although enzyme cleavage of DNA is widely used in biological sciences for the preparation of nonisotopic probes for nucleic acid hybridization assays, it has not been explored for biosensor immobilization. Thus, prior to immobilization (Scheme 1), the ds-DNA was modified via a bisulfite-catalyzed transamination of cytosine after endonuclease cleavage of plasmid DNA, resulting in N4-substituted labels [39]. In another report, we have shown that supramolecular immobilization of biotinylated dsDNA onto a self-assembled monolayer of avidin could provide a generic format for detecting small-molecular-weight organics [40].

Scheme 1 The reaction of biocytin hydrazide with cytosine (cytidine) residues of dsDNA.

With the recent progress in genetic engineering, proteins can be modified to introduce accessible moieties, which can further permit biomolecule immobilization onto the electrode surface in a controlled manner [17,18,41]. Some of these mutants are also modified to ensure better stability and specificity. Currently, many laboratories are focusing their efforts onto the development of new and innovative immobilization strategies. Most likely, these will result in enhanced analytical characteristics of the biosensors, especially with respect to the response time, stability, and lifetime. Thus, the successful practical application of biosensors is strongly dependent on how efficient the biological molecule is attached to the transducer surface.

BIOSENSORS FOR CLINICAL MONITORING

In the clinical sector, biosensors have enormous potential for real-time diagnosis of many diseases. When designed for clinical investigations, biosensors are expected to offer advantages of extra-labora-

tory analysis that can include hormones, steroids, or other drugs of abuse and metabolites [42–48]. The usefulness of a clinical test using a biosensor is also determined by its sensitivity, selectivity, and the ability to detect the disease with no false-positive results [4]. So far, the most successful biosensor for clinical analysis is the electrochemical glucose sensor for the determination of glucose in blood [43,44,48]. These biosensors are now commercialized in different configurations mostly available in single-use formats.

Commercial antibody-based biosensors or immunosensors have not been so successful. Due to their unique characteristics, immunosensors show great promises of complementing laboratory-based techniques and in providing rapid detection in clinical laboratories. Immunosensors are a subset of biosensors consisting of biological sensing elements or receptors [e.g., antibody (Ab) or antigen (Ag)] in close contact with physicochemical transducers (e.g., electrode or optical fiber). The measurement of a target analyte is achieved by the selective transduction of the receptor-target analyte reaction, resulting in a quantifiable electrical or optical signal. The expansion of biosensors for continuous monitoring of other clinical analytes have been reported [43,44,48]. Georganopoulou et al. described the two main strategies for using biosensors in clinical diagnosis: as directly implantable into the tissue or in the outflow of a microdialysis sampling probe [45].

Noninvasive biochemical systems for rapid clinical diagnosis of disease such as cancer, HIV, hepatitis, and skin or dental diseases are gaining increasing acceptance. For instance, Ivnitski et al. recently described the design of a new portable, noninvasive electrochemical hand-held biosensor as diagnostic indicator of dental diseases [46]. The sensor is based on functional relationship between the total level of salivary peroxidase, determined with the designed biosensor, and the clinical status of periodontal disease. Using a biosensor commercialized by Uppsala (BIACORE 100, Biacore AB) Gomara et al. detected Ab against hepatitis A (HAV) in human serum sample [47]. Biosensors for in vivo and in vitro clinical analysis need special requirements. These include sterilization protocols to avoid denaturation of immobilized biological element and the necessary diffusion of the analyte from undiluted sample into the active bioselective layer. In addition, clinical biosensors require periodical recalibration and long-term stability [45,48].

BIOSENSORS FOR HSA DETECTION

The determination of protein is of great importance in analytical chemistry and medicine. The rate of urinary excretion of human serum albumin (HSA) can be used to diagnose incipient renal disease, and its progression is one of the most studied proteins using biosensor devices [49–57]. Electrochemical techniques can provide rapid and direct method for HSA determination. In our laboratory, we have developed immunosensor based on the interaction between human serum albumin and its specific antibody (anti-HSA) immobilized within PPy membrane [50]. In this case, the Ab–Ag interaction was monitored by pulsed electrochemical detection, cyclic voltammetry and impedance spectroscopy using different potential routines. The feasibility of developing a generic system using a simple antibody-attachment procedure or simple electrolyte counterions on electrode surfaces has been investigated. When used in a flow-injection analysis mode, and with a pulsed amperometric detection, signals having adequate sensitivity (low parts per million, ppm, or sub parts-per-billion, ppb) are obtained. A further fascinating phenomenon observed with the system is the capacity to control antibody-antigen (Ab–Ag) interaction, thus making reversibility possible, and rendering the immunological sensors reusable. When compared with conventional enzyme-linked immunosorbent procedure, the time and efficiency of clinical measurements could be significantly reduced (Fig. 1).

In addition, the effects of variation in ion exchange, solution composition, and the condition of the synthesis have been used to examine the capacitive behavior of anti-HSA-containing PPy electrodes in the presence of HSA [51–53]. The performance of this system with respect to electrical signal generation, reusability, and reproducibility has been intensely studied and investigated. Also, interaction of anti-HSA–HSA was monitored using electrochemical quartz crystal microbalance (EQCM) [54,55].

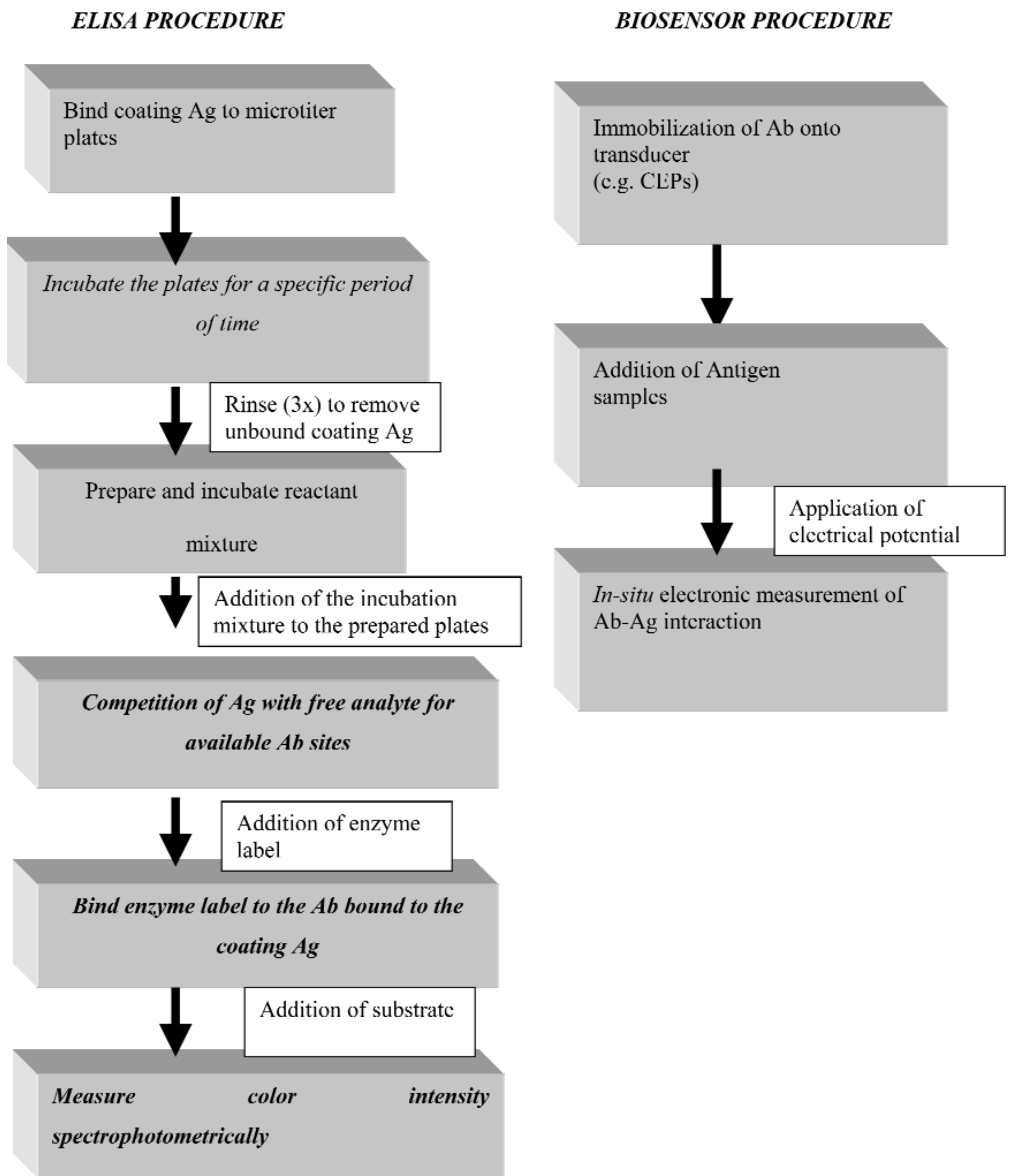

Fig. 1 Measurements steps involved in ELISA and pulsed electrochemical biosensor.

Riepl et al. developed a capacitive affinity biosensor for HSA determination using self-assembled monolayer technique for anti-HSA immobilization [56]. The occurrence of HSA, human fibrinogen (Fg), IgG, and IgM in the plasma deposits was studied with an optical biosensor by binding the respective antibodies onto the electrode surface by covalent cross-linking with glutaraldehyde (GA) [57]. Quantitative monitoring of urinary albumin was also achieved by using thick-film screen-printed electrodes on which anti-HSA was immobilized. The system is based on conductimetric detection utilizing colloidal gold modified with conducting polymer [49].

Potential applications for HIV

Currently, the standard diagnostic test for HIV infection is ELISA technique. In this approach, viral HIV antigens are adsorbed onto a solid interface, typically a microtiter plate. The immobilized antigens are then exposed to the HIV antibody (Fig. 1). The recognition of the HIV-antigen with antibody is registered via indirect enzymatic process using either a secondary antibody, or enzyme-linked proteins for detection. However, the multistep procedure involved, coupled with the less than adequate recovery of the sensing surface, necessitates the development of more reliable approaches for detecting and quantifying the human immunodeficiency virus type 1 (HIV-1). In the field of biosensors, the application of SPR-based optical techniques could contribute extensively to a greater understanding of the functional aspects of HIV infection and control [58]. The first application of commercial optical biosensor technology involved epitope mapping of monoclonal antibodies (mAbs) against the HIV capsid protein [59,60]. Markgren et al. reported SPR method on the recognition of immobilized HIV-1 protease by different inhibitors. The approach allows the characterization of inhibitors with $K_i > 5$ nM or identification of inhibitors with $K_i < 100$ mM [61,62]. Alterman et al. studied the interaction of a series of 17 structurally diverse inhibitors for the HIV-1 protease immobilized onto Biacore electrodes during SPR analysis [63]. Wang et al. developed an electrochemical biosensor for the detection of short DNA sequence related to HIV-1 [64]. The sensor relies on the immobilization and hybridization of the 21- or 42-mer single-stranded oligonucleotide from the HIV-1 U5 long terminal repeat (LRT) sequence at carbon paste or strip electrode. A detection limit of 4×10^{-9} M HIV-1 U5 LTR segment was obtained following 30 min hybridization. Cavic et al. evaluated the real-time detection of the binding of peptides to HIV-1 TAR RNP immobilized onto piezoelectric quartz crystals [65]. Different responses for different peptides adsorbed onto sensor surface were obtained. Aberl et al. developed a quartz crystal microbalance immunosensor for label-free determination of antibodies against HIV [66]. The application of biosensors for HIV research demonstrates how biosensors can be used in quantitative and qualitative mode to detect binding interactions.

Potential application of biosensors for cell cancer monitoring

Biosensors could be used by physicians and clinical workers for the early detection, diagnosis, and classification of different forms of cancer. In the context of cancer monitoring, biosensors can serve two major functions: (i) identifying and quantifying new biological molecules and (ii) studying biological processes involved in cancer development and progression. The possibility of using minimally invasive analytical instruments to monitor biochemical process within a single cell could also provide great advances in understanding cellular function and may offer an enormous potential in cell biology [67,68]. However, current research in this direction is at a very early stage and many efforts are necessary to obtain reliable instrumentation for intracellular measurements.

Vo-Dinh et al. [67] described the use of a fiber optic nano-immunosensor for intracellular quantitative detection of benzo[a]pyrene tetrol (BPT) inside the cytoplasm of two mammalian cell lines (human mammary carcinoma and liver epithelia cells). BPT is a biomarker for human exposure to the more potent carcinogen among the polynuclear aromatic hydrocarbons, the benzo[a]pyrene (BaP). The same research group designed an antibody-based fiber optic nanosensor for in situ monitoring of BaP in a single cell (MCF-7 human breast carcinoma cell line) [68]. Ayllot et al. recently highlighted current developments and practical challenges of optical nanosensors for intracellular measurements [69].

Schmidt et al. developed a biosensor system for measuring human telomerase activity, a eurokaryotic ribonucleoprotein complex, which is considered as an important component in the development of tumor cancer cells [70]. The sensor is based on total internal reflection fluorescence produced by the incorporation of fluorescence-labeled nucleotides. In this case, the PS-modified oligonucleotides were covalently immobilized onto a silanized fiber, which act as “the binder or as the substrate” for the telomerase. A DNA pencil-based biosensor described by Wang and Kawde was useful for the detection

of single-point mutation oligonucleotides in the BRCA1 breast cancer gene [71]. The applicability of impedance spectroscopy for studying alterations in the morphology of cell aggregates, apoptosis, or necrosis was also demonstrated by monitoring the electric behavior of membranes and extracellular space [72]. The sensor follows the model of a multicellular spheroid and offers a novel approach for anticancer therapies. In another paper, an amperometric reticulated vitreous carbon enzymatic sensor was designed for coulometric detection of $NADH/NAD^+$ in normal and cancerous cells. This research was based on the hypothesis of a difference in the NADH content in normal and cancerous cell tissue [73].

A novel electrochemical technique was designed to study the effect of several anticancer drugs (cisplatin, adriamycin, vinblastine) on the human ovarian carcinoma cell line A2780 and its adriamycin (A2780adr) and cisplatin (A2780cispt) resistant variant [74]. The sensor was used to detect and monitor real changes in cell behavior using changes in the electrochemical potential at the cell/surface interface in the extracellular microenvironment. Recently, scanning electrochemical microscopy (SECM) was developed as novel technique for monitoring living cells by measuring the intracellular redox activity [75]. The sensor was used to study charge-transfer reactions in normal and metastatic human breast cells and for cell differentiation.

BIOSENSORS FOR ENVIRONMENTAL MONITORING

Environmental monitoring typically involves several steps such as sampling, sample handling, and sample transportation to a specialized laboratory and finally a laboratory to determine the chemical composition and to establish the toxic effect. These conventional approaches are expensive, time-consuming, and require highly trained personnel. Thus, the multiple steps involved frequently prevent rapid information about the composition and/or the toxicity of the sample to be obtained in efficient manner. Thus, remediation efforts could take months or years. Consequently, the need for fast, sensitive, selective, and cheap alarm systems is becoming more apparent.

Presently, a great amount of scientific research has been devoted to designing such devices [76]. Many reports emphasized the effectiveness of biological sensors for real-time monitoring of important environmental analytes [77–85]. The most studied classes of analytes include pesticides, polychlorinated biphenyls, and heavy metals. Farre and Barcelo highlighted recent developments in biosensors based on acute toxicity measurements in wastewater and sewage sludge [86]. Recently, since epidemiological studies have shown that many synthetic chemicals, present in the environment, affect the body endocrine system, much effort is being focused on studying the behavior and development of sensitive detection methods for these compounds [87]. Other studies are focused on the determination of some bacterial toxins. For instance, electrochemical characterization of microcystin-LR [79] and the design of a new electrochemical, DNA biosensors for the detection of the *Microcystis* species have been reported [80]. The detection limit of this biosensor was 9×10^{-11} M target oligonucleotide related to *Microcystis* spp.

Biosensors for polychlorinated biphenyls (PCBs)

The persistence of polychlorinated biphenyls (PCBs) in the environment and their extensive usage in numerous industrial and commercial applications are currently of great concern. PCBs are mixtures of synthetic organic chemicals commercially manufactured under the name of Aroclors [31,88]. PCBs first became recognized as potential environmental hazards in 1970, and over the years, numerous regulatory agency activities led to a better and broader understanding of PCBs as an environmental issue. These compounds can be persistent environmental contaminants and may be accumulated through the environment to food and to humans. However, PCB toxicity and the real effects on human health continue to evolve. The limited information on the effect of PCBs indicated that these compounds might produce immunological abnormalities, reproductive dysfunction, or an increased thyroid volume, in-

creased prevalence of thyroid and liver disorders [88,89]. Nowadays, there is an increased interest to detect these environmental chemicals from both scientific and regulatory communities. Consequently, many efforts are concentrating in order to develop a fast and reliable method for their determination.

The analysis of PCBs is currently carried out using traditional analytical methods such as gas or liquid chromatography with electron capture detector or tandem GC/MS, mass spectrometers, and supplementary confirmatory techniques as infrared spectrometry [31,88–93]. In spite of their performances, these methods are too expensive for screening purposes [31] and generally require extensive sample preparation including extraction with organic solvents, clean-up, or separation [89–93]. In addition, these methods offer insufficient selectivity, and also, in some cases, the preparation steps are susceptible to insufficient recovery and/or contamination. However, increasing method sensitivity is costly and sometimes technically difficult with conventional analytical procedures. Fewer other expensive, but readily available techniques could be used for PCB detection. These include immunoassay (IA), enzyme (ELISA), fluorescence or radioimmunoassay (RIA) [89,93–95]. These techniques exploit the specific binding of a PCB to a unique antibody and are generally used for screening purposes since a large number of samples can be analyzed in a short time. Few colorimetric kits controlled by a portable photometer are now available in different configurations and could be used for the detection of PCB level.

Immunosensors have been reported to exhibit considerable potential for PCB detection [31,94,96]. For instance, we have developed a PCB immunosensor, constructed by immobilizing anti-PCB antibody into a conducting polypyrrole (PPy) membrane. Pulsed-accelerated immunoassay for signal generation in stationary cell or FIA by applying a pulsed waveform between +0.60 and –0.60 V and a pulse frequency of 120 and 480 ms. With the optimized sensor, a linearity of 0.3–100 μg/l and a detection limit of 3.3, 1.56, 0.39, and 1.66 ng/ml, respectively, were obtained for Aroclors 1242, 1248, 1245, and 1016 [31].

As low as 2.5 ng/ml PCB was detected using a system based on competitive immunoassay coupled with SPR [96]. In this case, the anti-PCB were immobilized onto a gold thin-layered sensor chip by covalent coupling using *N*-ethyl-*N*′(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCH)/*N*-hydroxysuccinimide (NHS). Other detection techniques such as quartz crystal piezoelectric or fiber optic have been also used for PCB detection. For instance, detection of 4,4′-dichlorobiphenyl was achieved using a piezoelectric biosensor with Ab immobilized onto the gold electrode using self-assembled monolayers of thiol [97]. A fiber optic immunosensor consisting of a quartz fiber coated with anti-PCB antibodies and subsequently bound with a fluorescein conjugate allowed the detection of various Aroclors, with different sensitivity and selectivity [98]. The biosensors detected 1 ppm Aroclors (tested: 1016, 1232, 1250, and 1262), but not polychlorinated pesticides, polychlorinated phenols, or trichlorobenzene. Roberts and Durst reported an immunosensor for rapid detection of PCBs based on the principle of immunospecific liposome migration [99]. The sensor was designed in two configurations: the first measures the competitive reaction between analyte-tagged liposomes and the sample analyte for immobilized Ab and allow detection of 0.4 nmol PCB in less than 8 min. The second configuration uses the principle of immuno-aggregation between anti-PCB antibodies and analyte-tagged liposome and is more sensitive than the first one with detection as low as 2.6 pmol PCB in less than 23 min. Recently, Laschi et al. reported the detection of PCB in food samples using an electrochemical immunosensor based on differential pulse voltammetry (DVP) and an enzyme label (alkaline phosphatase) [100]. The sensor was used to detect Aroclor mixture (1242 and 1248). The results demonstrated the possibility of using the biosensing devices as screening method in food sample as the results were comparable with the standard method, high-resolution gas chromatography (HRGC-LRMS) according to ISO 17025.

Most studies with immunosensors are carried out in aqueous solutions in which large molecules as Ab function ideally. However, PCBs, as well as other important environmental analytes, are poorly soluble in this medium. In addition, extraction and concentration of the sample are commonly carried out in organic solvents. In this context, a detection method for these compounds in the presence of or-

ganic medium is also required. In most cases, the immunological activity of immobilized Ab is generally lower in organic solvents compared to water. Significant improvement in this direction was achieved when Ab was encapsulated in reversed micelles [97]. Detection of PCBs was also achieved using DNA biosensors designed for environmental monitoring. In a recent work developed in our group, we reported a detection limit of 10 nM PCB using supramolecular dsDNA sensor on Ag-Au coated quartz crystal electrode with impedance spectroscopy [40]. Using an electrochemical system, Marrazza et al., detected as low as 0.2 mg/l PCBs using screen-printed disposable DNA biosensor [36]. In this case, determination was achieved by measuring changes of the electrochemical signal of guanine in calf thymus DNA extract immobilized onto the electrode surface.

Biosensors for endocrine-disrupting chemicals

In recent years, it has become evident that many environmental chemicals, including synthetic and endogenous estrogens, can mimic, block, or alter the action of endogenous steroid hormones and can interfere with hormone-regulated physiological processes [87,101–104]. These industrial and environmental chemicals are known as endocrine-disrupting chemicals (EDCs) and structurally resemble endogenous estrogens. They have been analyzed for many decades in numerous biological and medical investigations. Screening and confirmatory strategies for these steroids involve chemical or immunochemical methods followed by the complete instrumental confirmation of steroids by mass spectrometry. Until recently, the standard technique for analyses has been GC/MS. Moreover, the limits of detection were not sufficient to analyze steroids at low levels in urine and environmental samples. Also, these techniques typically require sample pretreatment, expensive apparatus, and skilled personnel. Recently, Lopez de Alda and Barcelo have discussed the current "state of the art" of available analytical methods for the determination of estrogens as environmental pollutants in wastewater [101]. The authors reviewed the more traditional techniques including a detailed analysis of all procedural steps for sample preparation. Due to estimated tens of thousands of chemicals under consideration for screening as potential endocrine disruptors, it is essential that rapid, sensitive, and reproducible high-throughput screening systems be developed.

Synthetic estrogens are characterized by the presence of phenolic functional groups, a common structural feature that is also found in natural estrogens. This structural feature could facilitate binding to estrogen receptor [101,104–107] and possibly generate receptor-induced transformations. Sadik et al. presented a summary of different approaches reported for EDCs [77] and also demonstrated the feasibility of in situ monitoring of the interaction between bisphenol A (BPhA) and dsDNA [40]. In a recent report, Ngundi et al. demonstrated the comparative electrochemical behavior of β-estradiol and selected EDCs, specifically alkylphenols, and proposed a possible link between the structure and their estrogenic activity [78]. Figure 2 shows a few examples of EDCs.

Recently, EDCs have been analyzed using fluorescence chemo-sensor, an impedance sensor, and an electrochemical sensor using electroactive 17β-estradiol labeled with daunomicin [108–110]. The extensive synthetic procedure required for labeling 17β-estradiol implies that alternative procedures be also considered. Matsunaga et al. reported a new fully automated immunoassay designed for the detection of alkylphenol ethylates (APEs), BPhA, and linear alkylbenzene sulphonates (LASs) using monoclonal antibodies, chemically conjugated to bacterial particle and alkaline phosphatase (ALP)-conjugated EDC [111]. Using this technique, concentrations of EDC were evaluated by the decrease in luminescence based on the competitive reaction of EDC and ALP-conjugated EDCs. Detection range recorded was between 6.6 ppb–66 ppm APEs, 2.3 ppt–2.3 ppm BphA, and 35 ppt–35 ppm LASs, respectively. Good correlation with the results was obtained with ELISA, and chromatographic GC/MS or LC/MS techniques. In another approach, the xenoestrogen BPhA and a natural phytoestrogen genistein were determined by fast impedance measurements [112]. The electrochemical system facilitates quantification of estrogen binding to the native receptor and allowed further characterization of the lipid bilayer structure by monitoring conformational changes in the membrane.

Bisphenol A

Nonylphenol

Diethylstilbestrol

Fig. 2 Examples of endocrine-disrupting chemicals.

The current report from our group demonstrated a promising approach for determining EDCs using a simple amperometric tyrosinase sensor (Tyr-CP). The detection principle is based on the ability of tyrosinase to catalyze the oxidation of the phenolic estrogens to *o*-diphenol and *o*-quinone, which is subsequently reduced at low potentials. Tyrosinase is a well-known enzyme that catalyzes the oxidation of phenols and diphenols. A tyrosinase-modified boron-doped diamond electrode was also developed for the determination of EDC BPhA and β-estradiol in a flow-injection system [113]. Carbon paste electrodes are preferably used, with respect to their simplicity and low cost, but also because of the low noise and background currents [114]. Application of Tyrosinase sensors has been restricted almost exclusively to phenol, catechol, and simple substituted phenolic compounds [114,115] such as chlorophenols, nitrophenols, and aminophenols, also important for environmental monitoring.

Figure 3 shows a typical current-time plot for the Tyr-CP sensor to consecutive injections of 1 μM BPhA to air-saturated 0.1 M phosphate buffer solution at pH 6.5 under continuous stirring. The reaction was monitored at an applied potential of –150 mV. As can be seen upon successive addition of phenolic derivative, a well-defined reduction current proportional to the concentration is observed, proving the effectiveness of the sensors for detecting BPhA. The mechanism for the detection of phenolic estrogens using tyrosinase most likely involves the tyrosinase-catalyzed oxidation of phenolic compounds at the B ring to yield the corresponding *o*-quinone product. Figure 3 (inset) illustrates linear calibration range of BphA with the error bars corresponding to the standard deviation for $n = 3$ measurements under the same experimental conditions. In terms of analytical performance, the sensor presents a linear range between 1–20 μM with a sensitivity of 29.69 mA/M and a detection limit of 0.15 μM BPhA (S/N = 3). Table 1 summarizes the results obtained with Tyr-CPE for phenol, catechol, and BPhA. The same sensor tested for other phenolic compounds has shown that the chemical structure of EDCs (the nature and the position of aryl ring substituents) as well as their spatial arrangement of the substituents could affect the detection limit and the sensitivity. These results will be described in details in another paper.

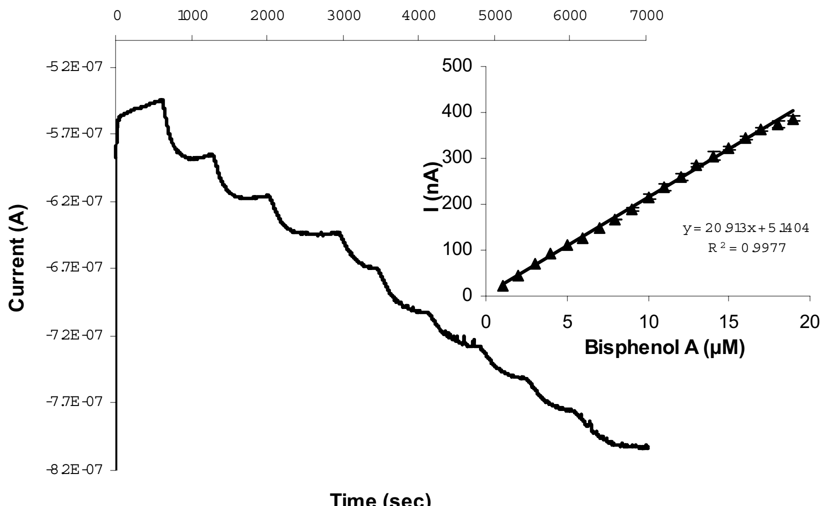

Fig. 3 Typical amperometric response of Tyr-CPE obtained at –150 mV vs. Ag/AgCl to successive addition of 1 μM BPhA. Inset graph: linear calibration range (n = 3).

Table 1 Analytical characteristics of Tyr-CPE for the detection of phenol, catechol, and BPhA.

Name	Linear range (μM)	R2	Detection limit* (μM)	Sensitivity** (mA/M)	Resp. time (min)
Bisphenol A	1-20	0.9977	0.15	20.91 (+/-0.99)	3
Phenol	1-15	0.9827	0.01	222.91 (+/-48)	2
Catechol	1-20	0.9939	0.025	208.53 (+/-42)	2

*DL = detection limit (corresponding to S/N = 3).
**n = 3 measurements with the same sensor under the same conditions.

Biosensors for detection of pathogenic bacteria

Bacterial pathogens are found widely in soil, food, and marine and estuarine waters, but also in intestinal tracts of humans and animals. Microbial diseases constitute one of the major causes of death in many developing countries. For this reason, significant efforts are needed to develop new systems for rapid detection of pathogenic bacteria in a variety of fields. Among these, enzyme, DNA, or immunosensors coupled with electrochemical, piezoelectric, optical, acoustic, and thermal detection represent viable alternatives [14,116,117]. The most studied bacteria are *Salmonella thypihmurium* and *enteritidis* [118,119], *Escherichia coli* (0157:H7 and other types) [120–123], *Listeria monocytogenes* [124], *Staphylococcus aureus* [125], and *Cryptosporidium parvum* [126]. Generally, using biosensors, pathogenic bacteria are detected in concentration range of 50–10^6 cells/ml [117]. Rowe et al. [127] developed a multianalyte fluorescent-based array immunosensor that is capable to simultaneous identification of bacterial, viral, and protein analytes. Thus, *Bacilus globigii*, *MS2 bacteri-*

opage and *Staphylococcal enterotoxin* were detected with the lower detection limits (LODs) of 105 cfu/ml, 107 pfu/ml, and 10 ng/ml, respectively. One of the first commercial applications of biosensors was dedicated to analyze pathogenic bacteria. SPR-based biosensing from BIAcore AB (Upsala, Sweden) commercialized a large range of biosensors, which includes several generation of first BIAcore [128].

FUTURE PERSPECTIVE

The current trends in biosensors development are conducted through small, easy to use, and fast sensors, so called "smart" systems. The key issue to be addressed in the future is the increasing demand for higher sensitivity and selectivity that will allow molecules to be monitored in real time at a minimal cost. The future biosensor is expected to function on the principle of "laboratory on a chip" [6], having all the essential components microfabricated on a chip with the aim to simplify and extend reliable monitoring of the analytes outside the central laboratory [5]. However, the real success in the development of a reliable biosensor for clinical and environmental applications depends on the results of combined efforts of scientists from many fields, including biologists, physicists, chemists, and engineers. In this context, considerable progress is expected in the field of bioelectronics that will facilitate efficient signal transduction from biological recognition element to electronic or optical device and vice versa. Future biosensors will require the development of new reliable devices or the improvement of the existing ones in order to allow superior transduction, amplification, processing, and conversion of the biological signal. Efficient biosensors will not necessarily function as a stand-alone detector, but will form an integral part of an analytical system. Compact and portable devices will constitute another future area of intensive multidisciplinary sensor research. Considerable progress is expected in genetic engineering for the production of enhanced stable and selective bioreceptors as components of efficient recognition elements, such as abzymes (antibody-possessing enzymatic activity) [3]. Synthetic peptides and protein nucleic acids will continue to contribute to the realization of practical biosensors. In the future, the principal application of biosensors in environmental monitoring will be as screening tools for multiple analytes. Progress biosensor research will enable the realization of automated diagnostic instruments for clinical analysis.

CONCLUSIONS

Biochemical sensors have emerged as a dynamic technique for qualitative and quantitative determination of different analytes for environmental, clinical, agricultural, food, or military applications. Despite the enormous potentials compared to laboratory-based analytical techniques, numerous problems still remained to be solved. Most biosensors have shown excellent characteristics for synthetic samples, pristine laboratory samples, they are not sufficiently robust in real samples. Most of the existing limitations could be directly related to operational and/or long-term stability of the biological receptor and/or the physical transducer. Other limitations could be attributed to poor reproducibility between sensors and selectivity in complex matrices. For practical applications, the most important obstacles are encountered once the sensor is used outside pristine laboratory conditions and is applied for in situ real sample monitoring.

Currently, there is no doubt that biosensors constitute the solution of many important problems encountered in conventional measuring techniques and may open new areas of modern analysis. But even if a real progress is achieved in this field, the biosensor market is still relatively small, requiring other optimization research studies in order to fully explore their real potentials. Today, more than 90 % of commercial biosensors are designated to glucose analysis [14]. Despite the availability of several commercial device, developed by principal manufacturers (Biacore AB: Biacore 1000, 2000, 3000, X, J, S51; Affinity Sensors: Iasys; IBIS Technologies: Ibis 1 and Ibis 2, Nippon Laser Electronics: SPR670 and SPR Cellia; Texas Instruments: Spreeta) [10], only very few analytes can be detected. As stated ear-

lier, this is mainly a consequence of insufficient reliability associated with poor stability of the biomaterial, multiple matrix effect, and also a dependence upon the physicochemical parameters and interferences within the transducers. Nevertheless, with respect to rapid, sensitive, and selective low- cost determination of a great variety of analytes, no suitable alternative exists for biosensors.

ACKNOWLEDGMENTS

This work was performed under NSF grant number CHE-0210968 and the U.S. Environmental Protection Agency grant number RD-83090601-0. OAS acknowledges the National Science Foundation for travel fund to attend the SEANAC conference. SA acknowledges the NSF-NATO for postdoctoral fellowship.

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