BIOSENSORS

1. Introduction

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. Chemical sensors usually contain two basic components connected in series: a chemical (molecular) recognition system (receptor) and a physicochemical transducer (1). Biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism (2,3). Historically, the term biosensor has been variously used to a number of devices either used to monitor living system or incorporating biotic elements. However, the current consensus is that this term should be reserved for sensors incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism, or cell where the biological element is in intimate contact with the transducer. A modern definition of a biosensor is as follows: *"A compact analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer. The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals that are proportional to a single analyte or a related group of analytes."* (4).

Figure 1 presents a generalized scheme of a biosensor.

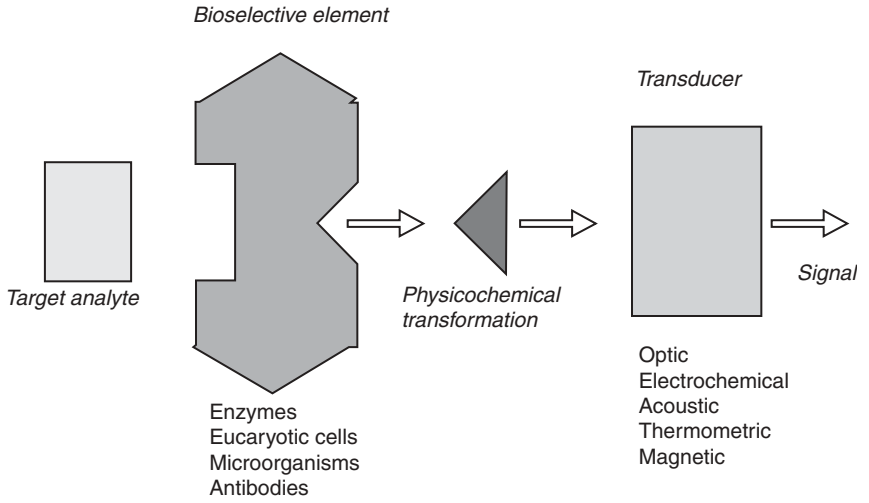

Fig. 1. Scheme of a generic biosensor.

The physicochemical transformation occurring in a biosensor due to the interaction between the biological element and the analyte target is converted into an usable signal by the transducer. The main purpose of the recognition system is to provide specificity to the biosensor, thus creating a device able to detect either a specific molecular target or a related family of compounds. Some biosensors are very selective for a single analyte (eg, glucose using glucose oxidase) and others are class specific, since they use eg, enzymes with broad substrate specificity (ie, phenolic biosensors based on tyrosinase activity) or intact microorganisms (eg, in BOD sensors).

In a biosensor, the biological component and the transducer are in intimate contact; this distinguishes this class of device from an analytical system that incorporates additional processing steps like separation processes or incubating chambers (ie, as in flow injection analysis). Thus, a biosensor should be a reagentless analytical device, although the presence of ambient cosubstrates (eg, oxygen for oxidoreductase) is often required for the detection of the analyte (1). Immobilization of the biological component can be performed using a variety of methods such as chemical or physical adsorption, physical entrapment within a membrane or gel, cross-linking of molecules, or covalent binding. The biological components used in biosensor construction can be divided into two broad categories: catalytic biosensors, where the primary sensing event results from a chemical transformation (catalyzed by an enzyme either isolated or retained in a microorganism or tissue); or affinity biosensors, in which the sensing event is dependent on an essentially irreversible binding of the target molecules (eg, affinity sensors based on antigen–antibody or nucleic acid interactions) (5). Recently, artificial receptors have emerged that may offer viable alternative recognition elements for biosensors, and this has become a fast-growing area for research (6). The transducer, or transducing microsystem, serves to transfer the signal from the recognition system to the electronic device, and it can be electrochemical, optical, thermometric, piezoelectric, or magnetic (7).

Catalytic and affinity-based biosensors have multifarious applications; eg, they can be used to measure blood glucose levels, detect pollutants and pesticides in the environment, monitor food-borne pathogens in the food supply, or to detect biological warfare agents. The future promises high density arrays of biomolecular sensors that rival microelectronics in size and capacity, deliver the recognition and discrimination of complex analytical equipment, and furnish scientists with biologically relevant information (8).

Biosensors may be categorized in different ways; it is possible to group biosensors according to the mode of signal transduction (ie, electrochemical, optical, or acoustic), or according to the biomolecule used (ie, biocatalytic or affinity based). In this article, we classified biosensors primarily on the basis of the biological element used.

2. Catalytic Biosensors

Catalytic biosensors are based on a reaction involving macromolecules that are present in their original biological environment, have been isolated previously, or have been manufactured (1). Biocatalytic elements normally used are enzymes (mono- or multisystems), whole cells from microorganisms such as bacteria or fungi, or tissues directly excised from the original organism. Considering a substrate S and the enzymatic product P, the reaction can be described (eg, 1):

$$S + S' \xrightarrow{cat} P + P' \quad (1)$$

S′ represents a second substrate involved in the biocatalytic reaction, which can be a reagent added directly during the reaction or something that is present in the environment (ie, oxygen dissolved in the solution). The reaction can be monitored by the transducer by measuring the consumption of S′, or the formation of the product P. Another product P′ can be formed from the reaction, and this can also be quantified. The biological components most commonly used to build biocatalytic biosensors are enzymes. There are a large number of enzymes commercially available, and increasing interest in enzymatic biosensors and assays has stimulated the number of enzymes that can readily be purchased. Advantages of the use of enzymes as biological elements are the possibility to operate in many environments such as aqueous solutions (9), organic phases (10,11) and air (12).

In the simplest type of catalytic biosensor, an enzyme is immobilized onto an electrode surface and the electrode is immersed in the test solution (Fig. 2). If the target analyte (ie, the enzymatic substrate) is present in the solution, a reaction occurs that can be monitored by, eg, amperometry. The signal is related to the substrate concentration.

Such enzyme-modified electrodes can be constructed using enzymes that catalyze oxidation–reduction reactions (where there is a passage of electrons between the enzyme and the electrode), or changes of pH or other ions in the solution tested. Once a substrate molecule has been transformed to product, the active site of the enzyme becomes available to react again with a new molecule.

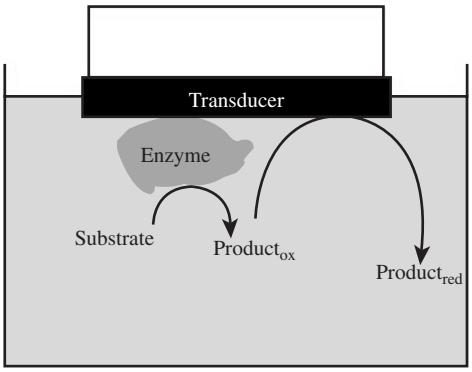

Fig. 2. Scheme of a biocatalytic biosensor based on enzyme catalysis. The example reported is regarding an electrochemical reduction.

The development of enzyme-based sensing devices, particularly those based on electrochemistry, continues to attract considerable attention. Of the electrochemical transducing systems available (amperometric, potentiometric, conductometric, and impedimetric), amperometric biosensors have dominated both research and commercial activity to date, largely because of their relative simplicity and flexibility (13–15). In general experimental amperometric systems employ a three-electrode design comprising a working electrode (to which a potential is applied in order to drive the electrochemical reaction), a reference electrode, and an auxiliary electrode. When a substance that can be oxidized or reduced at the potential applied is present in the solution, a current is generated. Electroactive species that can be detected using this type of approach include biochemicals such as reduced nicotinamide adenine denucleotide (NADH), inorganic species like molecular oxygen, or products of enzymatic reactions like hydrogen peroxide (produced by oxidases), benzoquinone (from phenol oxidation), or mediators (artificial electron acceptors such as ferrocyanide or ferrocene derivatives) involved in enzymatic reactions. The redox status of the biocatalyst's active center, cofactor, or prostethic group in the presence of substrate S can also be determined by using an immobilized mediator that reacts sufficiently rapidly with the biological element and for this reason can be easily detected at the transducer.

The most common example of an amperometric biosensor is the mediated amperometric glucose biosensor (16); this device involves the use of the enzyme glucose oxidase (GOD) as the biological recognition element, which catalyzes the following reactions (Fig. 3):

Initially, glucose reacts with the oxidized form of the enzyme to produce gluconolactone, which is then spontaneously hydrolized to gluconic acid. During this transition, two electrons and two protons are produced, with the concomitant reduction of the enzyme GOD. At this stage, a mediator such as a ferrocene derivative or quinone can shuttle electrons from the reduced enzyme to the electrode surface. There, the reduced form of the mediator is reoxidized to yield an amperometric signal.

Biocatalytic elements are also used in the preparation of potentiometric biosensors. These are based on the measurement of the difference in potential

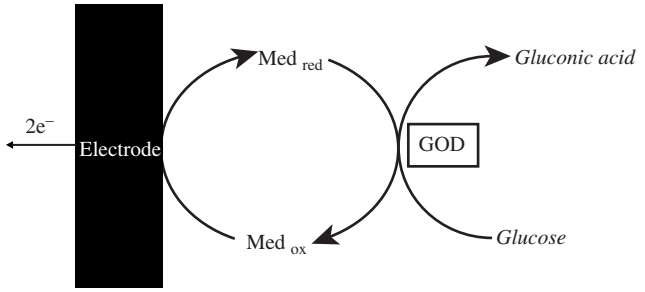

Fig. 3. Glucose oxidase-mediated electrochemistry.

between the working electrode and a reference electrode at near zero current flow. The potentiometric approach has largely been used to develop biosensors employing ion-selective electrodes modified with membranes. The most common potentiometric devices are pH electrodes, but several other ion (F^-, I^-, CN^-, Na^+, NH_4^+) or gas (CO_2, NH_3) specific electrodes can be used (1). In principle, the potential differences between the working and the reference electrodes varies logarithmically with the ion activity or gas concentration as described by the Nernst equation. This ideal condition is true when the selectivity of the membrane used is complete, or when the concentration of any interferent ions is very low and when the potential due to the contact between the various phases (solution, electrode surface, or membrane surface) is negligible or constant. Many examples of potentiometric catalytic biosensors can be found in the literature (17–19). One of the first applications of potentiometry in biosensors was for the detection of urea (20). In this work, a layer of a jack bean meal was used as a biocatalytic element. This native meal contains the enzyme urease, which catalyzes the following reaction (eg. 2):

$$\text{Urea} + H_2O \longrightarrow 2\,NH_3 + CO_2 \tag{2}$$

The CO_2 produced can be detected using a gas-sensing electrode. Similarly, a biosensor for pyruvate detection was constructed (21). In this case, plant tissue (corn kernels) was used, which contains pyruvate decarboxilase catalyzing the following reaction (eg. 3):

$$\text{Pyruvate} + H_2O \longrightarrow \text{Acetaldehyde} + CO_2 \tag{3}$$

As in equation 3, the CO_2 produced is correlated with the enzymatic reaction to generate a signal that is correlated to the analyte concentration.

The simplest enzyme electrodes employ a single enzyme, but this affords only a limited scope of potential analytes. More frequently now, two or more enzyme reactions are coupled. A recent example is the bienzymatic biosensor developed for oxalate detection (22). This biosensor involves two enzymes, oxalate oxidase (OXO) and horseradish peroxidase (HRP), incorporated into a carbon paste electrode modified with silica gel and coated with titanium oxide containing toluidine blue. The OXO was immobilized on silica gel modified with a titanium oxide surface using glutaraldehyde cross-linking. The HRP

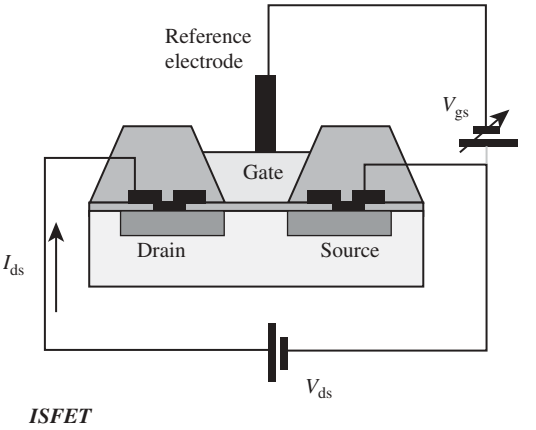

Fig. 4. Diagram of an ISFET.

was immobilized using covalent binding with carbodiimide on graphite powder. The biosensor showed a good performance with a linear response range between 0.1 and 2.0 m*M* of oxalate. It could be used for 80 determinations when stored in a succinate buffer at pH 3.8 in a refrigerator between measurements. The response time was 0.5 s.

An important transducer in the development of enzymatic biosensors has been the ion-selective field effect transistor (ISFET). A FET is a semiconductor device used for monitoring charge at the surface of an electrode, which has accumulated or been applied to a metal gate between the source and drain electrodes. The integration of an ISE with FET is realized in the ISFET (23) (Fig. 4). In the ISFET, the surface potential varies with the analyte concentration.

ISFETs can be combined with a biocatalytic element to create a biosensor. If the biological element is an enzyme, this is called ENFET and the term IMFET or immunoFET is used when antibodies are used. The invention of the ISFET in the early 1970s represented a major step forward in the design of chemical sensors (24). The pH-dependent potential at the interface between an electrolyte and a dielectric layer (eg, Si_3N_4 or Al_2O_3) is used to control the FET channel conductivity (25) of the ISFET. By immobilizing an enzyme to an ISFET gate, the ENFET (enzyme FET) was created (26). The enzyme catalyzes the conversion of a specific substrate (eg, urea, glucose, or penicillin) into a product (eg, a weak acid) that can be detected by the pH sensitive FET. The literature reports a pH-ISFET urea biosensor based on an enzyme-modified pH-sensitive Si_3N_4 gate ISFET with chemical immobilization of urease on the silicon nitride surface performed with the use of glutaraldehyde. The device obtained gave good results in terms of sensitivity and reproducibility and a linear range covering the physiological as well as pathophysiological ranges (27). Urea ENFETs have now been incorporated into commercial devices for bedside monitoring in critical care situations. ISFET technology has also been applied to develop immunosensors (IMMFET), but application of this technology has been more limited.

3. Affinity Biosensors: Immunosensors

Affinity biosensors are analytical devices comprising a biological affinity element such as an antibody, receptor protein, biomimetic material, or DNA, interfaced to a signal transducer, to convert the concentration of an analyte to a measurable electronic signal (28).

Because of their high affinity, versatility, and commercial availability, antibodies are the most widely reported biological recognition elements used in affinity-type biosensors; in this case, the affinity biosensor is known as an "immunosensor". Antibodies are polymers containing hundreds of individual amino acids arranged in a highly ordered sequence. These polypeptides are produced by immune system cells (B lymphocytes) when exposed to antigenic substances or molecules. Antibodies contain in their structure recognition/binding sites for specific molecular structures of the antigen. According to the "key-lock" model, an antibody interacts in a highly specific way with this unique antigen. The interaction is reversible, as determined by the law of mass action, and it is based on electrostatic forces, hydrogen bonding, and hydrophobic and van der Waals interactions.

Antibody-based biosensors (immunosensors), where the antibody or antigen is immobilized to the transducer, have been constructed in a variety of ways, but generally fall into one of three basic configurations. These formats involve direct noncompetitive assays, competitive (direct or indirect) assays, or sandwich-type assay formats (Fig. 5). In the case of the direct noncompetitive assay format, a unique optical or electrochemical property of the analyte of interest is observed as the target compound binds to the recognition site of the bioaffinity element and accumulates on the sensor surface.

Recent developments in electrochemical transducers have also allowed the noncompetitive detection of small molecular weight molecules. For these sensors, the formation of an antibody–antigen complex at the surface of a conductive thin film induces direct or indirect ionic movements resulting in a current flow through the conductive support. Although these types of format show a great deal of promise, one of their primary limitations involves nonspecific binding of nontarget compounds. More specifically, cocontaminants (any small molecules) that nonspecifically bind to the surface of the sensor cannot be distinguished from the analyte of interest.

The simplest biochemical immunosensor consists of an antibody or antigen immobilized to a transducer and results in a signal generated from the binding of an analyte to an antibody at the sensor surface. If the analyte (itself) can be detected by the signal transducer, this forms the basis of a noncompetitive assay. For example, the fluorescent analyte benzo(a)pyrene can be directly detected when binding to an antibody immobilized to a fiberoptic signal transducer (29). For these assay formats, the signal is directly proportional to the analyte concentration.

Because many sensors cannot detect the binding of an analyte, competitive formats are often used in conjunction with optical or electrochemical tracers. The primary advantages in the use of tracers in a direct immunoassay format are the high sensitivity and low potential for interference from matrix components afforded by these (usually fluorescent) molecules. The primary disadvantage in

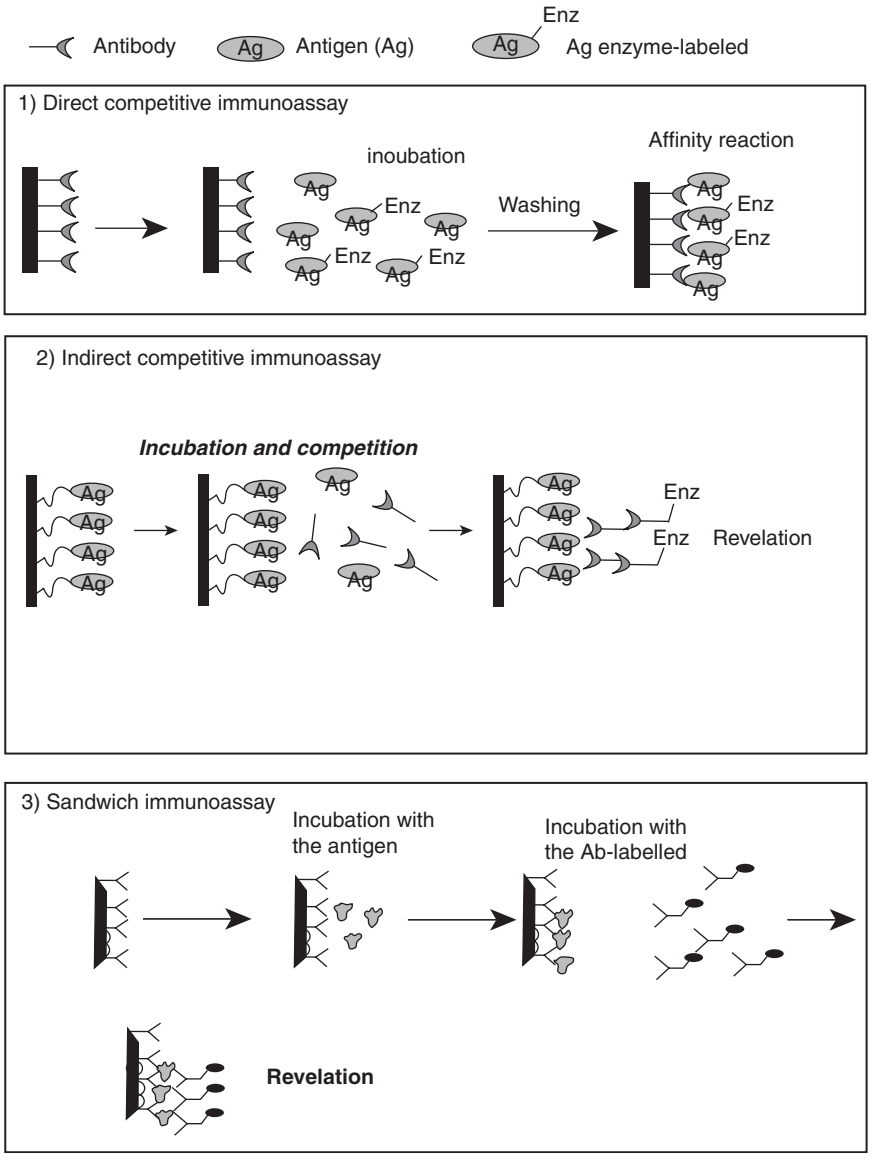

Fig. 5. Scheme of different enzyme immunoassay formats.

the use of tracers is the need to add a reagent other than the analyte of interest. More specifically, because the signal is inversely proportional to the concentration of analyte, it is often the case that the lowest analyte concentrations are measured as a small change in the maximum signal, which increases the relative signal noise.

Electrochemical immunosensors are highly diffuse. Most electrochemical immunosensors are based on enzyme-linked immunosorbent assay (ELISA) principles, where antibodies or antigens are labeled with, eg, peroxidase, alkaline

phosphatase, or GOD, and measured amperometrically by following the respective product iodine, *p*-aminophenol, or hydrogen peroxide. Glucose oxidase also has been used as an enzyme label in immunosensors development; a GOD labeled immunosensor for human serum albumin has been described using amperometric detection of hydrogen peroxide (30). In this system, nonspecific adsorption of enzyme-labeled antigen to the membrane was only 4.4% of specific binding, and the range of measurement, 0.5–200 mg L^{-1} of human serum albumin, was satisfactory for the diagnosis and for clinical applications.

Whereas the preferred method of measurement for catalytic biosensors is electrochemical, affinity biosensors have generally proved more amenable to optical detection methods (10). The commercialization of real-time bioaffinity monitors based on surface plasmon resonance (SPR) has provided a powerful new tool to the research community and to the pharmaceutical industry in particular. A surface plasmon is an evanescent electromagnetic field generated at the surface of a metal conductor (usually Ag or Au) excited from a light of an appropriate wavelength that impacts on the surface at a particular angle (θ_p). A sharp minimum in light reflectance can be observed when the angle of incidence is varied (31). The value of this "critical angle" is strictly dependent on the properties of the medium that is in contact with the metal layer, but is principally determined by their dielectric constant. When the metal surface interacts in an affinity reaction, the dielectric constant is altered and this can be quantified by measuring the change in the critical angle (Fig. 6).

This principle can be very useful in biosensor technology, because it allows the direct detection of a biological interaction. Surface plasmon resonance has been used to study the binding of immunoglobulin G (IgG) to gold and anti-IgG to immobilized IgG layers (32). In these studies, both a monoclonal mouse and polyclonal sheep IgG were used as receptor layers for anti-IgG. The kinetics of binding were investigated by monitoring the reflectivity of light at an angle close to plasmon resonance. Both the initial rate of change and final reflectivity

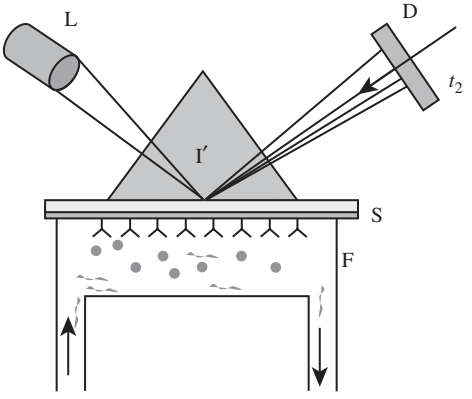

Fig. 6. Surface plasmon resonance detection unit. L: light source, D: photodiod array, P: prism, S: sensor surface, F: flow cell. The two dark lines in the reflected beam projected on to the detector symbolize the light intensity drop following the resonance phenomenon at time = t_1 and t_2. The line projected at t_1 corresponds to the situation before binding of antigens to the antibodies on the surface and t_2 is the position of resonance after binding.

were measured during and after protein binding. The amount of protein bound to the surface was found to be less for the monoclonal mouse IgG compared to the polyclonal sheep IgG, these two IgG nominally being of the same dimensions and molecular weight. Further, anti-IgG binding produced greater changes in reflectivity than the initial IgG layers. SPR was used also to investigate the effect of pesticides such as imazetaphyr, triazines, and parathion. Advances in the research and development of SPR detection of immunointeractions resulted in the introduction of commercially available instruments such as the BIAcore (Uppsala, Sweden). This instrument consists of a sensor chip, a SPR detector unit, and a liquid handling system that has two precision pumps and an integrated microfluidics cartridge (IFC). The autosampler and the liquid handling system together control delivery of sample plugs into a buffer stream that passes continuously across the sensor chip surface. The entire system is computer controlled, including data collection and analysis, resulting in a fully automated analytical system. The sensor chip (signal transducer) is a glass slide with a thin layer (50 nm) of gold deposited on one side. The gold film is in turn covered with a covalently bound matrix on which biomolecules can be immobilized. This commercial apparatus, available for coating with the desired antibody or antigen, was used, eg, to develop an SPR-based immunosensor for atrazine detection (33) together with other environmental applications.

Optical immunosensors based on fluorescence have also been realized. Sepaniak and co-workers (34) described the measurement of benzo(α)pyrene, which is itself fluorescencent; the benzopyrene is trapped by an antibody solution placed on the tip of an optical fiber and protected from the external environment by a membrane. Optical immunosensors have been developed for many environmental applications including pesticide analysis for analytes such as parathion (35) and triazine (36). Optical evanescent-wave technology has been used to streamline the design of affinity biosensors that contain a label or marker. For example, many immunoassays use a fluorescent marker to indicate when antibody binds antigen. An impressive recent example is provided by immunosensors designed to detect microbial warfare agents (37, 38). The integration of photochromic dyes into the antibodies of immunoassays has facilitated the production of high affinity sensors that can be optically switched to low affinity so that the devices can be regenerated and used again (39). A recent advance in affinity biosensor immunoassays is the introduction of paramagnetic particles attached to antibodies as the label. Binding of antibody to antigen attached to a solid substrate, can be detected with an electronic device that measures the magnetic field induced by the paramagnetic beads. The detector can be fabricated into a small hand-held device, and the approach offers the added bonus of providing a permanent record because the contents of the assay stick can be remeasured at any time, like a piece of magnetic recording tape.

Piezoelectric biosensors, based on the piezoelectric quartz crystal microbalance (QCM), have been reported for a range of applications in biochemistry and affinity biosensors construction (40). This type of sensor comprises an oscillator circuit and a thin slice of AT-cut ($\sim 35^\circ$ rotation angle) piezoelectric quartz crystal. Metal film (gold or platinum) electrodes are deposited onto both sides of the quartz and crystal. The sensor operates by observation of the propagation of an acoustic wave through the solid-state device. Sensing is usually achieved by

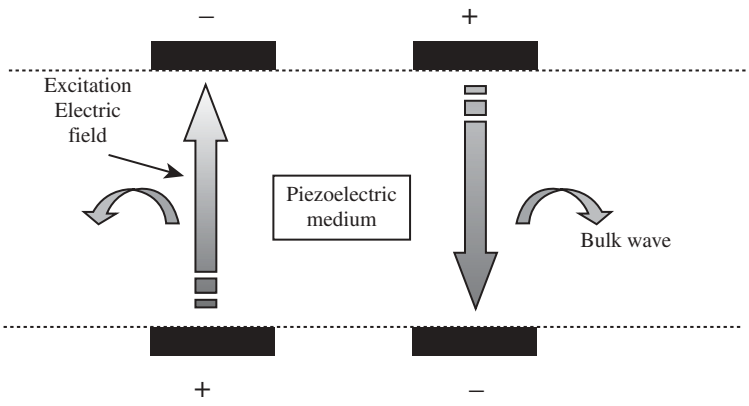

Fig. 7. Diagram of a piezoelectric system.

correlating the acoustic wave-propagation variations to the amount of analyte captured at the surface, and then to the amount or concentration of analyte present in the sample exposed to the sensor. Alternatively, changes in the physical properties of interfacial thin films in response to analyte may be measured (41). A diagram illustrating the principle of the measurement is shown in Figure 7.

The adsorption of a substance increases the rigid mass bound to the quartz crystal surface and, under dry conditions, causes the crystal to change its oscillation frequency according to the Sauerbrey equation (eg. 4) (42):

$$\Delta F = -2.26 \times 10^{-6} F^2 \, (\Delta m / A) \tag{4}$$

where ΔF (Hz) is the change in oscillation frequency of the coated quartz crystal, Δm (g), the change in the mass adsorbed onto the crystal, and A (cm^2) is the area of the coated quartz crystal, F (Hz), the resonance frequency of the quartz crystal. This approach can be used to precisely quantify analyte with a detection sensitivity in the nanogram range.

Piezoelectric crystals (Fig. 8) have been used as microbalances and microviscometers owing to their small size, high sensitivity, and stability, simplicity of construction and operation, light weight, and low power requirement. They have been applied in the determination of thin layer thickness, the study of general chemical species adsorption, and the analysis of the microrheology of liquid crystals and electrochemically polymerized thin films. A major application of

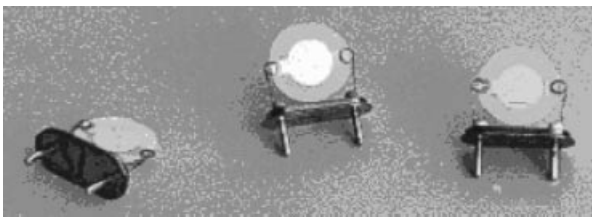

Fig. 8. Gold-modified piezoelectric crystals.

piezoelectric crystals has been in biosensor construction, especially immunosensors. By using these piezoelectric biosensor devices, it is possible to measure the immuno-interaction directly; such devices are often known as "microgravimetric immunosensors" (31). Recent developments in microgravimetric immunosensors and their application have been described in a number of reviews (43–45). A flow-through immunoassay system based on piezoelectric detection for label-free determination of antibodies against human immunodeficiency virus (HIV) has been described by Aberl and co-workers (46). This system involves a high level of automation and allows determination of target analytes in the concentration ranges found in HIV diagnostics. The piezoelectric detection method has been used in the determination of small molecules in environmental and clinical analysis. In a typical case, a competitive assay mechanism was used, employing haptens conjugated to a protein. A piezoelectric immunosensor for methamphetamine was realized by immobilizing the target molecule on the surface of the piezoelectric crystal. The assay procedure involved a competitive scheme, where both free antibodies and antigen were contained in the reaction medium (47). Antibodies binding with methamphetamine-modified immunosensor surface were in competition to bind with the target analyte.

4. Affinity Biosensors: DNA Biosensors

DNA biosensors represent a very important class of affinity biosensor. In this developing aspect of the field, DNA strands are used to detect the binding of oligonucleotides (gene probes). Such devices are known as "DNA biosensors" or more generally as "DNA Chips". The DNA biosensors present an enormous potential for early clinical diagnosis of genetically inherited diseases, on site detection of food contamination, forensic studies, and environmental monitoring. DNA biosensors involve the use of nucleic acids as biological recognition elements to explore novel hybridization probes, transduction strategies, and potential practical applications. Transduction strategies that have been reported include electrochemical, acoustic, and optical techniques. For each of these strategies, the biosensor format typically relies on immobilization of single stranded (ss) DNA to the sensor surface followed by hybridization of a complimentary sequence. Detection of the formation of double stranded (ds) DNA has been facilitated through the use of a variety of electrochemical and optical tracers that primarily bind or intercalate into ds DNA.

Electrochemical techniques have been used to differentiate between ss (prehybridized) and ds (hybridized) DNA using several approaches. A potentially major application of a DNA biosensor could be for the testing of water, food, soil, and plant samples for the presence of pathogenic microorganisms and for the presence of analytes (carcinogens, drugs, mutagenic pollutants, etc.) with binding affinities for the DNA molecule (Fig. 9). The detection of small molecule binding to DNA and general DNA damage resulting form ionizing radiation, dimethyl sulfate, etc has been described by following variation in the electrochemical signal derived from guanine. These approaches include the use of redox active intercalators that accumulate into ds DNA, transition metal complexes that mediate oxidation of the guanine base, or the direct oxidation of guanine.

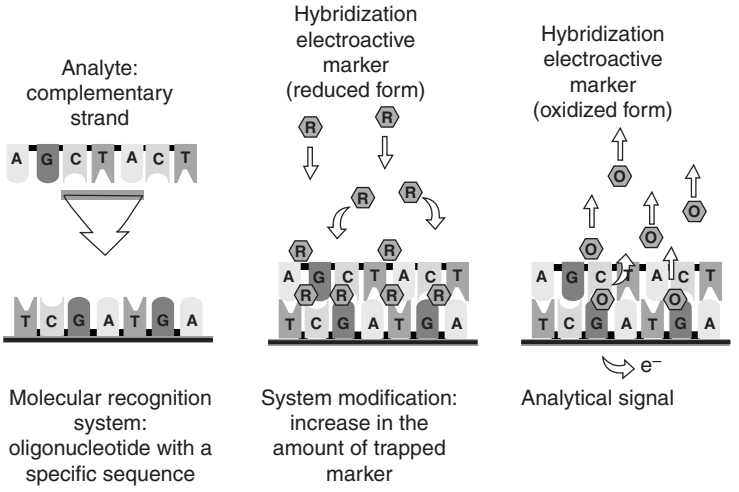

Fig. 9. Schematic of DNA biosensor for hybridization detection.

Marrazza and co-workers (48) reported the construction of disposable electrochemical biosensors for environmental applications based on intercalation and hybridization principles. For the former, a graphite screen-printed electrode was modified by the immobilization (as a probe) of specific synthetic oligonucleotides with a defined sequence for *Chlamydia trachomatis*, a bacterial species. The oligonucleotide immobilized on the surface was hybridized with different concentrations of the complementary sequences and the amount of hybrid was evaluated by chronopotentiometric stripping analysis (PSA) using daunomycin as an indicator of the hybridization reaction. The increase in the area of the daunomycin peak was used to detect the presence and the amount of the complementary sequence. This sensor could be used to detect the presence of bacteria following PCR (polymerase chain reaction) amplification of the sample. The latter DNA biosensor was realized by immobilizing calf thymus DNA at a fixed potential onto an electrode surface. The calf thymus DNA sensor was then immersed in a sample solution containing the analyte. After 2 min of interaction, a PSA was carried out to evaluate the oxidation of guanine residues on the electrode surface. In this case, it was possible to evaluate the electrochemical effects resulting from the presence of genotoxic compounds (49).

The mass change associated with DNA hybridization may also be detected by employing piezoelectric devices. By immobilization of a ss DNA onto quartz crystals, it is possible to detect the mass change after hybridization. Nucleic acid strands were covalently attached to the modified surface of piezoelectric crystal. When these immobilized probe strands were melted and incubated with complementary target strands in solution, association of probe and target to form duplexes resulted in an increase in mass that was detectable as a decrease of several hundred hertz in the crystal's resonance frequency relative to control crystals on which noncomplementary strands were attached.

A further example of piezoelectric DNA-based biosensors has been developed to detect bacteria toxicity in environmental samples (50). In this system,

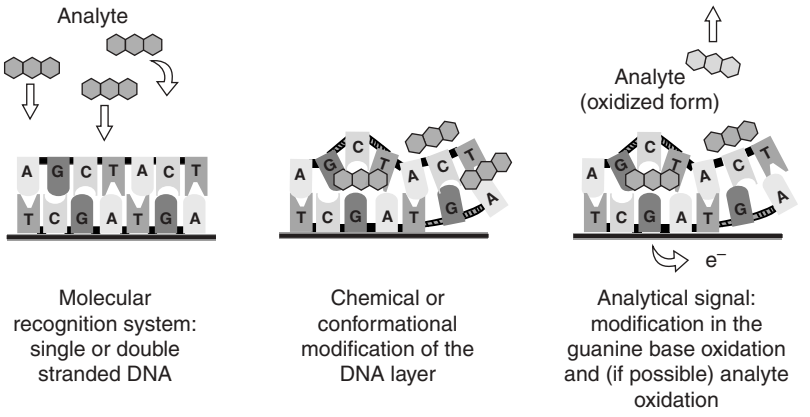

Fig. 10. Schematic of DNA biosensor to detect molecular intercalators.

a biotinylated oligonucleotide probe was immobilized on a streptavidin coated gold disk. Streptavidin was covalently linked to the thiol/carboxylated dextran modified gold surface. The immobilized probe was then reacted with a solution of the target oligonucleotide. The interactions of the immobilized DNA strand with a complementary and a noncomplementary sequence in solution were studied. The hybridization reaction was also performed on real samples of DNA extracted from different *Aeromonas* strains isolated from water, vegetables, or human specimens and amplified by PCR. These experiments showed that it was possible to distinguish between strains that contain the aerolysin gene and those that do not, hence furnishing an assay for the toxicity of the bacterium. The PCR has been widely used in conjunction with various sensor systems to

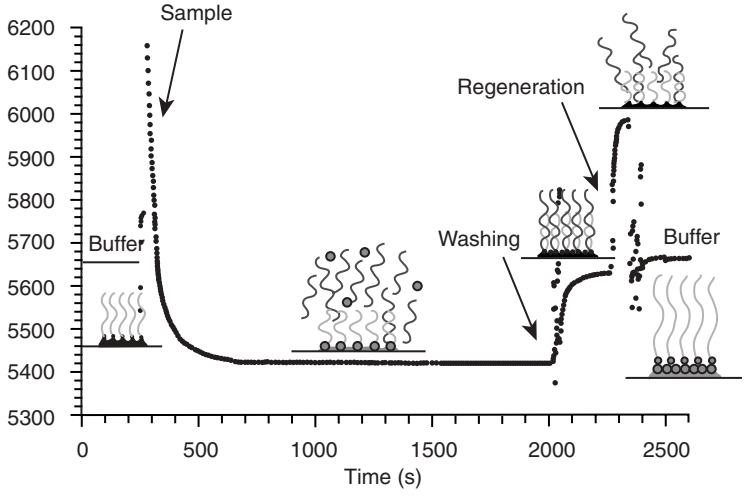

Fig. 11. Frequency profile for piezoelectric crystal modified in order to detect specific DNA sequences.

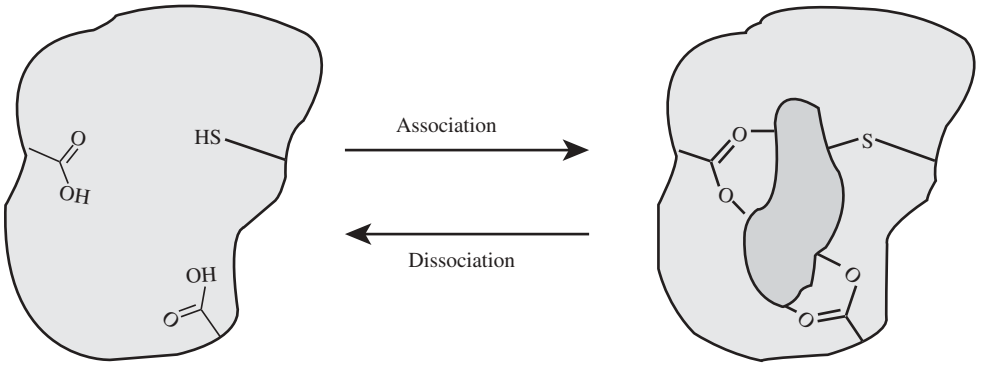

Fig. 12. Schematic representation of the association and dissociation of a molecule with the specific binding cavity generated in a molecularly imprinted polymer.

increase sensitivity by amplifying DNA in a sample, eg, for clinical applications, to detect apolipoprotein E polymorphisms (51).

5. Synthetic Receptors and Biomimetic Sensors

In biosensor technology, the recognition element is conventionally isolated from living systems, measuring interactions such as antigen–antibody or utilizing the substrate specificity obtained with enzymes. However, for a number of potential applications, no biocomponent is available or requirements such as stability cannot be met using biological molecule. For this reason, synthetic bioreceptors have attracted considerable attention as a potential new avenue for biosensor development. Semisynthetic receptors have been created by modifying enzymes and antibodies at both the pre- and posttranslational level. For example, antibody fragments have been produced and libraries of variants generated by random or point mutations at a genetic level. The design of semisynthetic receptors in biosensors may be superseded by totally synthetic ligands produced with the aid of computational chemistry, combinatorial chemistry, molecular imprinting, self-assembly, rational design, or combinations of one or more of these. For example, molecularly imprinted polymers (MIPs) have attracted a lot of attention because they can behave as artificial receptors for molecular recognition. This methodology is based on the prearrangement of print molecules and functional monomers prior to initiation of polymerization. In this way, a rigid cross-linked, macroporous polymer is formed that contains sites complementary to the print molecule both in shape and in the arrangement of functional groups (52). Removal of the print molecule by extraction leaves sites specific for print molecules and free for binding. Many imprinted polymers specific for different compounds have been prepared in several laboratories over the last few years, including specific polymers that have been used as antibody mimics. The striking resemblance of the binding properties (affinity and specificity) of MIPs to those of natural receptors, together with their inherent stability, low cost, and ease of preparation for industrial application have made them an attractive alternative

to antibody-based technologies. Conventional imprinted polymers are usually synthesized empirically or semiempirically using a limited number of common monomers in a trial-and-error approach. Three particular features have made MIPs the target of intense investigation: (*1*) their high affinity and selectivity, which are similar to those of natural receptors; (*2*) their unique stability that is superior to that demonstrated by natural biomolecules; and (*3*) the simplicity of their preparation and the ease of adaptation to different practical applications 3–5. A great variety of chemical compounds have been imprinted successfully. The resulting polymers are robust, inexpensive and, in many cases, possess an affinity and specificity that is suitable for industrial application.

Molecularly imprinted polymers are proposed for a number of applications including use as the stationary phase in high performance liquid chromatography (HPLC) and, in thin-layer chromatography (TLC) to separate chiral products and as a replacement for antibodies in biosensor construction. One example is a morphine-sensitive sensor (53). The method involved two steps. In the first step, morphine was bound selectively to the molecularly imprinted polymer in the sensor, a platinum wire electrode. In the second step, an electroinactive competitor (codeine) was added in excess, displacing some of the bound morphine. The released morphine was detected using an amperometric method. The system was able to measure morphine in the concentration range 0.1–10 μg/mL (morphine concentration of 0.5 μg/mL gave a peak current by oxidation of 4 nA) and was stable to autoclaving, demonstrated long-term stability, and was resistant to harsh chemical environments.

Molecularly imprinted polymer (MIP) technology has also been applied for environmental applications. Molecularly imprinted polymer membranes, containing artificial recognition sites for atrazine, have been prepared by photopolymerization using atrazine as a template, methacrylic acid as a functional monomer, and tri(ethylene glycol) dimethacrylate as a cross-linker (54). To obtain thin, flexible, and mechanically stable membranes, oligourethane acrylate was added to the monomer mixture. Reference membranes were prepared with the same monomer mixture but in the absence of the template. Imprinted membranes were tested as a recognition element for an atrazine-sensitive conductometric sensor. The influence of the polymer composition and type of solvent used as a porogen on the magnitude of the sensor response was investigated. The sensor developed demonstrated high selectivity and sensitivity with a detection limit of 5 n*M* for atrazine. The membranes synthesized exhibited the same recognition characteristics over a period of 6 months.

Transducing the binding event in molecularly imprinted polymers into a detectable signal has proved quite a challenge. However, creative solutions have been achieved with both optical and electrochemical configurations. In one example, a "bite-and-switch" approach has been used to produce sensors that detect creatine and creatinine in blood. In this two-step recognition process, a broadly specific chemical reaction is complemented by a three-dimensional recognition pocket to produce a strong "bite", which is followed by a "switch" to the fluorescent form of the indicator. A thioacetal reaction between the polymer and the amine groups in creatine and creatinine—results in the formation of a fluorescent isoindole complex; this reaction was made more specific for creatine and creatinine by molecular imprinting (55). In a further example of a

screen-printed design, an electrochemical sensor was developed that detected the herbicide 2,4-D by the displacement of homogentisic acid from a MIP (56).

6. Mass Production of Biosensors: Thick-Film Technology

Over the past two decades, there has been increasing interest in the application of simple, rapid, inexpensive, and disposable biosensors in fields such as clinical, environmental, or industrial analysis. The most common disposable biosensors are those produced by thick-film technology. A thick-film biosensor configuration is normally considered to be one that comprises layers of special inks or pastes deposited sequentially onto an insulating support or substrate. One of the key factors that distinguishes a thick-film technique is the method of film deposition, namely, screen printing, which is possibly one of the oldest forms of graphic art reproduction. The fabrication process of a screen-printed device requires the application of procedures and materials appropriate to the particular devices. To create a thick-film electrode, a conductive or dielectric film is applied to a substrate (57). The film is applied through a mask contacting the substrate and deposited films are obtained by pattern transfer from the mask. Conventionally, thick-film electrodes were baked at temperature ranging from 300 to 1200°C to drive off solvents from the applied paste and to cure the pattern paste. In commercial biosensors, this step is usually avoided since it would damage incorporated enzymes. Alternatives include cold-cured ink formulations and a photocured process using ultraviolet(uv) light.

More complex structures can be built up by repeating the print process using the materials appropriate to the specific design and a range of mask designs. A variety of screen-printed thick-film devices can thus be fabricated (Fig. 13) as a base for disposable electrochemical immunosensors, DNA sensors, and enzyme electrodes. These planar devices present many advantages including disposability, which is a very important characteristic when working with real samples, and small dimensions, which facilitates the design of portable measuring systems. The working electrode of the system can be fabricated using

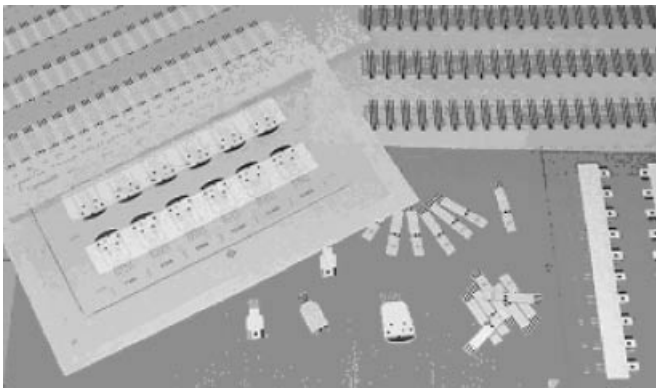

Fig. 13. Various screen-printed electrodes.

different kind of inks such as graphite, gold, or platinum, and then modified with the appropriate bioselective element.

Screen-printed electrodes have been used extensively in recent years to produce a wide range of sensors and biosensors. They have proved very versatile for work on real samples in environmental and clinical analysis. The most well-kown application of screen-printed electrode technology has been in the clinical analysis of blood glucose levels in people with diabetes. Historically, glucose sensing has dominated the biosensor literature and has delivered huge commercial successes to the field. The deceptively simple combination of a fungal enzyme (GOD) with an electrochemical detector has effectively met the needs of the 1–2% of the world's population that have diabetes. Complications associated with insulin-dependent diabetes such as blindness, kidney and heart failure, and gangrene (resulting in amputation) can be reduced by up to 60% through stringent personal control of blood glucose, including frequent monitoring of glucose levels. People with non-insulin-dependent (Type II) diabetes can also benefit from strict monitoring of glucose levels. Enzyme-based electrode biosensors have been used to test glucose levels in blood samples since 1975, but the emergence of a convenient, hand-held commercial format revolutionized their use (58). The commercial realization of the mediated glucose sensor came with the foundation of Genetics International (later to become MediSense) in Boston, with the launch of the pen-sized Exatech glucose sensors in 1987 (Fig. 14).

The system was invented, designed, and developed at Cranfield University in collaboration with Oxford University in the U.K. and consists of a small, disposable, single-use, glucose-sensitive electrode (based on a screen-printed mixture of GOD and mediator in a conductive carbon-paste binder) and a pen-sized (later designs adopted a credit card or computer mouse shape) meter containing the electronics and LCD display. Patients prick their finger and deposit the blood onto the sensor, and within 20–30 it is possible to quantify the glucose present in blood. The response of an amperometric biosensor is either a steady-state or a transient response, but it is never an equilibrium response, because the

Fig. 14. Pen-size glucose meter.

substrate has to diffuse into the sensor from the bulk solution, pass through the enzymatic membrane and react with the biocatalytic element.

Screen-printed electrodes have also been fabricated to detect environmental pollutants. A disposable amperometric sensor to detect hydrazines has been described (59) in which the working surface of the electrode was modified using cobalt phtalocyanine (CoPC) and a mixed-valent ruthenium cyanide. These mediators are able to catalyze the oxidation of various hydrazines, and then can be detected by amperometric measurement. The configuration of the sensor as a disposable strip facilitates on-site environmental and industrial monitoring.

Screen-printed electrodes can also be designed to incorporate bioaffinity molecules like antibodies, in order to obtain disposable immunosensors for clinical and environmental applications.The electrode constitutes both the solid phase for the immunoassay and the electrochemical transducer. A recent example is a disposable immunosensor (based on enzymatic labeling of one of the reagents) to detect polychlorinated biphenyls (PCBs) (60). In this case, the surface of the electrode was modified using different strategies to produce two different formats of immunochemical tests based on indirect and competitive assays. The graphite surface of the screen-printed electrodes was modified by immobilization of antibodies or antigens in order to obtain a selective surface for the realization of the immunological reaction. In the indirect competitive immunochemical test, a bovine serum albumin (BSA) conjugate (4,4′-dichlorobiphenyls-BSA) was used for the PCB immobilization procedure. The IgG anti-PCB reacts with the sample for a fixed time (30 min). Then a small amount (10 mL) was added to the electrode surface with immobilized PCB–BSA for the competition reaction. The IgG anti-PCB captured on the electrode surface was evaluated using a secondary, labeled HPR antibody. In the direct format, a fixed amount of anti-PCB were immobilized onto the solid phase. In this case, the competition was realized between the antigen in the sample and a limiting amount of the HRP-labeled congener added to it. In both cases, the extent of the affinity reaction was evaluated by chronoamperometric measurement of the products of the enzymatic activity of the label. Then, after molecular recognition, the electrodes were used as electrochemical cells.

7. Applications of Biosensors

The vast majority of commercial activity to date, in the field of biosensors, has been focused on medical applications and a substantial market now exists for such devices especially for home blood glucose measurement. Medically related opportunities for biosensors are expected to increase especially in the areas of pharmaceuticals and drug development and to serve the new genomics and proteomics industries. The second most reported application area for biosensors is for environmental analysis. This activity has mainly been supported by public money to underpin current and impending legislation to protect the environment. In the text above, these two application areas have dominated the examples cited. Less reported, but of enormous current interest, is the application of biosensors for defence (detection of biological and chemical warfare agents) and

security (detection of drugs, explosives, identity, etc). Biosensors are also expected to impact on the food and process industries. A steady but limited business has developed for food testing, eg, for sugars, amino acids, and organic acids. Expansion in the future is likely to be in the food safety and labeling area eg, to identify pathogens, toxins, allergens, and genetically manipulated crops.

Enzyme-based biosensors have generally been used to measure batch samples, but a number of attempts have been made to monitor processes on-line (61). In most cases, the analysis must be performed outside the reactor or process line *(ex situ)*. Consequently, aseptic on-line sample withdrawal from the bioreactor is necessary, because the biological component of biosensors is not easily sterilizable. Other disadvantages of biosensors for process monitoring are that they are invasive, temperature dependent, subject to fouling, and need to be recalibrated and regenerated frequently. Signal drift causes unreliability in these sensors, and the enzyme stability is also limited. Most biosensors are only able to measure one substrate at a time but, with a combination of different biosensors, various substances (glucose, ammonium, etc) can be measured simultaneously. Applications of on-line biosensors have included process optimization in pilot scale and control of animal cell culture where the product is very valuable. Bioluminescent sensors have also been developed for on-line applications and consist of a bioluminescent enzyme and an optical transducer. Optical biosensors provide a rapid and highly selective detection system. The use of optical biosensors is expanding (62; 63). Improving the reliability of *in situ* biosensors for industrial applications and the integration of biosensors into flow-injection analysis (FIA) is an important objective. Recent developments have been directed toward overcoming some of the common disadvantages of biosensors. An on-line resonant mirror optical biosensor was used to detect DNA–DNA hybridization in real time (64). Biotinylated oligonucleotide probes were immobilized on the sensor surface and hybridization of a complementary target oligonucleotide was monitored. The limit of detection was lower than other optical biosensors using surface-plasmon resonance, fiber-optic fluorescence, or light-activated potentiometric devices. An evanescent field biosensor was used to detect protein A produced by *Staphylococcus aureus* in blood-culture bottles in < 30 min (65). By using laser light at 488 nm, a plastic optical fiber and anti-protein-A antibodies conjugated with fluorescein isothiocyanate in a sandwich immunoassay adsorbed onto the fiber, a detection limit of 1 ng/mL was achieved.

Effective on-line sensing is arguably the most difficult technical hurdle facing biosensor technology. Progress in the related area of real-time *in vivo* has advanced significantly in recent years. Two new commercial devices have been launched for monitoring glucose *in vivo*. One offers a miniaturized, sterilizable, and implantable enzyme electrode produced using microfabrication technology, while the other is a transcutaneous device, which obtains a sample using reverse ionophoresis. It is clear that designs can be developed to deliver the required stability, accuracy, and robustness for this patient-centerd type of device. Future advances, however, are expected to be supported by developments in the biomimetic and imprinting areas, since these offer the hope of far greater stability and resistance to sterilization while maintaining the exquisite specificity and sensitivity associated with traditional biological elements.

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