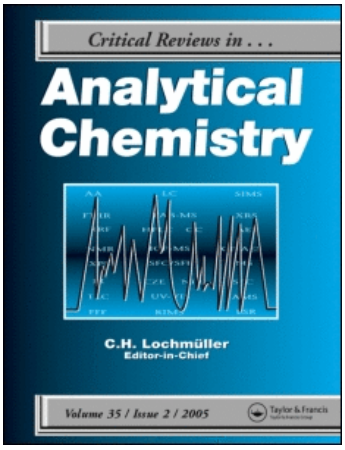

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Recent Advances in the Development and Analytical Applications of Biosensing Probes

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RECENT ADVANCES IN THE DEVELOPMENT AND ANALYTICAL APPLICATIONS OF BIOSENSING PROBES

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I. INTRODUCTION

The term "biosensor" has become a fashionable buzzword in recent analytical literature. While scientists have used the word to describe any number of innovative devices and instrumental systems, we believe that the two most widely accepted definitions are as follows:

1. A self-contained analytical device that responds selectively and reversibly to the concentration or activity of chemical species in biological samples
2. An analytical device which incorporates a biologically active material in intimate contact with an appropriate transduction element for the purpose of detecting (reversibly and selectively) the concentration or activity of chemical species in any type of sample

Definition 1 defines a biosensor in relation to the type of sample in which the device is used. Under this more general definition, a glass pH electrode, when used to measure the pH of blood or cell culture media, would be considered a biosensor. Similarly, a fiber optic probe which detects oxyhemoglobin in whole blood would fall into this broad category. On the other hand, definition 2 is much narrower in scope and only includes sensors that contain immobilized biological reagents/components (e.g., enzyme electrodes, immunosensors, etc.). Here, the "bio" part of the biosensor refers not to the type of sample, but to the nature of the chemical reaction that generates the analytical signal.

In this review, we restrict our discussions to sensors that fall under definition 2. Even within this more focused category, we need to make additional compromises regarding topics to be covered. For example, liquid membrane ion-selective electrodes that are based on natural ionophores (e.g., valinomycin, nonactin, etc.) are true biosensors under our working definition, but we believe that their inclusion here would be inappropriate owing to the extensive literature that already exists on this subject.

Our main objective is to present a concise, critical review of current biosensor technology. To this end, we only focus on sensors based on biocatalysts, bioreceptors, or complementary bioligands immobilized on a variety of detection elements (e.g., electrochemical, piezoelectric, fiber optic, etc.). Our coverage is not intended to be all inclusive. Several excellent reviews[1-13] and monographs[14-17] are available that provide more extensive literature surveys

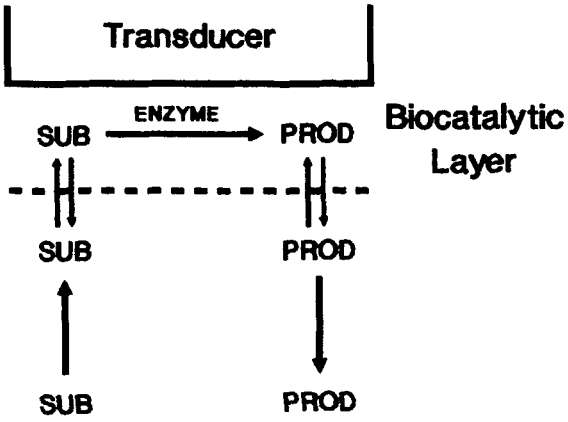


FIGURE 1. Schematic diagram of a biocatalytic-based biosensor.

on this topic. Our goal is to assess the relative merits of the most recent and significant advances in biosensor design and to identify obstacles that continue to impede the "real world" analytical application of these devices.

II. BIOCATALYTIC-BASED BIOSENSORS

A. General Configuration

A biocatalytic-based biosensor is configured with a biocatalyst immobilized at the sensing tip of a detection element. The biocatalyst mediates between the analyte of interest and the detector. Figure 1 shows a schematic representation of a biosensor. As shown, the analyte diffuses from the bulk solution to the biocatalytic layer where it is converted to a detector measurable species. A steady-state condition is established at the sensor tip when the rate of product generation is balanced by the rate of product removal. Alternatively, the consumption of a co-substrate can be detected. In both arrangements, a measured steady-state signal is related to the concentration of the analyte in the sample.

The sensor response is governed by the rate of product generation at the sensor tip. A combination of enzyme reaction kinetics and analyte mass transport control the overall kinetics at the solution/sensor interface. The sensor response is generally diffusion limited when the biocatalytic activity is high at the sensor tip (i.e., high enzyme loading). Low biocatalytic activity (i.e., low enzyme loading), on the other hand, causes the sensor to be limited by the reaction kinetics. Detailed treatments of the relation between sensor response and kinetic processes are available.[14,18,19] In general, a superior sensor response is attained with high enzyme loading. Greater selectivity, faster dynamic response, and longer useful lifetimes are attractive features frequently attributed to high enzyme loading.

B. The Biocatalyst

Several types of biocatalytic materials can be used for the construction of biosensors. These materials include isolated enzymes; subcellular fractions; and whole cell preparations from bacterial, plant, and mammalian sources. Several excellent reviews and monographs list many of the available systems along with their individual response characteristics.[13-15,17,20]

1. Isolated Enzymes

Many of the reported biosensors employ an isolated enzyme as the biocatalyst. Generally, the required enzyme is either purchased from a commercial supplier or purified by a known

procedure. Typically, a single isolated enzyme is employed; however, in some cases, multi-enzyme combinations are used to enhance the sensor response characteristics.

a. Single Enzyme Systems

Single enzyme systems rely on one enzyme to catalyze the required reaction. Selectivity, pH dependency, and useful lifetime are sensor response characteristics that depend directly on the properties of the immobilized enzyme.

Biosensor selectivity is partly influenced by the specificity of the enzyme. Many enzymes are selective in the reaction that they catalyze, while others work on a group of structurally related compounds. Examples are glutamate oxidase, which selectively catalyzes the oxidative deamination of L-glutamate,[21,22] and L-amino acid oxidase, which oxidatively deaminates many L-amino acids.[23] Clearly, the production or consumption of the monitored species by a substrate other than the analyte interferes with the measurement. Aside from the specificity properties of the immobilized enzyme, impurities in the enzyme preparation can result in additional biocatalytic activities at the sensor tip which can also cause this type of interference.

Enzyme inhibitors and activators are also potential interferences. A change in the effective amount of biocatalytic activity between sensor calibration and sample measurement can cause significant errors. An example is the effect of heavy metals. Depending on the enzyme/metal system, the metal ion can either enhance or inhibit the biocatalytic activity. Ions such as manganese (II) and magnesium are frequently required in the sample solution for optimal sensor response (maximum catalytic activity). On the other hand, ions such as copper (II) and mercury (II) can often adversely affect the sensor response by decreasing the effective biocatalytic activity. In extreme cases, the inhibitor can completely and nonreversibly inactivate the biocatalyst which renders the sensor useless. Often it is necessary to protect the biocatalyst from inhibitors by adding complexing or masking agents. For example, ethylenediaminetetraacetic acid (EDTA) is frequently added to complex inhibitory metal ions. The effects of activity modulation are minimized with high enzyme loading because the loss of a small fraction of the overall biocatalytic activity still leaves sufficient activity for normal operation. Activity modulation of this type can be used to determine the concentration of the modulator.[24] A low enzyme loading is preferred in this latter case so that maximal effect from the modulator is obtained.

All enzyme reactions are pH dependent and the solution pH must be adjusted often for maximum activity. The best pH for a particular application is generally a compromise between the pH dependencies of the biocatalyst, the detector element, and the sample.

A survey of the literature reveals an average lifetime of 28 d for biosensors that employ a potentiometric gas sensor as the detection element.[25] Lifetime values range from 0.4 to 240 d, however, which indicates that the effective sensor lifetime is heavily dependent on the stability of the biocatalyst. In addition, sensor lifetime is dependent on enzyme loading. With high enzyme loading, sufficient activity is still available for normal sensor operation after a fraction of the initial activity is lost owing to the natural degradation of the enzyme. Overall, the best conditions for enzyme stability must be established for each system. Experimental parameters of interest include pH, ionic strength, total protein concentration, temperature, and concentration of stabilizers such as dithiothreitol. In some cases, the best conditions for operation are not optimal for storage.[26]

Various methods are available to immobilize enzymes at the sensor tip. Immobilization procedures that involve covalent attachment, physical retainment, or entrapment in an inert protein matrix are commonly employed. The primary goal of any immobilization procedure is to retain a high activity of the biocatalyst at the sensor surface. Numerous reviews and monographs provide an excellent summary of this literature.[11,14,27,28] Several recent reports describe new methods of enzyme immobilization based on covalent attachment to inert

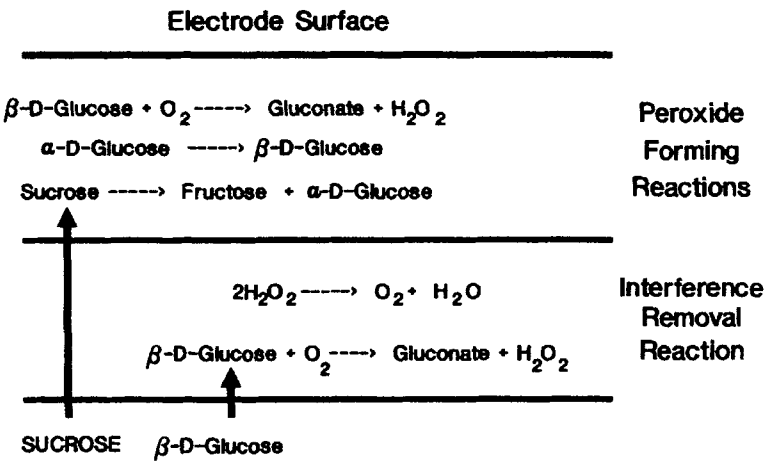


FIGURE 2. Selectivity enhancement scheme for a sucrose biosensor with an interferent removing pre-enzyme layer.

polymer membranes,[29-31] adsorption onto poly(vinyl chloride) membranes,[32] entrapment in fibrous cellulose triacetate structures,[33] and crosslinking with synthetic prepolymers.[34]

b. Multi-Enzyme Systems

The use of multiple enzymes can enhance the sensor response characteristics. For many years, catalase has been co-immobilized with oxidase enzymes to protect the enzyme from premature degradation by hydrogen peroxide. This strategy prolongs the operational lifetime of the sensor. In addition, enzymes can be combined to create new biosensors, to enhance sensor selectivity, and to amplify chemically the sensor response.

Multi-enzyme pathways can be used when a single enzyme system is not available. One example is the amperometric system for sucrose.[35] This system uses a combination of three enzymes at the surface of an oxygen electrode. The following three reactions are employed:

$$\text{sucrose} + H_2O \xrightarrow{} \alpha\text{-D-glucose} + \text{fructose}$$

$$\alpha\text{-D-glucose} \rightarrow \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \rightarrow \text{gluconate} + H_2O_2$$

where the first reaction is catalyzed by the enzyme invertase, the second by mutarotase, and the third by glucose oxidase. The presence of sucrose in the sample is measured amperometrically by following either a decrease in oxygen or an increase in hydrogen peroxide. A major problem for pathway-type sensors is interference by intermediates of the pathway. For example, endogenous β-D-glucose interferes with this sucrose biosensor.

An ingenious method of selectivity enhancement, which has been pioneered by Scheller and co-workers,[2,36] uses an additional enzyme to remove an interferent chemically. One example of this strategy is the fabrication of a sucrose biosensor composed of a prelayer that contains invertase, mutarotase, and glucose oxidase.[36] Figure 2 shows the reactions catalyzed and the relative positions of these two biocatalytic layers. The first enzyme layer effectively removes β-D-glucose from the sensor tip before it reaches the inner mediator region. The resulting sensor is not subject to interference by endogenous β-D-glucose. It is important to realize that the production of hydrogen peroxide must be monitored in this case because the interferent removing reaction consumes oxygen.

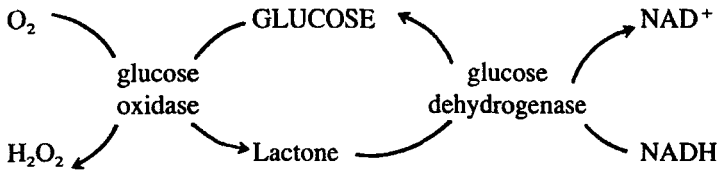
A second example is the creatinine biosensor[37] where creatinine deiminase is immobilized

at the surface of a potentiometric ammonia gas-sensor. Normal creatinine levels in serum are low and endogenous ammonia interferes with the measurement. A prelayer of glutamate dehydrogenase can be used to remove the interfering ammonia by the following reaction:

$$\alpha\text{-ketoglutarate} + \text{NADH} + NH_4^+ \rightarrow \text{glutamate} + NAD^+$$

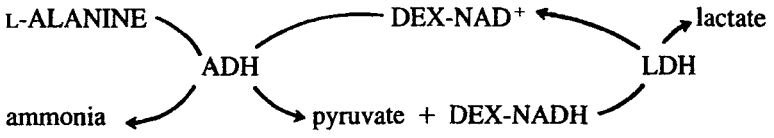
This selectivity enhancement reaction requires the addition of numerous reagents to the sample which diminishes the probe-type advantages of the original creatinine biosensor.

Multiple enzyme arrangements have also been constructed to amplify the signal by cycling the analyte.[38,39] An example is the glucose biosensor which uses the following reaction scheme:



Oxygen consumption is monitored amperometrically as glucose is continually cycled. The signal is amplified in comparison to the conventional single enzyme biosensor where 1 mol of oxygen is consumed for each mole of glucose. Overall, an improvement in detection limit is obtained. In general, amplification processes with substrate cycling require the addition of consumable reagents to the sample solution. For example, NADH is required for this glucose biosensor. Examples of nonenzymatic cycling and theoretical considerations of the amplification effects have been detailed.[40,41]

Alternatively, an immobilized cofactor can be cycled. An example is the L-alanine sensing probe[42] in which the following chemical reaction scheme is employed at the tip of a potentiometric ammonia electrode:



where ADH and LDH represent L-alanine dehydrogenase and lactate dehydrogenase, respectively. ADH produces ammonia from the analyte, and LDH regenerates the active form of the cofactor (NAD^+). Besides regenerating the cofactor, the LDH drives the analytical reaction by removing the products of the ADH reaction. NAD^+ and NADH cofactors are immobilized as dextran conjugates (DEX-NAD^+/DEX-NADH). Both the enzymes and cofactor conjugates are held at the sensor tip with a dialysis membrane. The resulting sensor is stable for 10 d. There is no need to add reagents with a cofactor immobilization and regeneration system such as this.

2. Alternative Biocatalytic Materials

Aside from isolated enzymes, various biocatalytic materials are available for the construction of biosensors.[43] Whole cell preparations from bacterial, mammalian, and plant sources, as well as subcellular fractions and artificial enzymes, have been proposed as biocatalytic materials.

Bacterial-based biosensors are constructed by physically retaining a slurry of freshly harvested bacterial cells at the surface of a detection element. Generally, the bacterial cells

are trapped behind a dialysis membrane or some other type of microporous membrane through which the analyte can penetrate. Enzymes located within the immobilized bacterial cells catalyze the required reaction. The bacterial cells simply serve to house the enzymes at the sensor tip.

Major advantages of bacterial cell biocatalysts include (1) the ability to use a biocatalytic activity that is not available in the form of an isolated enzyme; (2) the ability to use a complete, nature-optimized enzymatic pathway; (3) the increased stability of an enzyme that is maintained in its natural environment; and (4) the indefinite supply of fresh biocatalyst in the form of bacterial slants. Frequently, selectivity is a problem with bacterial cell-based biosensors. The presence of numerous enzymes within the bacterial cell can lead to positive interferences. Selectivity enhancement has been demonstrated by using enzyme and transport inhibitors.[44,45] Additional selectivity problems can be caused by compounds that alter the metabolism rate of the immobilized cells. This latter effect can be used effectively to monitor toxins and mutagens (for details see our discussion of environmental applications). Several excellent reviews are available that list specific examples of bacterial cell-based biosensors.[46-48] Recent advances include bacterial biosensors for sulfate,[49] monomethyl sulfate,[50] amino acids,[51-53] and ammonium ions.[54] In addition, improvements in sensor response characteristics have been demonstrated by purposely modifying the physical structure of the bacterial cell.[55] As an example, analyte transport into the immobilized cells can be facilitated by creating small openings in the outer bacterial cell membrane. Such permeabilized cells can be produced by freeze/thaw cycling or by brief exposure to various chemical reagents such as EDTA.[55a]

Sections of mammalian and plant tissues have been shown to be useful biocatalytic materials for biosensors.[56,57] Typically, a thin slice of the tissue is held at the sensor tip with a porous membrane. An example is the glutamine sensor where a thin slice of porcine kidney is held at the surface of an ammonia gas sensor with a nylon mesh (149-μm pore size).[58] Porcine kidney cortex cells contain a high level of the enzyme glutaminase which catalyzes the deamination of glutamine to form glutamate and ammonia.

As with bacterial systems, the tissue serves to house the enzyme in its natural environment. In some cases, the biocatalytic activity is more stable when maintained in the tissue matrix than in its isolated form. The lifetime of the tissue biosensor for glutamine, for example, is 30 d compared to only 1 d for the isolated enzyme-based glutamine sensor.[58] This lifetime enhancement is most likely due to an increase in stability provided by the tissue matrix. In comparison to bacterial systems, tissue biosensors tend to possess greater selectivity because fewer interfering enzymes are present in the tissue matrix. A selectivity enhancement scheme has been offered for cases when interfering enzymes are present.[59] This scheme is based on selective inhibition of the interfering activities.

Subcellular fractions of bacterial and tissue materials have also been employed as biocatalysts. Examples include a histidine biosensor based on an extract from *Lactobacillus 30a*[60] and a glutamine biosensor based on the mitochondrial fraction from porcine kidney cortex cells.[58] Fractionating the cells in this manner can effectively remove interfering enzymes and improve sensor selectivity.

Recently, Ho and Rechnitz have reported the first artificial enzyme-based biosensing probe.[61] Their example is a sensor for acetoacetic acid. The artificial enzyme is a poly(ethyleneimine) with 10% of the residues as primary amines. This polymer selectively catalyzes the decarboxylation of acetoacetic acid. The biosensor is constructed by retaining this artificial enzyme polymer at the surface of a carbon dioxide gas-sensing probe with a dialysis membrane. In comparison to the isolated enzyme-based acetoacetic acid biosensor (also based on a decarboxylation reaction), the artificial enzyme biosensor possesses a superior lifetime without the need for added cofactors. The artificial enzyme system operates with no changes in response for at least 6 months. In contrast, the lifetime for the isolated

enzyme system is less than 1 week. Selectivity of the artificial enzyme is excellent. Overall, artificial enzymes appear to be a viable substitute for natural biocatalysts. Recent advances in the development of artificial enzymes have been reviewed.[62,63]

C. Detection Systems

1. Amperometric

Oxidoreductase enzymes are commonly coupled with amperometric electrodes and either the consumption of oxygen or the production of hydrogen peroxide or NADH is measured. Alternatively, the direct or mediated electron transfer from a polarized electrode to the immobilized biocatalyst can be detected. The resulting steady-state current or the initial rate of current change (dI/dt)[64] is related to the concentration of the analyte in the sample solution.

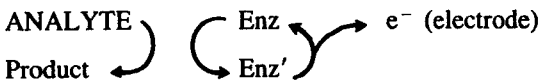
a. Oxygen Detection

Oxygen-consuming enzymes are typically coupled to a Clark-type amperometric oxygen gas electrode. Oxygen diffuses through a gas-permeable membrane, enters an internal solution, and is reduced at a platinum cathode. The measured steady-state current is directly proportional to the oxygen tension in the sample solution. By coupling this electrode with an oxygen-consuming enzyme, the resulting current is inversely proportional to the concentration of the enzyme substrate. A combination of enzyme specificity, membrane permeability, and electrode potential controls the overall selectivity of the biosensor. By using a gas-permeable membrane, nonvolatile endogenous electroactive materials do not interfere. Many examples of this type of biosensor have been reported.[2,3,12,14-17]

In general, the electrode current is proportional to the analyte concentration when the effective kinetics at the electrode surface are first order with respect to the analyte concentration. Under conditions of high analyte concentration and low oxygen levels, the electrode no longer responds to the analyte but to the oxygen.[65] One strategy to circumvent this problem is to design a sensor in which diffusion of the analyte into the biocatalytic layer is impeded while oxygen freely enters this layer. In this way, the effective concentration of oxygen in the reaction layer can be maintained high relative to that of the analyte. Gough and co-workers report such a system in which a two-dimensional enzyme electrode is employed.[66] Glucose oxidase is immobilized in a tubular-shaped chamber at the surface of a cylindrical oxygen electrode. The outer membrane of this tubular chamber is composed of hydrophobic silicon rubber which is highly permeable to oxygen, but impermeable to glucose. Figure 3 shows a schematic diagram of this electrode system. The entry of oxygen into the enzyme layer is allowed both from the open end and through the walls of the hydrophobic tubing. Glucose entry, on the other hand, is restricted to the open end. The cylindrical oxygen electrode measures the oxygen level throughout the tubular enzyme chamber. The resulting biosensor responds to glucose even under conditions of low oxygen levels.

b. Alternative Redox Processes

A second strategy to circumvent the problem of limited oxygen levels is to eliminate the need for oxygen by supplying an alternative mechanism for the redox reaction at the electrode surface. Direct electron transfer between the electrode and the enzyme is one approach. A second approach is to use an intermediary redox pair to mediate electron transfer from the enzyme to the electrode. The following reaction schemes are involved in these two cases:
Direct electron transfer:



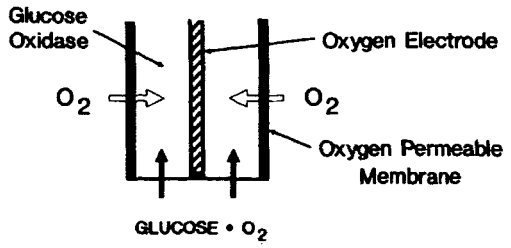


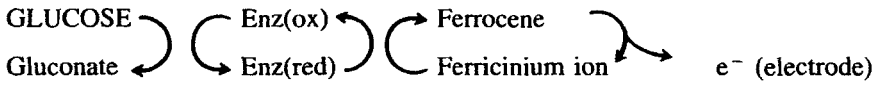
FIGURE 3. Cross-sectional view of a Gough-type two-dimensional glucose electrode.

Mediated electron transfer:

where Enz and Enz′ represent the oxidized and reduced forms of the enzyme, respectively, and Med(red) and Med(ox) represent the two forms of the mediator. The measured current associated with cycling either the enzyme or the mediator is related to the analyte concentration.

Direct electron transfer biosensors generally involve immobilization of the enzyme at the electrode surface through a covalently bound prosthetic group. The work of Wingard and co-workers is an example in which flavin adenine dinucleotide (FAD) is covalently attached to a carbon electrode.[67-69] Direct electron transfer from the FAD to the electrode has been demonstrated and further evidence suggests that direct electron transfer from the enzyme is possible. A second approach is to directly transfer an electron between a chemically modified electrode and an immobilized enzyme. Biosensors of this type have been prepared by using conducting organic salts such as tetrathiafulvalene tetracyanoquinodimethane (TTF^+ $TCNQ^-$) as the electrode material.[70-73] Efficient electron transport properties have been reported, particularly with flavin and heme dependent enzymes.[72]

Besides direct electron transfer, a mediator can be used to shuttle an electron from the enzyme to the electrode.[70,74-77] This latter approach has proven quite successful. The best example of such a mediated biosensor is the glucose sensor in which a ferrocene derivative serves as the mediator and glucose oxidase is the biocatalyst.[78] The reactions involved in this biosensor are as follows:



The enzyme catalyzes the oxidation of glucose to gluconic acid with the transfer of electrons to the enzyme. Subsequently, the enzyme transfers these electrons to the ferricinium ion to produce ferrocene. The ferrocene is then oxidized back to the ferricinium ion by the electrode and the anodic current is monitored and related to the glucose concentration. The ferrocene/ferricinium redox couple can be absorbed at the electrode surface in the stable ferrocene form. Partial dissolution of this absorbed material supplies the mediator at the electrode surface. An initial oxidation of the soluble ferrocene is performed to establish the required ferricinium ion. No differences in sensor response are observed under anaerobic and aerobic conditions with the ferrocene/ferricinium mediator system.

c. Hydrogen Peroxide Detection

Amperometric detection of enzymatically produced hydrogen peroxide is widely used for biosensors. The enzyme is immobilized directly on the surface of a polarized anode. In comparison to oxygen-sensing systems, hydrogen peroxide-based biosensors generally possess lower detection limits because the formation of hydrogen peroxide is detected on top of a zero background signal. In contrast, a decrement in oxygen relative to a high background level is measured with oxygen-based sensors. On the other hand, the selectivity of oxygen-based systems is generally better than for hydrogen peroxide-based sensors. Sample components that are easily oxidized can interfere with the hydrogen peroxide detection. Species such as ascorbate and uricate represent major interferences in clinical samples. Selectivity enhancement strategies have been proposed for hydrogen peroxide sensing.[79-81a] One common approach uses a cellulose acetate membrane that discriminates by size. The small hydrogen peroxide freely penetrates the membrane and is sensed by the electrode, while larger interferences cannot cross the membrane and are not detected.[17] Alternatively, an anionic membrane can be used to electrostatically repel common anionic interferences (i.e., ascorbate and uricate).[79] Recently, a combination of hexacyanoferrate (III) with laccase has been used to remove troubling interferences such as ascorbate.[81a] The interference effects from endogenous materials can also be eliminated by recording the difference between an enzyme-containing sensor and a blank or reference sensor.

d. NADH Detection

Several biosensors based on the amperometric detection of NADH have been reported.[70] A dehydrogenase enzyme is employed to catalyze the oxidation of the analyte with the concomitant reduction of NAD^+ to NADH. NAD^+ is regenerated electrochemically by oxidation of the NADH, and the resulting anodic current is measured. Unfortunately, when carried out with conventional electrodes, this oxidation process requires large overpotentials which decreases sensor selectivity. In addition, electrode fouling and the formation of products that are not enzymatically active severely limit the practicality of this direct oxidation approach. Recently, suitable modified electrodes have been developed which permit favorable oxidation of NADH.[71] These electrodes are based on conducting organic salts, such as tetrathiafulvalene tetracyanoquinodimethane (TTF^+ $TCNQ^-$). A mediatory mechanism has been proposed by Kulys for this electron transfer process.[72] Improved selectivity, increased efficiency in recycling, and less electrode fouling are observed.[71] A major problem with NADH-based biosensors is the need to add the cofactor to the sample. Attempts to co-immobilize the cofactor with the biocatalyst have not been particularly successful to date.

2. Potentiometric

Potentiometric-based biosensing probes have been developed for a wide variety of species. Glass pH electrodes and gas-sensing membrane electrodes have been used extensively in the fabrication of biosensors.[14,17,20]

a. pH Electrodes

Many enzymatic reactions produce or consume a proton. By immobilizing such an enzyme at the surface of a glass membrane pH electrode, the resulting pH change can be measured and related to the analyte concentration. An example is the penicillin biosensor in which the following reaction is used:

$$\text{penicillin} + H_2O \xrightarrow{\text{penicillinase}} \text{penicilloic acid}$$

A major problem with pH electrode-based biosensors is the pH dependency of the biocatalytic activity. Because the ability of an enzyme to catalyze a reaction is pH dependent,

the effective amount of enzyme at the sensor tip can change during sensor operation. The overall sensor response may or may not be repeatable depending on the pH stability of the biocatalyst.

In addition, the buffer capacity of the sample matrix strongly influences the sensor response. A high buffer capacity compensates for the formation or consumption of an acid and no pH change is detected. A low buffer capacity, on the other hand, can result in large pH changes which can inactivate the enzyme and limit the dynamic range of response. A compromise is required between the small signals over a wide concentration range for high buffer capacities and the large signals over a narrow concentration range for low buffer capacities. A difference in buffer capacity between the standards and the sample cannot be tolerated.

A pH-stat type sensor has been recently reported where protons are generated coulometrically to compensate for pH changes during the biocatalyzed reaction.[81b] The current required to maintain the pH level is measured as the analytical signal. This alternative approach still requires that the buffer capacities of the standards and sample be the same. Generally, biosensors based on pH measurements are not practical for measurements in complex biological samples.

b. Gas-Sensing Membrane Electrodes

Early in the development of potentiometric biosensors, glass, solid-state, and polymer membrane electrodes were used as the transducer element. For instance, urea sensors were prepared by immobilizing urease at the tip of cation-selective glass electrodes[82] and ammonium-selective polymer membrane electrodes.[83] The key problem for these first-generation sensors was selectivity. These early detection elements responded to many endogenous ionic species in the samples (i.e., sodium and potassium ions).

The advent of gas-sensing membrane electrodes for ammonia and carbon dioxide has made possible the development of potentiometric biosensors with sufficient selectivity for measurements in biological fluids. Typically, these electrodes are composed of a pH glass membrane electrode in conjunction with an internal electrolyte solution. For ammonia, this internal solution is composed of ammonium chloride. A thin gas-permeable membrane separates this solution from the sample solution. Ammonia in the sample diffuses across the membrane until the ammonia partial pressure is equal on both sides. The resulting ammonia in the internal solution determines the pH of this solution, and the resulting pH is measured with the pH glass electrode. The electrode potential is related to the sample ammonia concentration in a typical Nernstian fashion. Excellent selectivity is provided by the gas-permeable membrane because ionic substances in the sample cannot penetrate this membrane and alter the measured membrane potential. Biosensors are constructed by coupling ammonia and carbon dioxide gas sensors with deaminating and decarboxylating enzymes, respectively.

Biosensors based on ammonia and carbon dioxide gas-sensing electrodes suffer several common problems. First, endogenous ammonia or carbon dioxide must be considered. This is a particular problem with carbon dioxide-based biosensors because carbon dioxide levels can be substantial in many sample types. Gas dialysis[84] and enzymatic reaction[37] methods have been developed for removing endogenous interferences.

The pH dependency of gas electrodes can also be a problem. Because only the gaseous species is detected, gas sensors can only be used over a restricted pH range. The pH range for the electrode must overlap with that of the biocatalyst and the pH dependency of the resulting biosensor must be compatible with the sample. The pH of the sample must often be adjusted to match the optimal pH for the biosensor.

Perhaps the major problem with gas electrode-based biosensors is their slow dynamic response. This slow response is caused primarily by the slow response characteristics of the gas sensor itself.[85] Typical response times for the ammonia gas sensor range from 0.5 to

Table 1
FIBER OPTIC BIOCATALYTIC BIOSENSORS

Bioanalyte	Enzyme	Detected species	Optical measurement	Ref.
Ethanol	Alcohol oxidase	Oxygen	Fluorescence quenching	95
Ethanol	Alcohol dehydrogenase	NADH	Fluorescence	96
Glucose	Glucose oxidase	Oxygen	Fluorescence quenching	97
Glucose	Glucose oxidase	pH	Absorbance	98
Hydrogen peroxide	Peroxidase	Photon	Bioluminiescence	99, 100
Lactate	Lactate dehydrogenase	NADH	Fluorescence	101, 110
Lactate	Lactate oxidase	Oxygen	Fluorescence quenching	102
NADH	Bacterial luciferase	Photon	Bioluminescence	103
p-Nitrophenyl phosphate	Alkaline phosphatase	*p*-Nitrophenol	Absorbance	104
Penicillins	Penicillinase	pH	Absorbance	98
Penicillins	Penicillinase	pH	Fluorescence	105
Pyruvate	Lactate dehydrogenase	NADH	Fluorescence	101, 110
Urea	Urease	pH	Absorbance	98
Urea	Urease	Ammonia	Absorbance	101
Xanthine	Xanthine oxidase	Oxygen	Fluorescence quenching	102

2.0 min at high ammonia concentrations (i.e., > 0.1 m*M*) and from 5 to 10 min at low ammonia levels. The addition of a biocatalytic layer to the electrode surface can further slow the sensor response time. In addition, recovery times of these sensors are slow. The recovery time is the time required to reestablish the baseline potential between measurements. Recovery times as long as 45 to 60 min are required when starting from high ammonia levels.[85] Shorter times are needed when starting from lower concentrations. Inaccuracies are common when insufficient time is given for complete sensor response. Slow dynamic responses cause long analysis times, and they also limit the ability of these biosensors to monitor accurately the release or uptake of the analyte.

Attempts to improve the dynamic response of potentiometric gas sensors have centered on optimizing the composition and structure of the gas-permeable membrane[86,87] and the physical configuration of the sensing tip.[87] Guilbault has introduced an electrode configuration in which the internal electrolyte solution is replaced between measurements.[87] Although this arrangement dramatically reduces recovery times, it is a flow system that is not well suited for "probe" type applications.

3. Fiber Optic

Biosensors based on the immobilization of a biocatalyst at the distil tip of an optical fiber device have been recently introduced. These fiber optic biosensors can be classified into two catagories. The first class includes sensors that use a chemical reaction to mediate between the enzymatic reaction and the optical measurement. In the second class, the enzymatic reaction either consumes or generates an optically measurable species, and this species is directly monitored with an opto-electronic device through an optical fiber. Table 1 summarizes the fiber optic biosensors of both types that have been reported to date. A special issue of *Talanta* has been dedicated to the general topic of fiber optic chemical sensors,[88] and numerous excellent reviews[89-91] on this subject are available.

a. Chemically Mediated Fiber Optic Biosensors

Fiber optic chemical sensors for the measurement of oxygen, ammonia, carbon dioxide,

and pH can be coupled with enzymes to produce biosensors. These biosensors are analogous to the above-mentioned electrochemical-based biosensors with amperometric oxygen detection or potentiometric pH, ammonia, and carbon dioxide detection.

The fiber optic oxygen sensor is based on oxygen quenching of an appropriately selected indicator dye. Typically, dyes such as perylene dibutyrate[92] and pyrenebutyrate[93] are employed. An indicator solution composed of this fluorophore is separated from the sample solution by a thin gas-permeable membrane. Oxygen in the sample diffuses across this membrane and quenches the fluorescence of the indicator. A decrease in fluorescence intensity is monitored and related to the solution oxygen tension. The response of the resulting oxygen sensor can be described by the following Stern-Volmer equation:

$$\frac{I_o}{I} = 1 + KP_{O_2}$$

where I_o is the fluorescence intensity in the absence of the quenching agent (oxygen), I is the intensity in the presence of oxygen, K is the quenching coefficient, and P_{o2} is the oxygen partial pressure in the sample. Many fluorescent indicators for use in oxygen sensors have been described (see Reference 94 for a recent listing).

Lübbers and Opitz[95] first reported the construction of biosensors in which an oxygen-consuming enzyme is immobilized at the sensing tip of a fiber optic oxygen sensor. The relative intensity is measured and related to the sample concentration of the enzyme substrate. Sensors of this type have been described for ethanol, glucose, lactate, and xanthine (see Table 1).

A second class of chemically mediated fiber optic biosensors is based on immobilization of a deaminating enzyme at the tip of the fiber optic ammonia sensor.[101,106] This ammonia sensor uses an indicator solution that is composed of a pH indicator dye and ammonium chloride.[107,108] The indicator solution is separated from the sample solution by an ammonia-permeable membrane. Figure 4A shows a schematic representation of this sensor and Figure 4B details the various membrane phases involved in the sensor response. Ammonia in the sample partitions into the indicator solution until the partial pressure of ammonia is equal on both sides of the gas-permeable membrane. The addition of ammonia to the indicator solution changes the pH of this solution. By selecting an indicator with an appropriate acid dissociation constant, the change in pH can be followed optically through a set of optical fibers that are positioned in the indicator solution. Either absorbance or fluorescence measurements can be made. In the case where absorbance of the nonprotonated form of this indicator is monitored, the following expression relates the measured absorbance to the sample ammonia concentration:[107]

$$A = \frac{\epsilon b K_{In} C_{In} [NH_3]}{(K_{amm} C_{amm} - K_{amm}[NH_3] + K_{In}[NH_3])}$$

where ϵ is the molar absorptivity of the absorbing species, b is the effective pathlength of light at the sensor tip, K_{In} is the acid dissociation constant of the indicator, C_{In} is the total concentration of the indicator, K_{amm} is the acid dissociation constant for ammonia, C_{amm} is the total ammonium ion concentration in the indicator solution, and $[NH_3]$ is the concentration of ammonia in the sample solution.

By immobilizing a deaminating enzyme at the tip of this ammonia sensor, a biosensor for the substrate of the deaminase can be prepared. Figure 5 shows the response of a urea biosensor that has been constructed by immobilizing urease at the tip of the fiber optic ammonia sensor. A response function for biosensors of this type has been established. The solid line in Figure 5 shows the calculated response for this sensor. Although urea is the

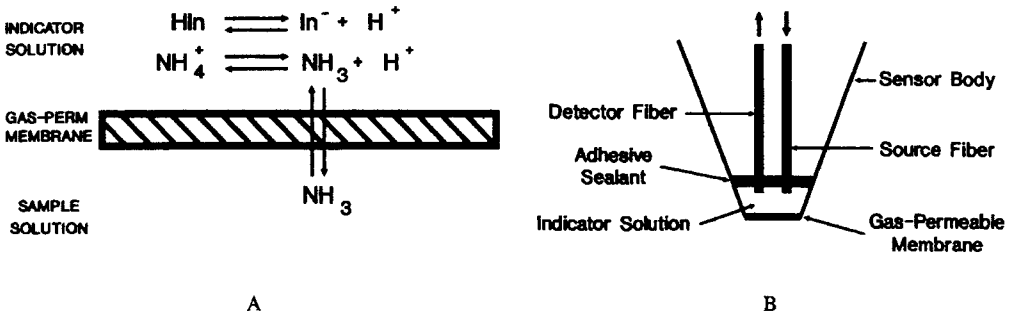


FIGURE 4. Schematic representation of the fiber optic ammonia gas sensor; (A) sensor components and (B) membrane phases.

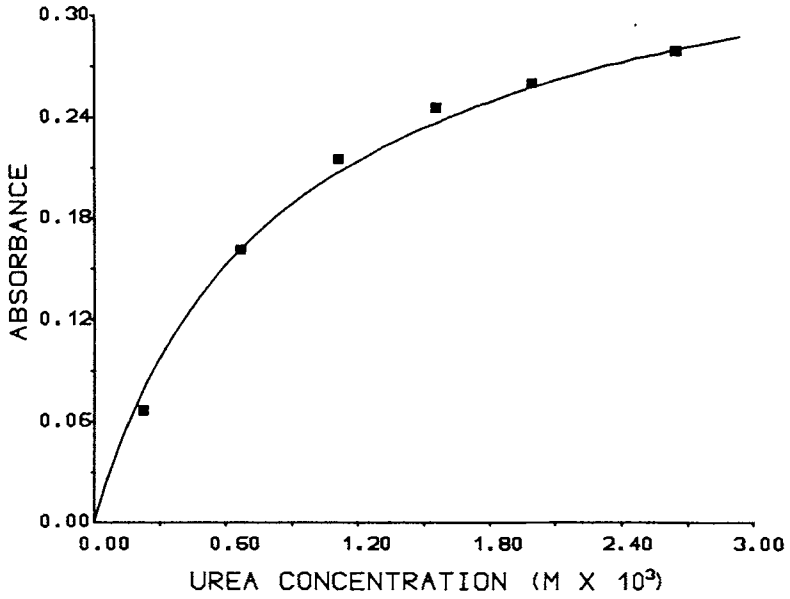


FIGURE 5. Typical response curve for fiber optic urea biosensor; (■) experimentally obtained values and (—) the predicted response.

only analyte for which this type of biosensor has been prepared to date, this strategy can be applied to all deaminating systems. Also, similar biosensors are possible by coupling fiber optic carbon dioxide sensors with decarboxylating enzymes.

Another type of chemically mediated fiber optic biosensor uses a fiber optic pH sensor as the internal sensing element. Action of the immobilized enzyme changes the local pH at the sensor tip. A suitable pH indicator dye is attached to the tip of an optical fiber device, and the enzyme-generated pH change is detected as a change in either absorbance or fluorescence.[98,105] As with pH electrode-based biosensors, the response of these pH-based fiber optic biosensors is heavily dependent on the buffer capacity of the solution, the pH stability of the biocatalyst, and the pH dependency of the biocatalytic activity. In addition, fiber optic pH sensors are subject to interference by quenching agents in solution and by changes in ionic strength and temperature. Overall, biosensors of this type will find limited use only in special situations where these parameters can be carefully controlled.

b. Nonmediated Fiber Optic Biosensors

In nonmediated fiber optic biosensors, the biocatalyzed reaction is followed by directly

monitoring either the production or consumption of an optically measurable species. Freeman and Seitz reported the first example of this type of sensor with a chemiluminescence-based hydrogen peroxide biosensor.[99] This sensor used a thin layer of peroxidase immobilized in a polyacrylamide gel at the tip of a fiber optic bundle. Peroxidase catalyzes the chemiluminescent reaction between hydrogen peroxide and luminol. The key product of this reaction is a photon. This photon is transmitted by the fiber optic bundle to a photomultiplier tube (PMT) detector. An increase in the concentration of hydrogen peroxide corresponds to a greater number of photons and a larger detector response. A similar sensor design has been reported by Aziawa and co-workers where the peroxidase is immobilized directly on the face of a photo-diode detector.[100] Aziawa and co-workers demonstrate that their hydrogen peroxide biosensor can be expanded to monitor other analytes by co-immobilizing a second enzyme that produces hydrogen peroxide. A glucose sensor with glucose oxidase illustrates this latter point. The Seitz sensor has a detection limit of 1 μM for hydrogen peroxide. The Aziawa system possesses detection limits of 2 and 50 mM for hydrogen peroxide and glucose, respectively. In both cases, sensor operation requires the addition of luminol to the sample.

The enzymatic production or consumption of a chromophore or fluorophore can be the basis for a biosensor. The first chromophore-based biosensor has been reported for *p*-nitrophenylphosphate.[104] This biosensor uses alkaline phosphatase covalently immobilized at the common end of a bifurcated fiber optic bundle. The biocatalyzed reaction converts *p*-nitrophenylphosphate to *p*-nitrophenol. In basic solutions, *p*-nitrophenol strongly absorbs 404 nm radiation. A steady-state concentration of this chromophore is established at the sensor tip, and the resulting steady-state absorbance is directly proportional to the substrate concentration in the sample.[104]

Biosensors based on the fluorometric detection of NADH have been recently introduced.[101,110] A dehydrogenase enzyme is immobilized at the tip of a fiber optic device and either the production or consumption of NADH is monitored. The first example of such an NADH-based biosensor uses a thin layer of lactate dehydrogenase at the common end of a bifurcated quartz fiber bundle. One arm of the bundle transmits the excitation radiation from a source to the sensor tip, and the other arm carries the emitted radiation from the sensor tip to a PMT detector. Interference filters are used to select the required wavelengths.

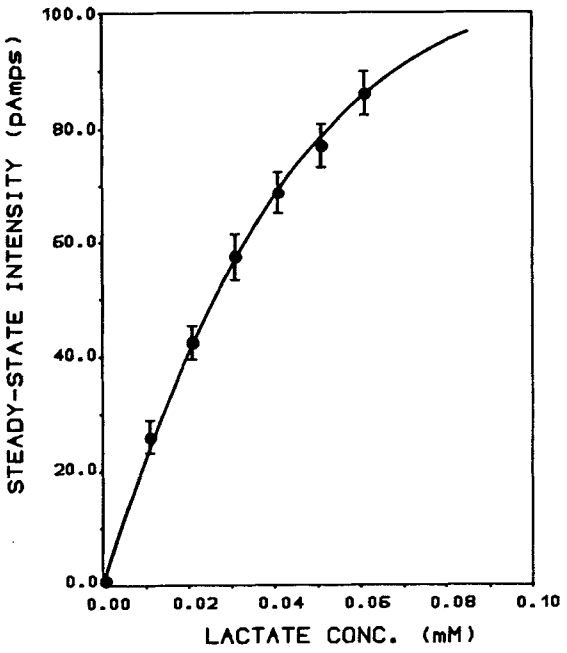
The following reaction is catalyzed by lactate dehydrogenase:

$$\text{lactate} + \text{NAD}^+ \leftrightharpoons \text{pyruvate} + \text{NADH} + \text{H}^+$$

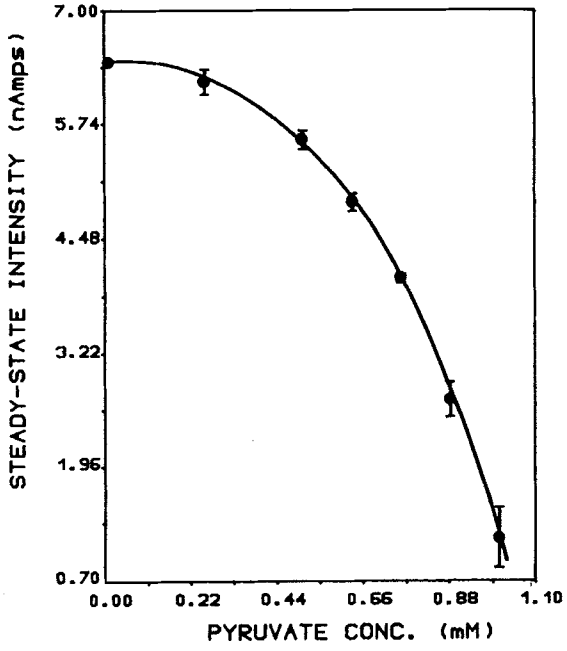
The production of NADH is measured in the lactate sensing mode which requires the addition of NAD^+ to the sample. Alternatively, the sensor can operate in a pyruvate sensing mode with the detection of NADH consumption. This latter arrangement requires that NADH be present in the solution. Figure 6 shows typical lactate and pyruvate calibration curves for this biosensor. As expected, an increase in the steady-state fluorescence intensity is obtained with an increase in lactate concentration owing to the production of NADH. The expected decrease in fluorescence intensity is measured with increasing pyruvate concentrations.

Many NADH/NAD^+ and NADPH/$NADP^+$ type biocatalytic reactions have been identified and the corresponding enzymes isolated. Numerous biosensors are possible based on these reactions. In comparison to the previously mentioned amperometric NADH-based biosensors, the fiber optic approach provides lower detection limits, and problems of electrode fouling are eliminated.

The primary advantage of fiber optic devices over electrochemical systems is the ability to monitor multiple wavelengths with a single optical fiber. Sensors for the detection of multiple analytes can be envisioned. Indeed, such a system has been recently introduced for blood gases.[111] In addition, a separate reference element is not required for the measurement. The analytical signal can be measured relative to the intensity of a reference wavelength.



A



B

FIGURE 6. Response curves for the NADH-based fiber optic biosensor with immobilized lactate dehydrogenase; (A) response to lactate in the NADH production mode and (B) response to pyruvate in the NADH consumption mode.

Intensity ratio measurements of this type provide stable readings and minimize the need for sensor recalibration.

4. Thermal Detectors

Enzymatic reactions are generally exothermic, and the heat produced during the reaction can be sensed by a temperature sensitive transducer. Such calorimetric-based biosensors have been reported with enzymes immobilized directly on either a thermistor[112] or transistor[113] device. Typically, measurements are made relative to a blank sensor (a sensor with no enzyme or with inactive enzyme) to account for ambient temperature fluctuations. Because the heat of reaction can be measured for any enzyme-catalyzed reaction, this approach is quite general and can lead to the development of biosensors for numerous analytes.

Unfortunately, the generated heat rapidly dissipates into the surrounding solution which results in small signals and low sensitivities. An alternative arrangement has been pioneered by Mosbach and Danielsson where a thermistor detector is positioned at the end of an immobilized enzyme reaction column. As the sample passes through the column, the biocatalyzed reaction takes place, and the resulting change in solution temperature is subsequently measured by the thermistor detector.[114,115] Reviews of this type of sensing system are available.[112,116,117]

5. Miniature Detectors

Micro-fabrication methods are being devised for the mass fabrication of miniature biosensors (i.e., sensing tips with diameters between 0.1 and 1 mm). Enzymes can be coupled with pH-sensitive field effect transistors (FETs), gas-sensitive transistors, and amperometric-based oxygen and hydrogen peroxide-sensitive devices. In addition, tip sizes from 100 to 500 μm are possible with the above-described fiber optic biosensors. Techniques for construction of biosensors with micron sensing tips are also being developed by covalently immobilizing enzymes at the tips of micro-electrodes.

The most common type of miniature biosensor reported to date involves the immobilization of an enzyme at the pH-sensitive silicon nitride surface of a FET device. Such enzyme field effect transistors (ENFETs)[118] are generally operated in a differential mode with an enzyme-free pH-sensitive FET as the pseudo-reference element. ENFETs based on pH detection have been reported for glucose,[119-121] penicillin,[122] adenosine triphosphate,[123] hypoxanthine,[124] and urea.[125] Each of these biosensors is strongly dependent on the buffer capacity of the sample which makes them unsuitable for most applications.

Gas-sensitive transistors for ammonia have been coupled with deaminating enzymes to produce biosensors for urea,[126,127] creatinine,[126,127] histidine,[126] and alanine.[127] In addition, amperometric oxygen and hydrogen peroxide sensitive devices have been used for the fabrication of miniature glucose sensors.[125,128]

Micro-biosensors with sensing tips between 1 and 100 μm have been reported with enzymes immobilized at the tips of micro electrodes for pH, hydrogen peroxide, ammonia, and NADH. Micro-biosensors for urea[129] and acetylcholine[130] have been prepared by immobilizing urease and acetylcholinesterase, respectively, at the tip of micro pH electrodes. The urea sensor employs an antimony wire and the acetylcholine sensor employs a micro-glass electrode. In addition, micro-urea biosensors based on ammonia gas-sensors with tip sizes of 10 μm have been reported.[131,132] Finally, a micro-amperometric sensor for pyruvate has been prepared with lactate dehydrogenase coupled with the oxidation of NADH at a micro-pyrolytic carbon fiber electrode.[133] Sensors of this size might be useful for intracellular measurements.

D. Current and Potential Analytical Applications

1. Clinical

The application of biosensors in clinical chemistry centers around the development of

devices for alternate site and critical care measurements. Systems for alternate site measurements are designed for use in settings such as a physician's office, an operating room, an emergency room, an ambulance, and a patient's home. Critical care devices are those designed to continuously monitor the blood chemistry of a critically ill patient. Such continuous monitors will allow fast response to sudden changes in the patient's condition. Specific requirements for alternate site and critical care monitors are presented in a recent chapter by Home and Alberti.[134]

There is a trend to design clinical chemistry instruments based on a single type of detection element (i.e., amperometric, potentiometric, or fiber optic). Many of the clinically important analytes, such as glucose, lactate, and ethanol, can be measured amperometrically by combination of available oxidase enzymes with oxygen electrodes. Unfortunately, suitable oxidase enzymes are not available for all clinically significant species, such as urea and creatinine. Often deaminase-based biosensors are available for these other important analytes. An amperometric ammonia probe is required before a totally amperometric approach can be developed. Karube and co-workers have described an amperometric ammonia electrode that uses a layer of nitrifying bacterial cells at the surface of an oxygen electrode.[134,135] Alternatively, a totally potentiometric approach requires either biosensors based on potentiometric oxygen detection or biosensors based on measuring redox potentials with inert electrodes.[136] No examples of suitable potentiometric oxygen electrodes have appeared, and the proposed redox potential system is severely limited by selectivity problems. Perhaps a system based on spectroscopy is the only practical approach. Fiber optic biosensors for all commonly considered clinical analytes are possible by coupling the appropriate enzyme with fiber optic sensors for oxygen, ammonia, carbon dioxide, or NADH.

Considerable effort has been given to the development of implantable biosensors. Of primary importance is an implantable glucose sensor that can be used in conjunction with an artificial pancreas. Such a device would be invaluable in the control of diabetes. Unfortunately, several significant barriers still impede the development of implantable biosensors. Issues of biocompatibility, biocatalyst stability, and *in vivo* calibration continue to demand attention.[137-139] Significant progress, however, has been made in the development of a glucose sensing system for a bedside, *ex vivo* artificial pancreas[139,140] and a short-term, closed-loop system for diabetes treatment.[141]

2. Biomedical

Little attention has been given to the application of biosensors to biomedical research. In many instances, a small, selective sensor could be used to follow continuously the concentration of a biomolecule during fundamental investigations. Intra-cellular, and site-specific extra-cellular measurements of important biomolecules could be made with micro-biosensors.

The use of micro-biosensors to measure putative amino acid neurotransmitters is one example of such an application. Separation of nonsynaptic and synaptic pools of these amino acids during perfusion experiments has been difficult with conventional procedures.[143] Generally, high amino acid concentrations in the nonsynaptic pool overshadow the lower concentrations in the synaptic pool. Micro-biosensors that are selective for the amino acid under investigation could be positioned to measure the release or uptake at a specific synaptic location on the tissue matrix. In this way, the two amino acid pools can be effectively separated and selective neurochemical measurements can be made. A recent attempt to apply a glutamate selective biosensor to study the neurochemical properties of this amino acid reveals that considerable developmental work is necessary before this approach is practical.[144] Nevertheless, successful development of selective micro-biosensors for amino acids and other biomolecules will have a tremendous impact on many areas of biomedical research.

3. Environmental

Biosensors can be used to detect and identify environmentally toxic and mutagenic sub-

stances. Inhibition of the activity of an immobilized biocatalyst can be measured as a decrease in the rate of the biocatalyzed reaction. The extent of inhibition can be related to the concentration of a particular toxin. Generally, an isolated enzyme biocatalyst can be used to sense a class of compounds. An example is the detection of organophosphates based on the inhibition of acetylcholinesterase. The enzyme reaction can be monitored with a pH electrode, and the rate of reaction is inversely proportional to the concentration of the organophosphate. Of course, the substrate for the catalyzed reaction must be added to the sample to initiate the measurement. In addition, inhibition by the toxin might not be reversible which would require a new sensor for each measurement. A wide variety of substances, such as drugs, heavy metals, and pesticides,[145] can be measured based on enzyme activity modulation. Specific examples include systems for phosphates,[146] copper (II) and zinc (II) ions,[147] organophosphates,[148,149] and carbamates.[149]

Bacterial electrodes have been used to measure general toxicity of a solution. Here, the metabolic rate of living bacterial cells is monitored with an oxygen electrode. As the toxicity level increases, the respiration rate of these immobilized cells decreases and less oxygen is consumed. Overall, an increase in oxygen is detected with an increase in toxicity. The extent of metabolism decrease indicates the magnitude of toxicity. Sensors of this type are not selective for a particular toxin or for a class of toxins, but they provide a measure of the overall toxicity level of the sample. Of course, nutrients for microbe respiration must be present in the sample. Bacterial electrodes of this type have also been used as sensors for antibiotics.[46]

Mutagen screening is also possible with bacterial cell electrodes.[150] Karube and co-workers have demonstrated this principle with sensors based on immobilized strains of *Bacillus subtilius*[151] and *Salmonella typhimurium*.[152]

The *B. subtilius* system is based on a differential measurement between a bacterial electrode with immobilized *B. subtilius* (Rec^-) and a second electrode with immobilized *B. subtilius* (Rec^+). These two bacterial strains are immobilized at the surface of oxygen electrodes and the difference in respiration is monitored. The measurement is based on the different response of these bacterial strains to a mutagenic species. *B. subtilius* (Rec^-) is a designed strain that cannot repair damaged DNA. *B. subtilius* (Rec^+), on the other hand, is a wild strain that can repair damaged DNA. Initially, the two sensors are placed in a solution with the required nutrients and a current difference is measured. Both sensors are then exposed to the mutagen and a change in the current difference is detected. The mutagen destroys the DNA of both cells, but the cells of the wild strain can repair this damage while the (Rec^-) cells cannot. The death of the (Rec^-) cells is detected as an increase in current (decrease in respiration). The magnitude of the current change is linearly related to the concentration of the mutagen. Chemicals that affect cell respiration in other ways, such as antibiotic, bactericides, etc., do not elicit a response because a difference measurement is utilized.

A second mutagen screening biosensor has been reported with *S. typhimurium* (TA-100) cells immobilized on an oxygen electrode. This particular bacterial strain requires histidine in the growth medium for cell respiration and growth. In the presence of a mutagen, this strain is converted to the wild form of *S. typhimurium* that does not require histidine. Initially, the sensor is placed in a histidine-free growth medium. The mutagen is added and an increase in respiration is measured as a decrease in current. Respiration increases because of a conversion from histidine-dependent to histidine-independent cells.

The primary advantage of mutagenicity biosensors over conventional assays is the rapid analysis time. Biosensors require less time because microbial respiration is measured in a thin layer of cells at an electrode tip as opposed to the measurement of bulk cell growth. Measurements can be made within hours with the sensor approach, whereas days are required with conventional methods (i.e., the Ames test). In addition, biosensors can detect lower concentrations of a mutagen. Detection limits of 0.001, 1.6, and 10 μg/ml are reported for

Table 2
EXAMPLES OF RECENT INDUSTRIAL APPLICATIONS OF BIOSENSORS

Analyte	Application	Ref.
Arginine	Peanut maturity	153b
Lysine	Cereal grains	154
Lysine	Bioreactor	155
Lactate	Tissue culture medium	156
Glutamine	Mammalian cell bioreactors	157
Tyramine	Meat freshness	158
Assimilable sugars	Fermentation broths	159
Lactose/ethanol	Foodstuffs	160
Glucose	Foodstuffs	161
Ethanol	Wine	162
Inosine monophosphate	Fish tissue	163
Adenosine monophosphate	Fish tissue	164
Ethanol	Aerobic yeast suspensions	165
Hypoxanthine/inosine	Fish	166
Carbohydrates/amino acids	Tea plants	167
D-Galactose	Milk/sugar products	168
Microbial respiration	Fish freshness	169
Sulfite	Foodstuffs	170

the *S. typhimurium*-based biosensor, *B. subtilius*-based biosensor, and Ames test, respectively.[151,152] It must be noted that these sensors are not reversible. A new sensor must be constructed before each measurement.

Respiration-type biosensors can be developed for other systems as well. For example, biosensors with the well-established tumor model (mouse leukemia L12010) immobilized on an oxygen electrode can be used to identify possible anti-tumor agents.[153a] Overall, many exciting possibilities exist for using biosensors based on cellular respiration.

4. Industrial

Biosensors are finding applications in the food and biotechnology industries. Table 2 summarizes some of the recently reported examples of biosensor applications in these areas. Selective, rapid, and reversible responses make biosensors ideally suited for such industrial applications.

Application of biosensors as industrial process monitors [171] is an important and expanding area of development. Biosensors can be used as continuous monitors to control a given process either directly through a feedback mechanism or indirectly through operator intervention. Such monitors can also be used for continuous quality control measurements along the process stream. Benefits of such process monitors are well recognized[172] and include greater efficiency, better product quality, improved product consistency, and lower operation costs.

In-line monitors for bacterial and mammalian cell bioreactors require sensors that are free from microbial contaminates. Development of sterilizable biosensors is desirable for this reason. Unfortunately, sterilization conditions are generally too harsh for the immobilized biocatalyst. An alternative arrangement uses the biosensor in an on-line manner where the sample is (1) automatically removed from the process stream, (2) conditioned for the analysis, and (3) subjected to the sensor for the measurement.[173] Afterward, the sample can be either discarded or taken for further analysis.

On-line arrangements can be configured with the enzyme either immobilized on the detection element or in an enzyme column through which the sample passes. In the latter

arrangement, the product generated or co-substrate consumed during passage through the enzyme column is subsequently measured with an unmodified sensing element. The enzyme column approach would seem to have the advantage that conditions could be optimized individually for the biocatalytic reaction and the detector measurement. By using separate components, units can be replaced individually as needed. Reactor columns with large amounts of enzyme can be employed to minimize frequency of replacement. Finally, the biosensing probe approach is a kinetic method based on the establishment of a steady-state condition. The modular approach, on the other hand, can be configured so that the biocatalyzed reaction goes to completion and an equilibrium measurement is employed. The modular approach can, therefore, take advantage of the merits of equilibrium methods. Interestingly, Stulik and co-workers conclude from their direct comparison of enzyme reactor vs. enzyme probe systems for glucose and tyrosine that the probe arrangement gives superior sensitivity, precision, and response rate.[174] Dispersion caused by the enzyme column is thought to be responsible for the poorer analytical performance of the enzyme reactor arrangement.

III. RECEPTOR-BASED BIOSENSORS

A. Types of Molecular Receptors

Receptor-based biosensors gain their selectivity from the natural affinity of certain proteins, or fragments of proteins, toward specific target species (complementary ligands). Unlike biocatalyst-based sensors, no chemical reaction need take place after the molecular recognition process. In general terms, the receptor selectively interacts with a given ligand to form a thermodynamically stable complex in accordance with the scheme:

$$R + L \rightleftharpoons RL$$

with an affinity constant of K_{RL}. This association is governed by size and shape (so-called lock-and-key type mechanism) of the receptor pocket and complementary ligand, as well as charge-charge interactions, hydrogen bonding, and van der Waals interactions. Biosensor selectivity over related analyte species (e.g., L′, L″, etc.) is generally proportional to the affinity of the receptor toward these other species relative to the primary analyte ($K_{RL'}/K_{RL}$, $K_{RL''}/K_{RL}$, etc.). A number of different biological receptor type molecules have been employed for the construction of biosensors. In this section, we briefly review the nature and properties of these biomolecules.

1. Antibodies

The high specificity and affinity of antibody-antigen binding reactions have prompted considerable efforts to use these immunochemical reactions to design biosensors. Most mammalian antibodies fall into the immuno-gamma-globulin class (IgG). Such protein molecules have molecular weights of about 160,000 Da, two antigen-binding sites per molecule, and the ability to bind tightly to their respective antigens (with K_{RL} values in the range of 10^6 to 10^{11} l/mol). When using IgG antibodies for the design of biosensors, two types of preparations are possible: polyclonal and monoclonal. Both types need to be elicited through an initial immunization of an animal with an appropriate high molecular weight antigen. When preparing antibodies toward low molecular weight species (drugs, steroids, hormones, etc.) such haptenic substances must first be covalently attached to a suitable high molecular weight antigen before the immunization step (immunogen). The immunization process results in a large population of IgG molecules in the serum of the animals, each with different affinities and selectivities for the desired analyte ligand. Use of such heterogeneous binding preparations (polyclonal) can cause difficulties in biosensor applications. Therefore, the

utilization of modern monoclonal antibody technology provides biosensor researchers with IgG receptor molecules with more desirable homogeneous binding properties.

In certain applications, it may be necessary to reduce the size of the IgG molecule and, at the same time, eliminate its divalent binding character. This can easily be accomplished by enzymatically cleaving IgG molecules into pieces, two of which are identical but independent protein molecules possessing the same receptor binding sites as the original IgG molecules. These smaller binding fragments are termed "FAbs".

2. Endogenous Binding Proteins and Lectins

These water-soluble binding proteins are usually analogous to antibodies in terms of their interactions with target molecules. However, there is no need to elicit their production via immunizaton procedures. Indeed, normal physiological fluids and the intracellular cytoplasm of animal, plant, or bacteria cells are the primary sources of these naturally selective receptors. As endogenous binders, these proteins usually offer a homogeneous population of binding sites (analogous to monoclonal antibodies). Molecular weights and valences can vary considerably, as can the affinity constants for complex formation with their target ligands (e.g., up to 10^{15} l/mol for avidin/biotin system). The nature of the complementary ligand is also quite variable, including proteins, drugs, steroids, smaller ions (e.g., $HOP\overline{\overline{4}}$), and gases (e.g., hemoglobin serves as a natural receptor for oxygen).

Lectins are a special class of natural binding proteins that recognize certain carbohydrate units. Thus, such proteins can complex with polysaccharide structures, smaller sugar molecules (e.g., glucose, fructose, etc.), or larger proteins which possess innate carbohydrate substructures (glycoproteins). For sensing purposes, lectins offer a potential advantage when compared to antibodies and other natural binders in that their affinity constants are somewhat lower than the other classes of receptor proteins (10^3 to 10^6 l/mol). In most instances this implies faster dissociative kinetics, enabling sensors based on these proteins to be truly reversible. At the same time, however, lower affinity can comprise the ultimate detection limits of the sensor.

3. Membrane Bound Chemoreceptors

Endogenous binders which cannot be removed from their normal biological environment fall under this broad category. Since the structures of such proteins are often dependent on the integrity of the biomembranes in which they reside, if completely isolated, these receptors would lose all biological binding activity. Therefore, to be utilized in the design of biosensors, researchers must find ways to use whole intact cells which contain these receptors, or reincorporate the receptors into synthetic biomembranes after their initial isolation from the cells (phospholipid bilayers or Langmuir-Blodgett films). Natural binders within this class that have been studied for biosensing purposes have included receptors for neurotransmitters, opiates, and amino acids.

4. Enzymes

We have previously described how enzymes can be used to devise analytically useful biocatalytic probes. However, aside from binding substrates and catalyzing reactions in solution, many enzymes possess binding affinities for other important species without concomitant catalytic activity. The binding sites for these species may be at the same site as the substrate(s) or at a completely different location within the structure of the enzyme (allosteric sites, cofactor binding sites, etc.). The various binding regions of the enzyme may be useful for molecular recognition of competitive and noncompetitive inhibitors, cofactors, substrate analogs, etc. In addition, in certain sensor configurations (gas phase measurements), enzymes can apparently be used to bind and detect natural substrates without the simultaneous catalysis of these normal solution phase species.

B. General Detection Schemes

A wide variety of detection schemes have been proposed for the development of receptor-based biosensors. We restrict our coverage to those systems in which either the receptors or their complementary ligands are in intimate contact with a detection element (not flow-through reactors, etc.). When the receptor is immobilized, the device is generally intended to detect the complementary ligand. When the ligand or an analog of the ligand is immobilized, the device responds to the concentration of the receptor. This latter scheme can also be used to detect free analyte ligand levels via a competitive binding method (add fixed and known amounts of receptor to the sample). Two general approaches may be used to detect interactions of receptors and their complementary ligand species at the surface of a biosensor device. These two approaches are outlined below.

1. Direct Sensing

This class refers to sensors in which the interactions of the receptor and its complementary ligand result in a directly detectable physical or chemical signal without the addition of external reagents. For example, upon binding of the receptor to the ligand, the spectral properties of the ligand or receptor can change (e.g., fluorescence, absorption, etc.) or heat can be generated or consumed (exo- and endothermic reactions). Similarly, when one component of the interacting pair is immobilized, the binding interaction will result in a mass change at the surface on which the immobilized species resides. Such spectral, thermal, or mass changes can be sensed by the appropriate transduction elements. This is schematically illustrated in Figure 7. Although such direct sensing schemes are the most desirable, there have been only a handful of literature reports in which this approach has been applied successfully for the development of functional biosensors.

2. Indirect Sensing Schemes

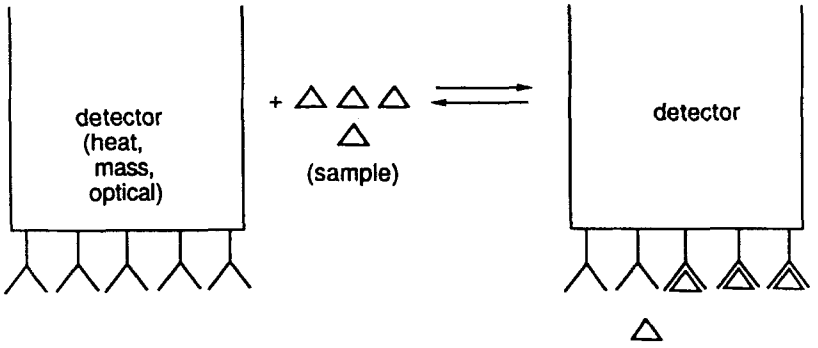
By far, most receptor-based biosensors reported to date fall into this category. Here, the binding reaction is detected by following a secondary reaction or process (i.e., the signal is not generated from the receptor or ligand themselves). Sensors in this category can be further divided into two subclasses: those that use specific labels (enzymatic, electrochemical, fluorescent, etc.) to follow the binding reaction, and those that rely on modulation of signals (usually electrochemical) arising from background ionic components of the sample medium.

a. Labels

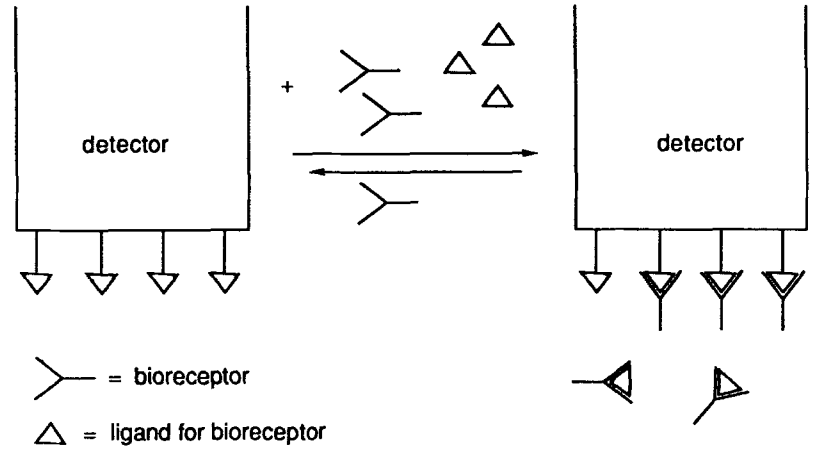
Generally, when labels are used, one component of the receptor ligand pair is immobilized on or near the detector, while the second component is covalently tagged with a chemical tracer that can be detected at low levels. Sensing is normally accomplished by using a competitive binding approach, whereby the degree of binding between a fixed amount of the labeled and unlabeled components is perturbed by the presence of the analyte. This is illustrated in Figure 8 for the case where the receptor is the immobilized component. Usually, such sensors can only be used for discrete sample measurements and not for continuous monitoring. Indeed, after incubating the labeled component with the sample and the sensor, the unbound label must be washed away prior to measuring the bound label concentration. Subsequent discrete measurements with the same probe require fresh aliquots of the labeled component. To fabricate truly reversible sensors, both the labeled and unlabeled receptor/ligand species must be immobilized or retained in a region adjacent to the detection element.

b. Modulation of Secondary Ionic Processes

Indirect sensors of this type are typically electrochemical devices in which potentials, currents, capacitances, or resistances (conductance) are the measured parameters. Changes in these parameters can occur when the receptor interacts with its complementary ligand. The best example of this sensing scheme may be found in nature. Many chemoreceptors



A



B

FIGURE 7. Schematic representation of biosensors that are based on direct detection of the interaction between the immobilized receptor and sample ligand (A), or an immobilized ligand and receptor in the sample (B).

function by altering the permeability of living cell membranes to ions as a result of the receptor-ligand binding interaction. When ion permeabilities of the membranes change, measurement of cell membrane potentials or conductivities provides a means of detecting this binding reaction. However, detection is critically dependent on bathing the cells in a medium with known ionic composition.

C. Specific Designs Classified According to Detection Element

In the previous two sections, we have summarized the types of receptors and the general detection approaches which may be used to design receptor-based biosensors. In this section, we cite and critically review specific examples taken from the literature. For convenience, we have classified these examples according to the type of detection example employed rather than the detection scheme.

1. Electrochemical

Many of the initial efforts to develop receptor-based biosensors involved the use of potentiometric measurement systems. For example, Janata[175] reported on "an immunoelec-

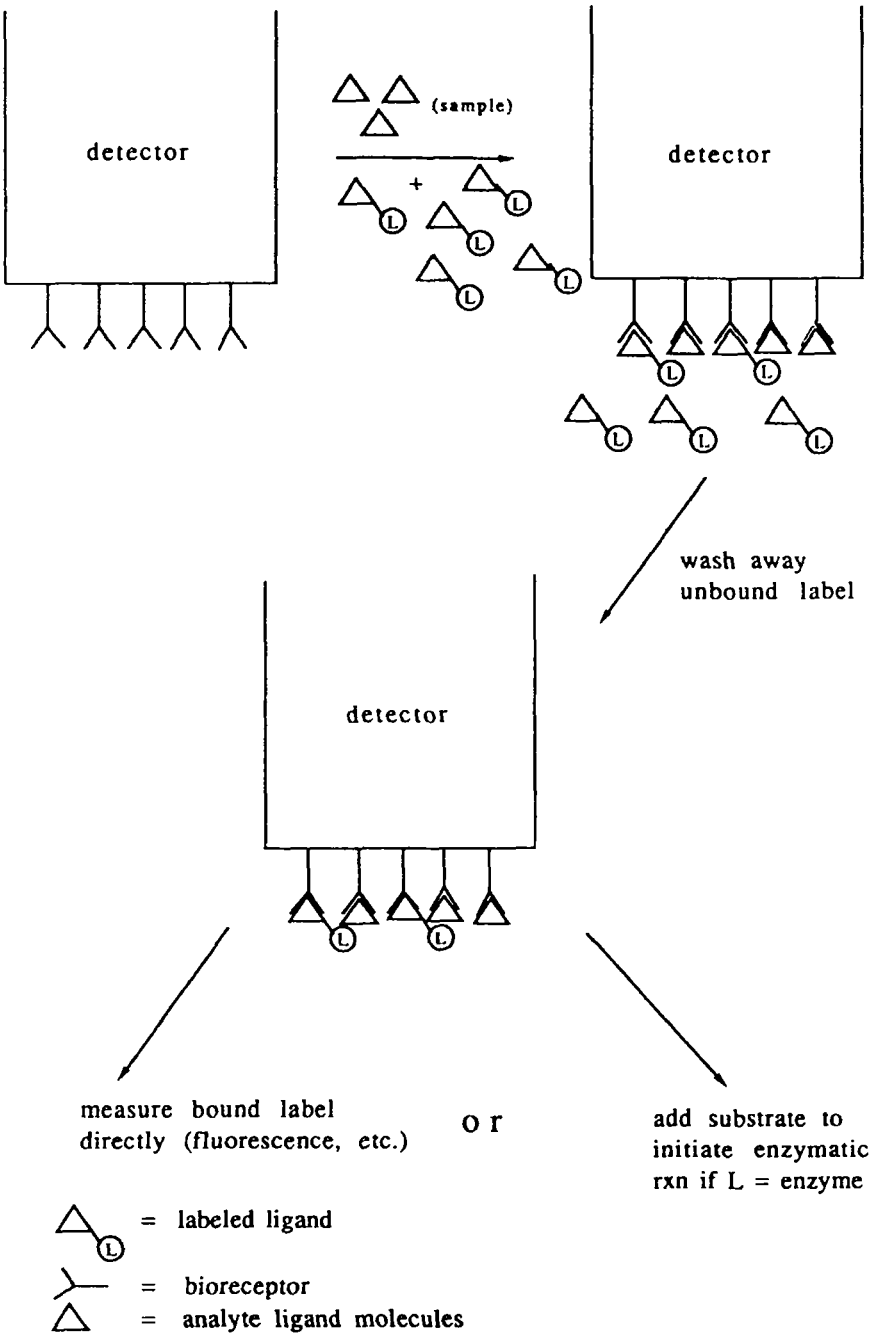


FIGURE 8. Representation of the design and detection steps involved in using receptor-based biosensors employing immobilized receptors and labeled ligands.

trode" in which Concanavalin A (a lectin) was covalently immobilized to the surface of a polymeric film deposited on a platinum wire. Large cell emf changes were observed when the sensor was exposed to yeast mannan, a polysaccharide that forms a strong complex with the lectin. While much of this response was attributed to nonspecific binding of the polysaccharide to the polymer coating, differential measurements, in which the normal Ag/AgCl reference electrode was replaced by a polymer coated wire without immobilized Concona-

valin A, still yielded significant responses to mannan (e.g., 10 to 15 mV). This suggested a selective interaction was indeed causing the potential change. This concept was subsequently extended to true antibody-antigen interactions with the immobilization of rabbit anti-human IgG onto the polymeric film.[176] In an appropriate bathing buffer, this sensor exhibited selective potentiometric response to human IgG proteins at very high concentrations. Further extensions of this general approach have been proposed, including the development of immunochemically sensitive field effect transistors (IMFETs), which are responsive to the Wasserman antigen (Syphilis test), human serum albumin, and other proteins.[177] These devices employ appropriate antibodies immobilized on PVC films directly coated on the metal oxide gates of field effect transistors.

Similar devices have been proposed in which the receptor or ligand is immobilized without benefit of an intermediary polymeric film. For example, Yamamoto et al.[178] covalently attached anti-human chorionic gonadotropic (HCG) or HCG itself to a titanium oxide wire and then monitored emf changes when the antigen (HCG) or anti-HCG were added to a buffered sample solution. Lowe[179] immobilized the dye Cibacron Blue F3G-A on the same type of titanium wire and observed significant (17 mV at 10 μg/ml HSA) and selective responses to human serum albumin, a protein that is known to bind to certain dyes with high affinity.

In these early examples of potentiometric receptor-based biosensors, researchers postulated that the observed emf responses were caused by a change in electrode surface charge resulting from the receptor-ligand interaction directly (i.e., a direct sensing scheme). In subsequent work, however, Collins and Janata[180] critically evaluated the potential determining processes in polymer membrane-type receptor-based probes. In this important study, these workers clearly showed that the potential changes observed in such probes resulted from a modulation in the ability of small bathing ions to enter the polymeric membrane following the surface reaction between the receptor and its ligand. Others have reached the same conclusion.[181,181a] An analogous assessment can be made with regard to the sensors prepared by immobilizing ligands or receptors on titanium oxide wires. Since such wires are by themselves potentiometric pH sensors, it is likely that receptor or ligand response occurs because the receptor-based binding interaction perturbs the normal exchange current density for proton equilibration with the oxide layer. In short, direct sensing of innate charges on receptors or bioligands can only be accomplished if the interface involved (where the immobilized species is) is completely blocked in terms of medium ion exchange processes. In practice, such completely blocked interfaces are extremely difficult to achieve, and thus, almost all potentiometric receptor-based sensors reported to date are, in reality, indirect probes. The entire issue of blocked vs. unblocked interfaces in connection with potentiometric immunosensors is covered in detail in an excellent review by Buck.[182]

Classical unblocked interfaces would include neutral carrier or ion-exchanger-based polymeric ion-selective membranes. Here, the high exchange current density for selected ions is provided by an appropriate ionophore incorporated within the membrane. Solsky and Rechnitz[183,184] took advantage of this fact to demonstrate a rather novel potentiometric immunosensor concept. In their original work,[183] they covalently coupled dinitrophenol (DNP) to a neutral carrier crown compound (dibenzo-18-crown-6) and impregnated this carrier-ligand conjugate into a PVC membrane. When used in a standard ion-selective membrane electrode measurement arrangement, the emf to the cell changed as the concentration of anti-DNP antibody increased in the sample solution. The sensor apparently was selective since antibodies toward other compounds induced only negligible effects on the cell emf. In further work, analogous systems were described for detecting antibodies toward digoxin[185,186] (using benzyl-15-crown-5-digoxin conjugates), prostaglandins,[187,188] and cortisol.[189] In addition, these sensors can be used to detect the corresponding ligands (e.g., digoxin, etc.) via a competitive binding approach. More recent efforts suggest that ligands

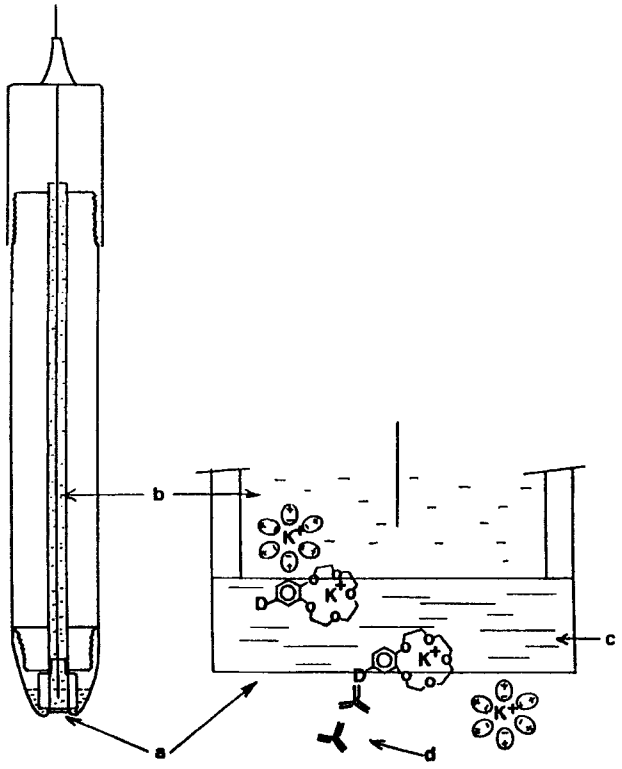


FIGURE 9. Pictorial diagram showing the membrane electrode used and an expanded view of the processes that take place at the membrane/sample interface of a sensor based on the PIMIA concept; (a) PVC membrane containing ligand-crown ether carrier (D = drug as ligand), (b) internal electrolyte solution, (c) membrane plasticizer, and (d) anti-drug antibodies. (From Keating, M. Y. and Rechnitz, G. A., *Anal. Chem.*, 36, 801, 1984. With permission.)

with innate ionophoric properties can be used in place of ligand-ionophore conjugates within polymeric membranes to devise similar antibody-responsive sensors.[190,191]

The concept of employing ligand conjugates of membrane-active ion-carriers to devise biosensors is schematically illustrated in Figure 9. Upon binding of the receptor (antibody in most cases) to the carrier-ligand conjugate at the membrane sample interface, the ion binding selectivity of the ionophore is apparently altered,[184] changing the equilibrium partitioning of bathing ions (e.g., K^+, Na^+) into the membrane phase. This approach has been termed potentiometric ionophore mediated immunoassay (PIMIA).[185] If the concentration of the carrier-ligand conjugate in the membrane is lowered, much greater potentiometric responses to given antibody levels are observed. This is illustrated in Figure 10, for the digoxin-anti-digoxin system.[185]

Normally, PIMIA experiments are performed by adding the antibodies to the bulk sample solution. Reuse of the electrode requires rinsing the surface with a low pH buffer to dissociate the antibody-ligand complex. In a recent paper,[192] however, Bush and Rechnitz described how this concept can be extended to devise a truly reversible immunosensor. This can be accomplished by entrapping a fixed amount of monoclonal antibody in a layer adjacent to the polymeric membrane (using a simple dialysis membrane). In their prototype sensor, Bush and Rechnitz immobilized anti-2,4-dinitrophenol antibodies in a layer adjacent to a polymer membrane doped with 2,4-dinitrophenol. Such a membrane displays potentiometric response to potassium ions. In the presence of anti-DNP, this response in altered.[191] When

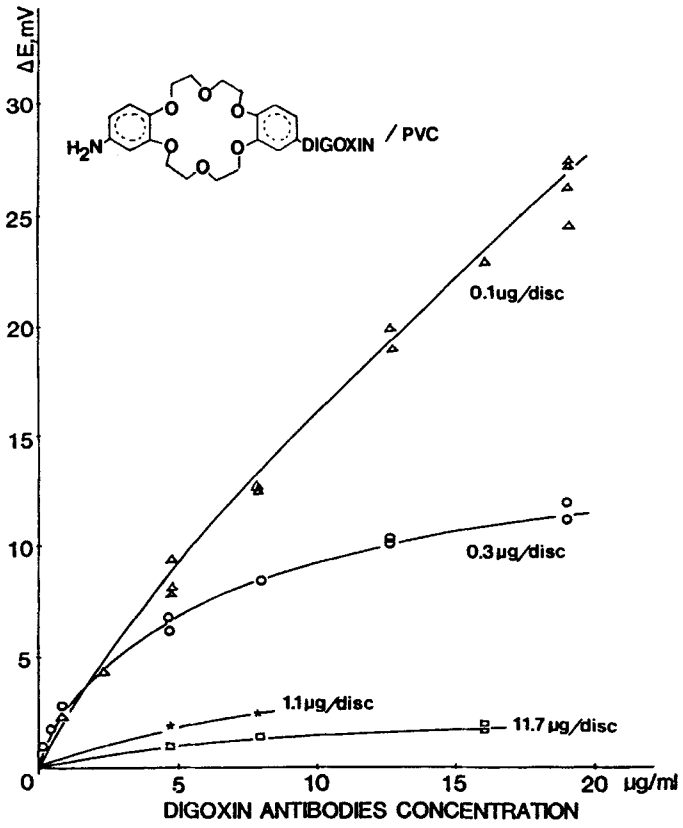


FIGURE 10. Effect of digoxin-dibenzo-18-crown-6 concentration in PVC membrane on the net potential responses to rabbit anti-digoxin antibodies in Tris-HCl buffer containing 3 m*M* KCl; 11.7 µg per membrane (□), 1.1 µg per membrane (★), 0.3 µg per membrane (○), and 0.1 µg per membrane (△). (From Keating, M. Y. and Rechnitz, G. A., *Anal. Chem.*, 36, 801, 1984. With permission.)

2,4-DNP is present in the sample, it diffuses into the antibody layer and competes with the membrane DNP for antibody binding sites. Figure 11 illustrates how the response of the sensor to 2,4-DNP levels is reversible. Connel and Sanders[188] also speculated about this layered scheme in which analyte ligands can diffuse into the antibody layer and compete with the membrane-bound ionophores or ionophore-ligand conjugates for antibody binding sites. The affinity of the antibody is important. If the affinity is too high, the system will not be truly reversible on a relevant time scale (e.g., 5 to 10 min). Initial studies suggest that affinity constants need to be on the order of 10^6 M^{-1} to gain reasonable rates of reversibility.[193]

Another indirect electrochemical sensing approach involves using receptor-ligand interactions to modulate the transmembrane potential of hydrophilic membranes such as cellulose acetate.[194] For example, Aizawa et al. described a dual membrane differential cell arrangement for the detection of antibodies toward the Wasserman antigen (Syphilis test).[195] When the Wasserman antigen was immobilized on one membrane, the cell potential changed 1 to 4 mV upon addition of various dilutions of Syphilis-positive serum to the sample compartment of the cell. No change in potential was observed with negative control serum. Similar membrane-based sensors have been proposed for the selective detection of HSA and other antigens with immobilized antibodies.[194] Yao and Rechnitz[196] recently extended this approach

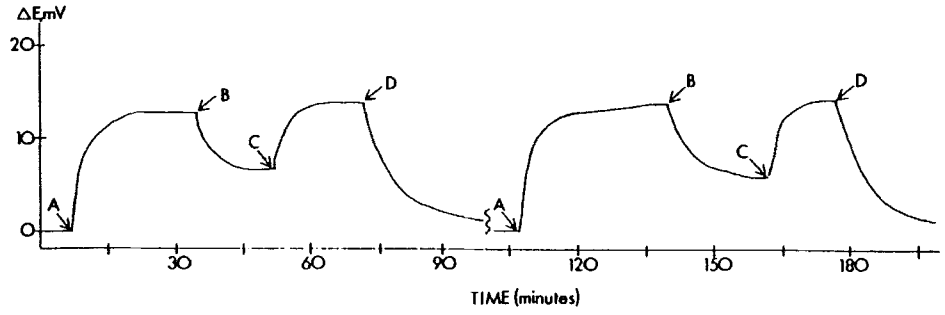


FIGURE 11. Typical time vs. potential tracing for the continuous monitoring of dinitro-phenol (DNP) with a PIMIA type sensor employing immobilized anti-DNP monoclonal antibodies. Changes in DNP concentrations at lettered points were as follows: (A) zero to 25 μ*M*; (B) 25 μ*M* to 5 μ*M*; (C) 5 μ*M* to 25 μ*M*; (D) 25 μ*M* to zero. All measurements were made in a background Tris-HCl buffer containing 1 m*M* KCl. (From Bush, D. L. and Rechnitz, G. A., *Anal. Lett.*, 20, 1781, 1987. With permission.)

to the detection of low molecular weight ligands (riboflavin) by immobilizing positively charged acriflavin or negatively charged flavin adenine dinucleotide to both sides of a cellulose acetate membrane. When such membranes were conditioned in a solution of riboflavin binding protein (RBP), this protein complexed the flavin analogs on the sample side of the membrane. Upon mounting the membranes in a conventional electrode body and measuring the membrane potential vs. a normal SCE reference, relatively large potential shifts (up to 10 to 15 mV) were observed when riboflavin (analyte) was added to the sample solution. These potential shifts were in different directions, depending on which riboflavin analog was attached to the membrane. Such changes occurred due to the competitive dissociation of RBP from one side of the membrane upon the addition of free riboflavin. As little as 0.2 μ*M* riboflavin could be detected with high selectivity over FAD and FMN.

Potentiometric sensors based on the immobilization of receptors or ligand on hydrophilic membranes respond in a theoretically predictable manner. In simplest terms, such membranes can be viewed merely as liquid junction potential points within the overall cell. The ion transference and thus the electrical potentials across these membranes are dependent on the nature and concentration of electrolyte solutions on each side of the membrane as well as the density and nature (+ or −) of charged sites on the surface of the membrane.[194] When changes in the surface charge density occur as a result of ligand-receptor interactions or dissociation (as in the case of the riboflavin sensor[196]), this effectively changes the liquid junction or membrane potential by altering the relative rates of bathing ion transference through the hydrophilic membrane. Hence, as with all of the indirect potentiometric measurement schemes described thus far, practical application of these sensing devices requires that sample solutions have known and fixed ionic compositions, since it is the modulation of response to these background sample ions that is actually measured.

Modulation of ionic transference through membranes is also the underlying mechanism of some of the latest and more speculative receptor-based biosensor research. It is known that the resistances and/or membrane potentials of living cell membranes change due to the binding interactions between membrane-bound receptors and their complementary ligands. Similar observations have been made with artificial phospholipid bilayer membranes.[197] In principle, such changes can be used for designing sensors, either with intact cells or the artificial membranes. An interesting example regarding the use of intact cells may be that found in the recent work of Belli and Rechnitz,[198] who utilized immobilized antennule structures from blue crabs to devise a prototype biosensor for glutamate. Nerve fibers of the antennules were contacted to a platinum indicator electrode, while the base regions, containing amino acid chemoreceptors, were in contact with the buffered sample medium.

Action potentials of the nerve cells were monitored by recording the potential between the platinum indicator electrode and a Ag/AgCl reference within the saline solution that bathed the remaining nerve fibers. In the absence of glutamate, there was an infrequent firing of the nerves. In the presence of glutamate, the frequency of nerve firings and the amplitude of the action potentials observed increased significantly. Integrated responses were directly proportional to the logarithm of the glutamate concentration in contact with the base region of the antennules.

Reports of chemoreceptor-based sensing with artificial bilayer membranes have also appeared. Generally, these efforts have focused on measuring the conductances of the membranes in the presence of appropriate ligands and/or receptors. For example, Smuda and deLevie[199] showed that, when partially purified opiate receptors from bovine brain cell were incorporated into synthetic membranes, the conductance of these membranes changed in the presence of trace levels of leucine-enkephalalin. While no quantitative data were presented, this work points to the possibility of generating analytically useful electrical signals from artificial membranes doped with chemically selective receptors. Others have also speculated on this approach,[200] particularly the use of reconstituted acetylcholine and GABA (gamma-amino butyric acid) receptors for detecting neurotoxins and GABA antagonists.[201] The main problems have been the difficulties in isolating the biologically active receptors and the fragility of the lipid bilayer membranes. The latter represents the most serious obstacle impeding the design of reliable biosensor structures based on chemoreceptor-ligand interactions.

The use of enzyme labels in conjunction with immobilized bioreceptors or ligands to design electrode-based biosensors has received considerable attention. Several researchers have used the generalized scheme shown in Figure 8 in which the selective receptor is immobilized on a membrane adjacent to the surface of an appropriate electrochemical detector. When such receptor membranes contact a solution of enzyme-labeled ligand, the enzyme becomes immobilized at the electrode surface as a result of the receptor-ligand binding interaction. If unlabeled ligand and enzyme-labeled ligand are allowed to compete for the receptor binding sites, the amount of enzyme bound to the surface of the electrode decreases in proportion to the concentration of the analyte present. After washing away the unbound enzyme-labeled ligand and sample, the bound enzyme activity can be determined simply by immersing the electrode into a substrate solution and either monitoring the rate of substrate depletion or product formation at the surface of the probe. Alternate schemes in which the ligand is immobilized on the electrode surface and the receptor is the enzyme-labeled species have also been proposed. When the analyte is a protein, sandwich-type ELISA detection methods can be employed. In this case, an anti-protein antibody is bound to the electrode surface and exposed to the sample. After an equilibration period, the electrode is rinsed and exposed to a solution containing an antibody-enzyme conjugate. This conjugate will bind to the surface of the electrode only if there is analyte protein already bound to the immobilized antibody. After a second washing step, the activity of the bound enzyme conjugate is directly proportional to the concentration of protein analyte in the original sample. These sandwich assays tend to yield linear calibration curves (increasing signal with increasing analyte), whereas competitive binding methods result in classical sigmodial logarithmic responses with analytical signals inversely proportional to the analyte concentration.

Various detector electrodes and labeling enzymes have been used in the above enzyme-linked receptor-based sensors. Boitieux et al. have employed an iodide selective electrode in conjunction with horseradish peroxidase.[202-204] The enzyme catalyzes the oxidation of I^- to I_2 in the presence of H_2O_2. Thus, when iodide and peroxide levels are fixed in the final assay buffer, enzyme activity is detected at the surface of the electrode by monitoring a decrease in I^-, potentiometrically. The selective antibodies were immobilized on gelatin membranes which were held in contact with the iodide selective membrane of the electrode.

In one case, oestradiol-17B was detected in the sub-nmol/l range using immobilized anti-oestradiol-17B and a peroxidase-oeastradiol-17B conjugate (competitive binding arrangement).[203] These same workers employed a sandwich assay scheme to detect Hepatitis B surface antigen at levels below 1 μg/l.[202] In related work, Mascini et al. used a glass membrane pH electrode, membrane-bound anti-human chorionic gonadotropin (HCG), and an acetylcholinesterase-HCG conjugate to detect free HCG at levels around 1 U/ml.[205]

Amperometric electrodes also have been used to develop various enzyme-linked receptor-based sensors. For example, Aizawa et al. have devised a host of sensors with a classical Clark-type oxygen electrode as the base sensor and catalase as the labeling enzyme.[206] Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen. Thus, when the enzyme is bound to the oxygen sensor as a result of the interaction between an immobilized receptor and an enzyme-ligand conjugate, an increase in output current is observed (increase in pO_2 at the sensor surface). Initial efforts focused on using a standard competitive binding scheme where appropriate antibodies were immobilized on a cellulose acetate membrane placed over the gas permeable membrane of the oxygen probe. Species detected in this manner have included HCG,[207] alpha-fetoprotein (AFB),[208] and thyroxine,[209] among others. Oxygen sensors have also been used in conjunction with glucose-oxidase-labeled antibodies to develop solid-phase sandwich-type devices for detecting Hepatitis B surface antigen[210] and Factor VIII related antigen.[211] In related work, low molecular weight ligands, rather than the bioreceptors, have been immobilized on the membranes affixed to oxygen probes. For example, Ikariyama et al.[212] described a novel competitive binding sensor for biotin based on the interaction of membrane bound analogs of biotin and a catalase-avidin conjugate (i.e., avidin is a natural bioreceptor for biotin). Analogs of biotin were used instead of native biotin to lower the association constant of the labeled avidin with the surface of the sensor. The assays of biotin were performed by pre-equilibrating the membrane of the sensor with the catalase-avidin conjugate. Upon exposing this loaded membrane to biotin, the conjugate dissociated because the avidin binds with higher affinity to native biotin in the sample. This results in less catalase activity at the surface of the electrode. After rinsing away the dissociated conjugate, the remaining bound enzyme activity was detected by adding hydrogen peroxide to a fixed volume of buffer and monitoring the increase in O_2. As little as 1 ng/ml of biotin could be detected.

While the enzyme-linked bioreceptor-based sensors are intriguing from a scientific standpoint, in practice such devices have many disadvantages. First, these electrodes are not true sensors, since they do not respond in a reversible fashion. Indeed, after a single use, the membranes must be regenerated by rinsing with a buffer that dissociates the bioreceptor-ligand complex. Second, the fixed levels of external reagents in the form of enzyme substrates must be added to obtain the analytical signals. Third, the entire measurement process is rather slow, typically 1 to 2 h to achieve equilibrium between the immobilized species and the solution phase conjugates. This equilibration period must be followed by subsequent washing, activity determination, and regeneration steps. Thus, with a single sensor, relatively few assays can be run in a day. Given that modern ELISA (enzyme-linked immuno-sorbent assays) microtiter plate-based solid phase assays can handle 96 samples and/or standards at one time, it is unlikely that the electrode-based ELISA approaches described above will have significant analytical impact. In our view, the real advantages of using electrochemical detection to monitor bioreceptor-ligand interactions are only realized when the sample can remain in contact with the sensor during the measurement step (not washed away).

2. Optical Sensors

While most of the recent advances in optical bioreceptor-based sensors have been made using indirect techniques employing fluorescent labels, there have been several noteworthy efforts to use direct sensing approaches. For example, Zhajun and Seitz[213] described a novel

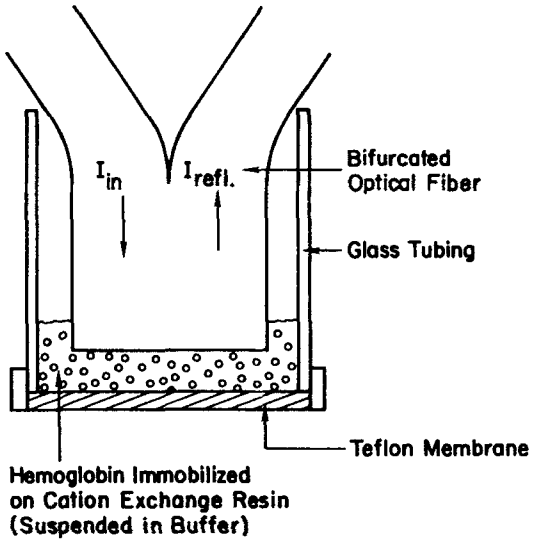


FIGURE 12. Schematic diagram of new optical oxygen sensor based on immobilized hemoglobin.

optical sensor for oxygen in which immobilized hemoglobin was used as the natural bioreceptor. Upon binding oxygen, the absorption spectrum of immobilized hemoglobin (immobilized on ion-exchange resin) changes significantly (blue shift of heme soret band). As in red blood cells, this process is fully reversible. Absorption at 405 nm correlates to the concentration of oxyhemoglobin, while that at 435 nm corresponds to deoxyhemoglobin. When the immobilized bioreceptor is retained behind a gas-permeable membrane, and is in contact with a bifurcated fiber, oxygen can permeate into the hemoglobin layer and change the optical absorption spectrum. Figure 12 illustrates this design. By monitoring the reflected intensity of light through the optical fibers at the two indicator wavelengths and ratioing these two values, a useful response to pO_2 is obtained. Figure 13 illustrates typical calibration curves obtained using the ratio mode as well as single wavelength detection at 405 and 435 nm. Although this particular sensor lacks adequate stability for long-term use (i.e., hemoglobin eventually oxidizes to methemoglobin as well as denatures), it should be possible to solve these problems through the use of synthetic metalloporphyrins or organometallic Schiff bases, which reversibly bind oxygen at room temperature.

Another approach to direct optical sensing of receptor-ligand interactions involves the use of internal reflectance spectroscopy (IRS) to probe the chemical components in regions immediately adjacent to optical waveguides (evanescent wave; within one wavelength). For direct sensing, attentuated total reflectance (ATR) techniques have been employed.[214] In one of the earliest studies,[215] albumin was adsorbed onto the surface of a germanium waveguide and IR radiation was used to probe the region. When this interface was exposed to a solution of anti-albumin antibodies, there was a decrease in the transmission intensity at 1650 cm^{-1}, presumably due to the increase in the concentration of amide bonds resulting from the selective association of anti-albumin antibodies at the waveguide/sample interface. More recently, an analogous direct sensing arrangement for detection of methotrexate was reported by Sutherland et al.[216] In this effort, anti-methotrexate antibodies were covalently bound to the surface of a quartz wave guide (quartz slide). When the waveguide contacted a solution containing methotrexate, there was a decrease in light transmission through the wave guide at 310 nm. Since methotrexate absorbs strongly at 310 nm, the observed decrease in intensity was due to the preconcentration of this drug by its association with the antibody within the region sensed by the evanescent wave. Increasing the concentration of methotrexate in the

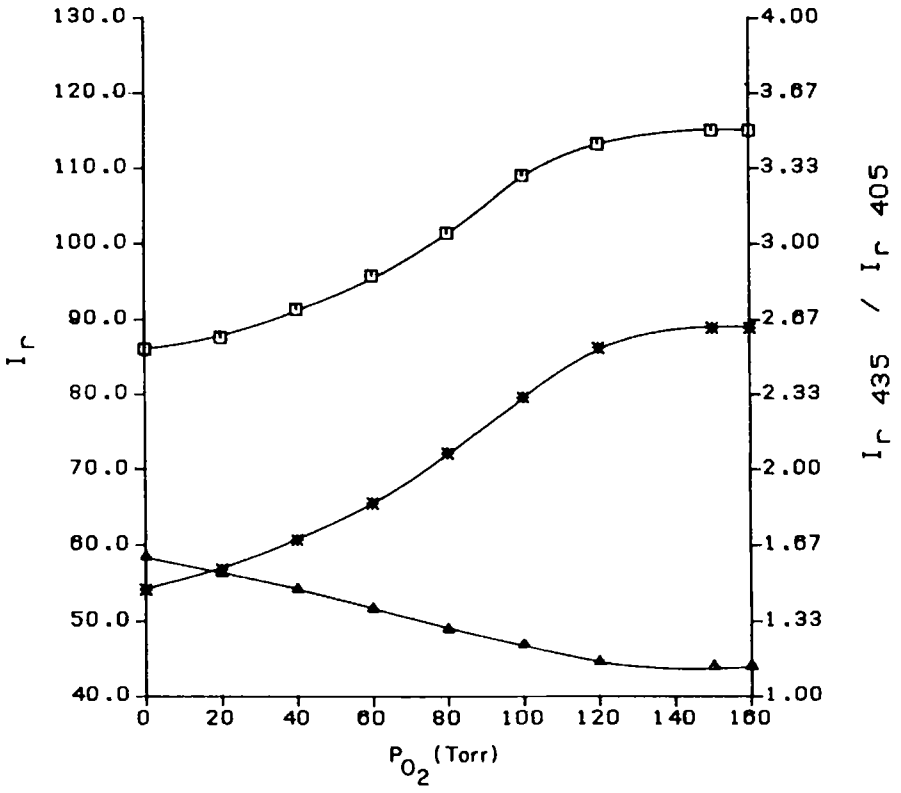


FIGURE 13. Typical response curves for optical oxygen sensor based on immobilized hemoglobin. Relative reflected intensity, I_r, as a function of oxygen partial pressure at 435 nm (□) and 405 nm (△); the intensity ratio at these two wavelengths (★). (From Zhajun, Z. and Seitz, W. R., *Anal. Chem.*, 58, 220, 1986. With permission.)

sample solution increases the amount bound to the immobilized antibodies and further decreases the transmittance through the quartz waveguide. The detection limit using this approach was determined to be 270 n*M* methotrexate.

The direct sensing ATR approach is interesting in that it converts an essentially nonselective optical measurement system into a more selective one by using bioreceptor-ligand interactions. This is accomplished by selectively separating the analyte (from the remaining components of the sample) into the region that is probed by the evanescent wave. Therefore, without the use of indirect labeling techniques, the analyte must have some unique optical property that allows it to be detected in the microscopic region adjacent to the waveguide. While there are other significant obstacles to overcome from a practical sensing standpoint (including reversibility of the surface interactions, the requirement for more complex optical instrumentation, and nonspecific adsorption problems), the technique does appear to offer some promise for designing useful sensors for a limited number of important biomolecules.

As previously stated, the majority of work to date in the area of receptor-based optical sensors has involved the use of labels, particularly fluorescent labels. Clearly, the most innovative of all the recent efforts has been the optical affinity glucose sensor described by Schultz et al.[217,218] This device is based on the reversible competition between glucose and fluorescein-dextran for a limited number of concanavilin-A (Con-A) receptor sites. As shown in Figure 14, the receptor (a lectin) is immobilized on the inner wall of a narrow hollow dialysis fiber. The high molecular weight fluorescein-dextran conjugate is entrapped within the confines of the hollow fiber. Glucose in the sample diffuses freely through the walls of this fiber. Excitation rediation from a light source enters the hollow fiber through an optical

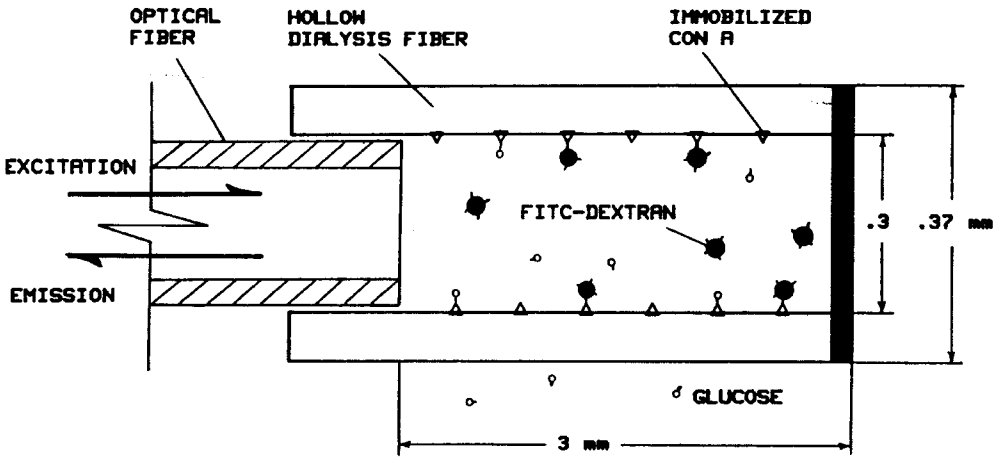


FIGURE 14. Schematic diagram of optical affinity sensor for glucose based on immobilized concanavalin-A and fluorescein-labeled dextran. (From Schultz, J. S., Mansouri, S., and Goldstein, I. J., *Diabetes Care*, 5, 245, 1982. With permission.)

fiber and causes the fluorescein to fluoresce. The emitted light is detected by the same optical fiber with an appropriate beam splitter, optical filter, and photon detector arrangement. When the glucose concentration in the sample is low, most of the fluorescein-dextran conjugate is bound to the Con-A along the walls of the fiber and the fluorophore is spatially removed from the path of the excitation light. Consequently, fluorescence intensity is low. At high glucose levels, the fluorescein-dextran conjugate is displaced to the center of the fiber via a competitive reaction and detected fluorescence increases. As shown in Figure 15, equilibrium response to changes in glucose occur within 6 to 12 min, and this response is completely reversible, making it a true biosensor. In this case, reversibility occurs at reasonable rates owing to the relatively weak affinity constants of Con-A toward glucose ($4 \times 10^2\ M^{-1}$) and dextran ($1.5 \times 10^4\ M^{-1}$), and the fast dissociation rate constants. By using this novel design, it should be possible to devise analogous reversible affinity biosensors for other important biomolecules, provided that the affinity of the binder is kept relatively low. Modern monoclonal antibody technology may provide binders with these desired characteristics.

Single-use indirect probes based on fluorescent-labeled receptors or ligands have also been devised using internal reflectance spectroscopic approaches. Total internal reflection fluorescence (TIRF) has been the technique most often employed, either using glass microscope slides or more modern optical fibers as waveguides. The technique is analogous to ATR, except that that evanescent wave (at wavelength 1) is now used to excite molecules within the waveguide/sample interface region. Fluorescent light (wavelength 2) from these molecules can pass back into the optical waveguide and be carried to an appropriate detector. In the earliest work, Kronick and Little[219] immobilized low molecular weight ligands such as morphine or phenylarsonic acid on the surface of quartz microscope slides. When the surface came in contact with fluorescein-labeled anti-ligand antibodies, fluorescence intensity from the waveguide/slide interface increases (if the evanescent wave energy is the proper wavelength for the fluorescein label). Upon addition of free ligand to the sample solution, the binding rate of the fluorescein-anti-ligand to the surface is reduced in an amount proportional to the concentration of ligand added. In the case of morphine, as little as 0.2 μmol/l could be detected. Sutherland et al. also used a microscope slide TIRF arrangement in conjunction with immobilized anti-IgG antibodies and fluorescein-labeled anti-IgG to develop a noncompetitive sandwich-type assay to detect human IgG at levels as low as 30 nmol/l.[221]

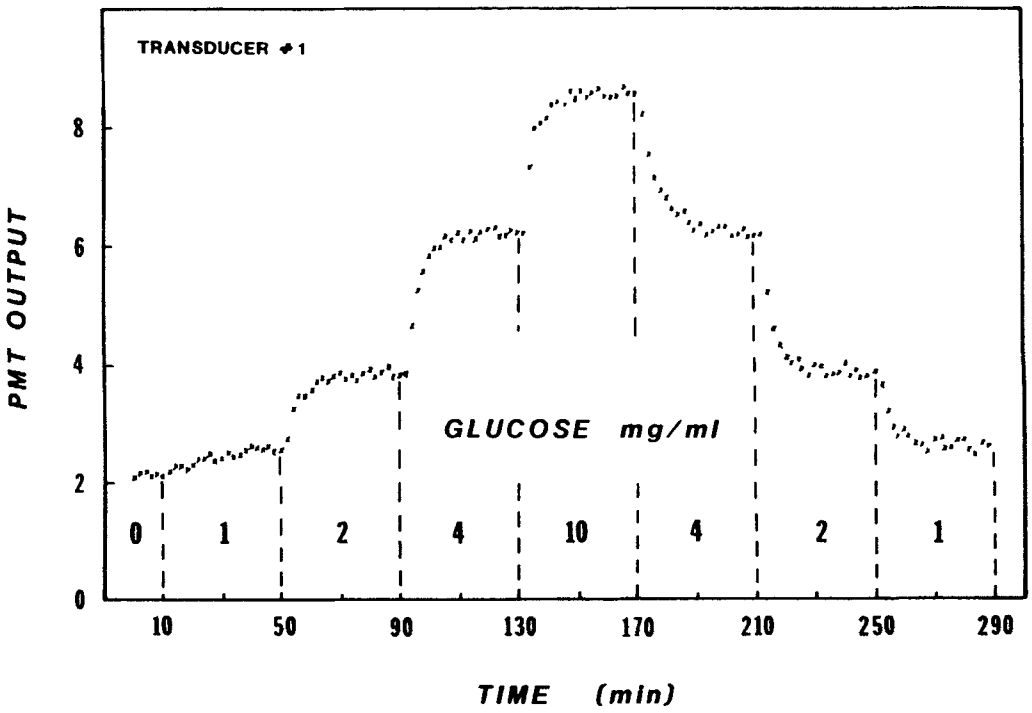


FIGURE 15. Typical transient response of optical affinity sensor at 24° after step changes in glucose concentration. (From Mansouri, S. and Schultz, J. S., *Bio/Tech*, October, 885, 1984. With permission.)

More recently, the TIRF approach has been further refined by the late Tomas Hirschfeld and others[222-225] so that it can be implemented by using a single optical fiber as the waveguide, and a small surrounding capillary tube as the sample chamber. In this instance, the immobilized component, usually an antibody, is covalently attached to the outer surface of the optical fiber material (normally quartz). In some instances, the immobilized binder is preloaded with fluorescein-labeled ligand. In the presence of unlabeled ligand in the sample introduced into the capillary tube surrounding the optical fiber, a decrease in fluorescence occurs due to competition for antibody binding sites. Slovacek et al.[226] have adapted this basic design for the detection of ferritin and digoxin in blood samples. However, unlike Hirschfeld et al., the labeled component was added to the sample (solution phase). In the case of ferritin, a sandwich assay was used in which the rate of binding of fluorescein-labeled anti-ferritin antibody to a fiber coated with anti-ferritin antibody is directly proportional to the ferritin concentration in the sample. As little as 5 ng/ml of ferritin could be detected. For the digoxin assay, fluorescein-digoxin competes with unlabeled digoxin for a limited number of anti-digoxin sites immobilized on the fiber. The rate of conjugate binding was inversely proportional to the concentration digoxin. As little as 0.5 ng/ml could be detected in a short 2-min kinetic assay.

Optical fiber sensors based on fluorescent-labeled receptor or ligand reagents have also been accomplished without using the evanescent wave approach. Indeed, Tromberg et al.[227] immobilized rabbit IgG on the distal sensing tip of a quartz optical fiber. When the tip of the fiber was exposed to fluorescein-labeled anti-IgG, an increase in fluorescence occurred due to the binding of the labeled receptor at the tip. Unlike the TIRF systems, to observe this increase, unbound labeled reagent must be washed away prior to the fluorescent measurement. In the presence of unlabeled anti-IgG, the amount of bound label (after washing) decreased owing to the competition for ligand (IgG) sites on the tip of the fiber. With very

small volumes of samples, as little as 25 fmol of unlabeled anti-IgG could be detected with a relatively short 20-min incubation period. Unfortunately, the need for washing steps and the fact that a new fiber had to be used for each measurement (nonregenerable system) makes this approach less attractive than some of the ATR and TIRF efforts described above.

3. Thermal and Mass Detection

Receptor-ligand binding reactions can be endo- or exothermic (either directly or through secondary effects, e.g., displacement of solvent molecules). Thus, in principle, it should be possible to devise direct sensing schemes that are based on detecting the heat generated or consumed by these selective reactions. Early efforts suggested that the measurement of such changes with thermistors was indeed possible. For example, Jordan[228] showed that thermometric titration techniques could be used to sense antibody-antigen interactions directly. However, unlike biocatalytic systems, using this enthalpic approach in a true sensor arrangement (in which one component is immobilized on the thermistor) is not feasible because the reaction is not continuous. Indeed, the heat consumed or liberated rapidly dissipates into the surrounding medium, and steady-state changes in temperature cannot be recorded. Consequently, there have been no recent efforts aimed at devising receptor-based thermometric sensors.

On the other hand, the detection of mass changes at interfaces after the interaction of receptors and ligands does lead to a finite and constant signal perturbation. By using piezoelectric crystal mass detectors,[229] several groups have demonstrated that direct monitoring of receptor-ligand interactions in the gas and liquid phases is feasible. The gas-phase work is particularly exciting since the resulting devices appear to be truly reversible. For example, Guilbault immobilized the enzyme formaldehyde dehydrogenase on the surface of an AT quartz crystal.[230] When the crystal was exposed to a gas phase containing formaldehyde, the resonance frequency of the crystal changed (decreased) as a result of formaldehyde molecules selectively binding to the active site of the enzyme on the surface of the crystal (changing the surface mass). Surprisingly, this interaction was reversible without the formation of products, provided that the activated crystal was not continuously exposed to the substrate for more than about 1 min. The device could detect gas-phase formaldehyde at levels ranging from 1 to 10 ppm with high selectivity over other aldehydes and volatile alcohols. In more recent work, Ngeh-Ngwainbi et al.[231] demonstrated that antibodies could also be used as the selective receptors in such gas-phase microgravimetic biosensors. Indeed, these workers cleverly immobilized anti-parathion antibodies at the surface of a quartz crystal. In the presence of gas phase parathion at only 35 ppb, significant changes in the resonant frequency occurred. As shown in Figure 16, this change was reversible within 2 min upon exposing the device to a gas phase stream without parathion. While the specificity of the antibody binding in the gas phase was substantially less than that observed in solution, the device did exhibit some selectivity over related species (e.g., malathion, methyl parathion, etc.). Although there are still fundamental questions to be answered regarding the exact mechanism of the response of these sensors and the effects of various atmospheric components (e.g., humidity, etc.), the preliminary experiments reported to date are very encouraging.

Operating piezoelectric crystal mass detectors in solution phase appears to be more difficult. Here, nonspecific absorption of ions and solvent molecules, as well as the viscosity and surface tension of the bathing medium, can cause severe problems. Nonetheless, several biosensors based on this approach have been reported. For example, Roederer and Bastiaans[232] used a differential measurement scheme in an effort to devise a sensor for human IgG. Anti-IgG antibodies were covalently immobilized on the surface of a quartz crystal. A second crystal without the immobilized receptor was used as a pseudo-reference element. The ratio of resonant frequency shifts between the two crystals was taken as the analytical signal. The crystal with immobilized antibody displayed a much larger frequency shift (4 ×) than the

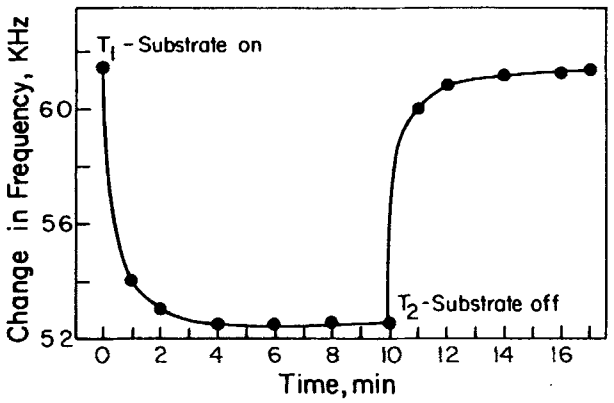


FIGURE 16. Typical response of piezoelectric crystal coated with anti-parathion antibodies to a parathion (substrate) saturated carrier gas, 35 ppb, at 30°C. (From Ngeh-Ngwainbi, J., Foley, P. H., Kuan, S. S., and Guilbault, G. G., *J. Am. Chem. Soc.*, 108, 5444, 1986. With permission.)

reference crystal when each was exposed to buffered solutions containing human IgG. Thus, while nonspecific absorption of protein to the surface of the crystal does occur, the presence of selective antibodies on the surface of the indicator crystal causes a larger amount of IgG to be specifically adsorbed. By using the ratio signal method, a linear response to IgG was observed in the 0.0225 to 2.25 mg/ml range of human IgG. As with many other receptor-based sensors, reuse of the piezocrystal immunosensor required that the crystal be immersed in a high ionic strength buffer to dissociate the antibody-antigen complex. Similar systems have been reported for the detection of IgG using immobilized protein A (a natural binding protein for IgG)[233] and for *Candida albicans* microbes using immobilized antibodies.[234] The latter work is particularly interesting, since the authors claim that the sensor responded to the candida cells in the range of 10^6 to 10^8 cells per ml while displaying no response to other microbes. However, it should be noted that the actual resonant frequency measurements were not made in solution, but rather in air after the crystal was exposed to the candida cells and then thoroughly dried.

Thompson et al.[235] provided some very interesting results from a fundamental study regarding the behavior of liquid-phase receptor-based piezoelectric crystal devices. In their work, these researches showed that quartz crystals coated with anti-IgG do indeed undergo reproducible resonant frequency shifts when in contact with solutions containing IgG. However, these researchers concluded that "the frequency shifts observed are not necessarily associated with the classical microgravimetric signal." This statement was made after they found that, for crystals where the antibody was covalently attached directly to the quartz, the resonant frequency actually increased rather than decreased in the presence of IgG. This observation completely contradicts the behavior reported by Roederer and Bastians[232] (described above) for essentially the same system (anti-IgG immobilized directly to quartz crystal). The unexpected behavior was ascribed to nonequilibrium viscosity effects at the quartz/solution interface as a result of the antibody-antigen interaction. Grabbe et al. also found nonquantitative behavior with similar devices.[235a] Whatever the cause of these particular results, it is clear that more basic research will be required to completely understand how surface receptor-ligand interactions alter the resonant frequencies of the crystals when operated in solution. From a practical standpoint, however, given all the nonspecific effects (e.g., protein adsorption, sample density and viscosity, etc.) that can occur, it is unlikely that solution phase piezoelectric biosensors will ever provide enough accuracy and precision to become widely used as analytical devices.

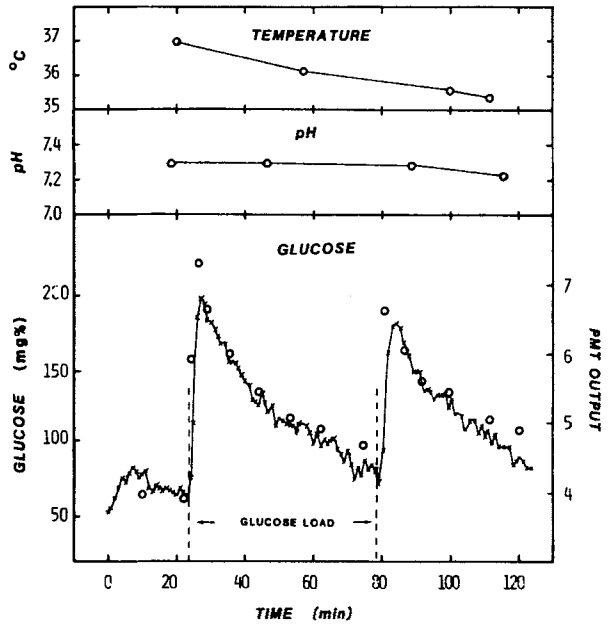


FIGURE 17. *In vivo* comparison of optical glucose sensor response (×) and sampled blood glucose tests (○) after intravenous glucose tolerance tests. (From Mansouri, S. and Shultz, J. S., *Bio/Tech*, October, 885, 1984. With permission.)

D. Analytical Applications

The development of receptor-based biosensors is still very much at the basic research stage. Thus, it is not surprising to find that none of these sensors has yet achieved widespread analytical use. However, several of the sensors described above, particularly the ones that respond selectively and reversibly, do offer much promise for the future. Indeed, if the sensor is truly reversible, then the device can be used for continuous monitoring purposes. To date, the most elegant example of this application of a bioreceptor-based sensor may be found in the work of Mansouri and Schultz,[218] who demonstrated that their new affinity optical glucose sensor could be used to continuously monitor glucose in whole blood. Figure 17 illustrates the results obtained when the sensor was used in dogs to follow levels of glucose after intravenous injections of glucose (glucose tolerance test). Excellent correlation between the continuously monitored and sampled blood glucose values was observed. Clearly, if this novel sensing concept could be extended to other analytes via the use of other receptors, a large number of very useful sensors would result. Such reversible devices would have applications in biomedical monitoring (e.g., drugs) as well as for continuous detection of critical components in modern bioreactors.

For those receptor-based sensors that are not reversible, regardless of how effective (accuracy, etc.) they are at detecting specific biomolecules (ligands or receptors), it is difficult to envision what advantages they will offer when applied to real analytical problems. In view of the numerous homogeneous and heterogeneous immunoassays already in use for routine bioanalysis, simply adapting these assays to a "sensor" or "probe" type arrangement is not likely to have much analytical impact unless such devices respond in a selective, fully reversible, and reproducible manner.

IV. FUTURE PROSPECTS

A. Designer Biocatalytic and Receptor Proteins

Recent advances in several areas of biochemistry can be used to develop novel biomolecules with specific physical and chemical properties. Many of the current limitations of both biocatalytic and receptor-based biosensors can potentially be overcome by designing protein structures that meet the specific requirements of biosensors.

Protein engineering can be used to prepare custom-designed enzyme and receptor protein mutants. For enzymes, significant progress in developing modified biocatalysts by systematically changing the tertiary structure of an enzyme has been reported.[236] Enzyme mutants with improved stability and enhanced selectivity can be developed. Development of enzyme mutants that are insensitive to common activity modulators might also be possible, and this approach can minimize sample pretreatment or reagent addition requirements. Alternatively, an enzyme mutant that is highly sensitive to a particular activity modulator can be developed and used for toxin and mutagen measurements. Perhaps an enzyme mutant that is highly sensitive to substances that complex human DNA can be developed for use as a sensitive predictor of mutagenicity. Protein engineering might also be used to provide an attachment site for immobilization. The location of this site on the enzyme can be selected to control the orientation of the enzyme on the solid support which can maximize the amount of bound activity.

As noted above, the major problem with receptor-based biosensors is the lack of reversibility. Monoclonal antibodies with rapid dissociation kinetics are currently being evaluated for sensor applications.[192] The micromolar detection limits that are possible with these antibodies are acceptable for many analytical applications. However, in the future, a combination of protein engineering and monoclonal antibody technologies might yield antibodies with rapid dissociation kinetics and higher binding constants. In this way, reversible immunosensors with nanomolar detection limits will be possible.

Enzyme engineering can provide large quantities of enzyme by cloning such enzymes in host bacterial cells. Entire enzyme-lipid membrane structures can also be cloned to provide stable biocatalytic structures. The idea is to clone not just the protein, but the entire protein-membrane complex for cases where the membrane component helps to stabilize the activity.

Artificial or synthetic enzymes have been shown to provide extremely stable biocatalytic activities.[61] Considerable progress has been made in the development of selective artificial enzymes.[237-239] In addition, catalytic antibodies[240] can be used to provide novel biocatalytic activities. Here, antibodies can be developed against a transition state analog of a desired reaction. By selecting the appropriate analog, catalytic antibodies for new reactions can be produced and the corresponding biosensors can be prepared.

Finally, the search for new enzyme activities cannot be overlooked as a source for needed biocatalysts. A recent example is the isolation of glutamate oxidase from *Streptomyces* sp. X-119-6.[21] This enzyme selectively catalyzes the oxidative deamination of glutamate in the presence of oxygen. In contrast, common amino acid oxidase enzymes are not selective. Glutamate selective biosensing probes have been constructed by coupling this new enzyme with an oxygen-sensing element.[241] The need for a glutamate biosensor in the food industry[241] and as a continuous monitor for mammalian cell bioreactors[157] is fulfilled because of this newly discovered enzyme. Efforts to screen exotic microbes, such as thermophilic bacterial strains,[242] for enzymes with the selectivity and stability properties required for biosensors must be encouraged.

B. Biocompatibility

A major issue in the application of biosensing probes for *in situ* or *in vivo* monitoring is biocompatibility. Materials for sensor construction must be compatible with the surrounding

medium to the extent that the sensor accuracy is not adversely affected over the time frame of the measurement. Short-term sensor applications, such as during emergency room situations, surgical operations, and biomedical research experiments, require biocompatibility over periods from minutes to hours. On the other hand biocompatibility for periods from days to years are required in critical care monitoring and implantable devices.

Work is required to develop sensors based on materials that elicit minimal biologic response or to develop a system in which the sensor is isolated from the surrounding thrombogenic process by a protective envelope. The major concern is changes in sensor calibration as the immediate environment around the sensor changes. Unless a system can be developed whereby the biologic reaction has absolutely no effect on the sensor response, the sensor must be amenable to *in situ* calibration to account for such variations. A checking system to detect when the sensor is completely isolated from the sample site (e.g., fouled, coated, etc.) is desirable for long-term applications.

C. Reagentless Probes

Application of the above-mentioned biocatalytic biosensors often requires that the sample be modified. Common modifications include the addition of a cofactor or activator, the removal of an inhibitor, or the adjustment of pH. These required modifications severely limit the *in situ* and *in vivo* application because it is not possible to deliver the required reagents to the sample site. Likewise, implantable sensors cannot rely on the addition of reagents for long-term operation.

Cofactor immobilization is one approach to eliminate or minimize reagent addition problems. Biocatalysts that are insensitive to modulators and that do not require reagents for protection are possible with the above-mentioned protein engineering or synthetic enzyme strategies. For short-term applications, a reagent-dispensing probe can be developed where reagents are slowly dispensed just at the measurement site. Cleland and Enfors[243] have introduced such a system in which a buffer solution with the required reagents is continuously pumped to the sensor tip. A dialysis membrane separates this buffer solution from the sample. The analyte diffuses across the dialysis membrane and is sensed by the biosensor in the buffer solution. Although this dialysis membrane approach is not well suited for the development of micro-probes, it can be used to construct probes for *in line* sensing of bioreactors. Of course, perturbation of the system under investigation by the added reagents or the consumption of substrate[244] must always be considered.

A novel reagentless probe design has been recently introduced in which an "internal enzyme" concept is employed. Internal enzyme biosensors employ a perm-selective membrane to separate the sample from an enzyme-containing internal solution. The analyte selectively passes through the membrane and enters the internal solution where the analytical reaction is catalyzed. The rate of this reaction is directly proportional to the analyte concentration in the sample solution. The enzyme is never directly exposed to the sample solution and this allows for the use of optimal solution conditions without reagent addition. Internal enzyme biosensors for ethanol have been reported in which a gas-permeable membrane separates the enzyme solution from the sample. Alcohol dehydrogenase[101] and alcohol oxidase[245,246] based systems have been reported.

Overall, the development of biosensors has progressed considerably since the original paper by Clark in 1962.[247] Further advances in the development and application of both biocatalytic- and receptor-based biosensors are expected. We hope this critical review stimulates fresh ideas and new approaches that will help resolve the remaining problems in this field.

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