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Immobilization and Stabilization of Biomaterials for Biosensor Applications

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Abstract

Biosensors are finding applications in a variety of analytical fields. A biosensor basically consists of a transducer in conjunction with a biologically active molecule that converts a biochemical signal into a quantifiable electric response. The specificity of the biosensor depends on the selection of the biomaterial. Enzymes, antibodies, DNA, receptors, organelles, microorganisms as well as animal and plant cells or tissues have been used as biologic sensing materials. Advances in biochemistry, molecular biology, and immunochemistry are expected to lead to a rapid expansion in the range of biologic recognition elements to be used in the field of biosensors. Biomaterials that are stable and function even in highly acidic, alkaline, hydrophobic, or oxidizing environments as well as stable to high temperature and immune to toxic substrates in the processing stream will play an important role. Techniques for immobilization of the biomaterials have played a significant role in the biosensor field. Immobilization not only brings about the intimate contact of the biologic catalysts with the transducer, but also helps in the stabilization of the biologic system, thus enhancing its operational and storage stability. A number of techniques have been developed in our laboratory for the immobilization of enzymes, multienzyme systems, cells, and enzyme-cell conjugates. Some of these aspects that are of significance in biosensor applications have been highlighted.

Index Entries: Biosensors; immobilization; stabilization; permeabilized cells; microbial sensors.

Introduction

Biosensors provide a rapid and convenient alternative to conventional methods for monitoring chemical substances in fields as diverse as medicine, environment, fermentation, and food processing *(1–3)*. In medicine, biosensors can be used for the monitoring of blood glucose, urea, cholesterol,

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lactate, neurotransmitters, and so on. They are also gaining importance in monitoring the assimilable nutrients, intermediates, and end products of fermentation and in food industry for shelf-life assessment (freshness) of fish, meat, and vegetables, microbial contamination, and olfaction *(1,2)*. Biosensors will play a major role in environmental monitoring *(3,4)*, such as for estimation of BOD (Biological Oxygen Demand) *(5)* and for assay of explosives such as TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) *(6)* as well as other toxic and mutagenic environmental chemicals including pesticides *(7)*, herbicides, and heavy metals *(3,4)*.

A biosensor is a device that detects, transmits, and records information regarding a physiologic or biochemical change. Technically it is a probe that integrates a biologic component with an electronic transducer, thus converting a biochemical signal into a quantifiable electric response to yield a measurable signal. Biosensors come in different sizes and shapes and make use of a variety of transducers such as pH- or ion-selective electrodes, thermistors, optical fibers, and piezoelectric crystals *(1,8)*. The function of a biosensor will depend on the biochemical specificity of the biologically active material.

The objective of this article is to review briefly some aspects on the selection, production, immobilization, and stabilization of biomaterials for applications in the biosensor field. Emphasis has been laid on studies carried out in my laboratory.

Biomaterials

As biologic sensing elements, enzymes, antibodies, DNA, receptors, organelles, and microorganisms as well as animal and plant cells or tissues have been used. The choice of the biologic material depends on a number of factors including the specificity, storage, and operational and environmental stability. The choice of the biologic material also depends on the analyte to be detected, such as chemical compounds, antigens, microbes, hormones, nucleic acids, or any subjective parameters such as smell and taste. A number of novel and stable enzymes may be required in the future for the analysis of a variety of complex chemicals contained in varied environmental conditions encountered in industrial processing as well as waste management. Highly thermostable enzymes that can withstand even autoclaving temperatures may be required for the *in situ* monitoring of fermentors and other high-temperature processes *(9–11)*.

Currently, there is also an interest in cold active enzymes. These enzymes have a low temperature optima, or they exhibit high activities even at low temperatures *(12)*. The basic advantage of such enzymes is the possibility of carrying out process monitoring even under chilled storage conditions normally practiced in food and allied industries. These enzymes can be obtained from cold-adapted microbes or fish. Recently, a biosensor based on this concept has been reported for the measurement of sugars that makes use of a psychrophilic strain of *Deinococcus (13)*.

In addition to thermal stability, other important environmental conditions often required are the ability to act in highly acidic, alkaline, hydrophobic (organic solvent), or oxidizing environments. In this direction a variety of extremophiles such as the thermophiles, alkalophiles, and halophiles have gained importance in the production of enzymes for use in biosensors and other applications *(14)*. Selective screening methods for discovering the rare genera and species of microbes for the production of novel analytically useful enzymes and also the need for an integrated approach to combine classic microbiology with developments in modern biotechnology for sustained advances in research and development are essential for future developments *(15)*.

Purified enzymes have been most commonly used in the construction of biosensors. The major advantage of using a pure enzyme is its high analytical specificity. A crude enzyme mixture can often result in side reactions, thus decreasing the overall specificity of the system. Moreover, because of the very high specific activities of purified enzymes as compared to crude enzyme preparations, a large amount of the enzyme activity can be loaded on a small surface area of the transducer. For the miniaturization of biosensors, more active enzyme preparations may be required in the future. Developments in efficient downstream processing systems such as membrane filtration, aqueous two phase, reverse micelles, and biospecific affinity techniques can play an important role in the extraction, purification, and concentration of analytically useful enzymes *(16,17)*. In this direction, a rapid method based on hydrophobic chromatography has been developed in my laboratory for the purification of yeast D-amino acid oxidase that can find application in the fabrication of biosensors for the detection of D-amino acids *(18)*.

The purified enzymes, in general, are often expensive and unstable, thus limiting their applications in the industrial as well as biosensor fields. In this respect, for the utilization of intracellular enzymes, immobilization of whole cells has been shown to be a better alternative to immobilization of purified enzymes *(19–21)*. Doing so avoids the lengthy and expensive operations of enzyme purification and preserves the enzyme in its natural environment, thus protecting it from inactivation either during immobilization or its subsequent reuse. It may also provide a multipurpose catalyst, especially when the process requires the participation of a number of enzymes in sequence. The major limitations that may need to be addressed while using such cells are the diffusion of substrate and products through the cell wall and unwanted side reactions owing to the presence of other enzymes. These problems could now be obviated by the use of permeabilized cells as a source of enzymes *(19–21)*.

The cells can be permeabilized using physical, chemical, and enzymatic approaches. The most common technique is the use of organic solvents or detergents. Such treatment removes some of the lipids from the cell membranes, thus creating minute pores, allowing the free diffusion of small molecular weight substrates/products across the cell membrane while

retaining most of the macromolecular compounds such as the enzymes inside the cell. Permeabilization renders the cell nonviable and also empties it of most of the small molecular weight cofactors, thus minimizing the unwanted side reactions *(19,21)*. Side reactions, which can occur owing to the presence of other enzymes in a cell, can be minimized by inactivating such enzymes *(22)*. Several techniques have been developed in my laboratory for obtaining permeabilized cells containing catalase *(23)*, invertase *(24)*, alcohol dehydrogenase *(25)*, D-amino acid oxidase *(26)*, and some enzymes from halophilic organisms *(27)*. Permeabilized nonviable cell systems can serve as an economic source of intracellular enzymes for simple biosensor applications that do not require cofactor regeneration or metabolic respiration, such as glucose oxidase, amino acid oxidase, and urease *(1)*. In some cases in which the enzyme in a cell is present in the periplasmic site, the whole cells can be used without permeabilization as in the case of invertase in yeast *(24)* and urease in bacterial cells *(28)*. A typical example is the whole-cell amperometric glucose biosensor based on *Aspergillus niger* containing glucose oxidase *(29)*.

Immobilized viable cells have gained considerable importance recently in the fabrication of biosensors *(1,30)*. Viable cells are mainly used when the overall substrate assimilation capacity of microorganisms is taken as an index of respiratory metabolic activity, which essentially requires multienzymes and cofactor regeneration as in the case of the estimation of BOD *(5)*. Another mechanism used for the viable microorganism-based biosensor involves the inhibition of microbial respiration by the analyte of interest, such as environmental pollutants *(30)*. Whole-cell biosensors have also been developed using genetically engineered microorganisms for the monitoring of organic and pesticide contamination *(1,31)*. The microorganisms used in these biosensors are typically produced with a constructed plasmid in which genes that code for luciferase are placed under the control of a promoter that recognizes the analyte of interest. When such microbes (bioreporters) metabolize the organic pollutants, the genetic control mechanism also turns on the synthesis of luciferase, which produces light in the presence of oxygen *(31)*.

Quite often a single cell may not contain all the enzymes necessary for a sequential reaction. In such cases, mixed microbial cultures have shown promise. Thus, *Gluconobacter oxydans* containing glucose oxidase has been used in conjunction with *Saccharomyces cerevisiae* cells containing periplasmic invertase or permeabilized *Kluyveromyces marxianus* cells containing intracellular β-galactosidase in the fabrication of a sucrose and lactose biosensor, respectively *(32)*. Alternatively, studies from my laboratory have shown the possibility of introducing a deficient enzyme into a cell by directly binding it on its cell-wall surface like binding of glucose oxidase using lectins (concanavalin A) or polyethylenimine (PEI) on microbial cells induced for invertase *(33)* or β-galactosidase *(34)*. Whole cells have also been often replaced by plant or animal tissues. Some examples include the

use of cucumber peel as a source of ascorbate oxidase and kidney slices as a source of amino acid oxidase *(1,35)*.

Immunosensors have gained importance in the recent past because of the availability of monoclonal and polyclonal antibodies directed toward a wide range of analytes as well as the relative affinity and selectivity of these recognition proteins for a specific compound *(1,36,37)*. Nucleic acid–based affinity biosensors are being studied for the detection of chemically induced DNA damage *(38)* and detection of microorganisms through the hybridization of species-specific sequences of DNA *(2,39)*.

Immobilization of Biomaterials

The basic requirement of a biosensor is that the biologic material bring the physicochemical changes in close proximity to a transducer. In this direction, immobilized enzyme technology has played a major role. Immobilization not only helps in forming the required close proximity of the biomaterial with the transducer but also in stabilizing it for reuse. The biologic material has been immobilized directly on the transducer or, in most cases, in membranes, which can subsequently be mounted on the transducer *(1)*. Biomaterials can be immobilized either through adsorption, entrapment, covalent binding, crosslinking, or a combination of all these techniques *(19–21,40)*. Selection of a technique and support depends on the nature of enzyme, nature of substrate, and configuration of the transducer used. The choice of support and technique for the preparation of membranes often has been dictated by the low diffusional resistance of the membrane. The choice of technique also depends on the biomaterial of interest. Thus, enzymes and antibodies have normally been immobilized either by covalent binding or through adsorption followed by crosslinking. When covalent binding or crosslinking is used, precaution needs to be taken, so as to bind the enzyme without significantly affecting its conformational flexibility, since minor alterations in conformational changes on binding a ligand to a protein molecule can often be used as a criteria in analytical determinations. In this direction, novel $P(CH_2OH)_3$-polyetheramine-derived polymeric films designed to retain maximal enzyme activity have been recently reported *(41)*. Analytically useful glycoprotein enzymes such as glucose oxidase, peroxides, and invertase can also be covalently bound via their carbohydrate moiety *(42,43)*. Such an approach often results in better retention of enzyme activity, because it avoids the chemical modification of functional groups in the protein moiety of the enzyme.

Entrapment and adsorption techniques are more useful when cells or cellular organelles are used. Passive trapping of cells into the pores of membranes made up of cellulose or other synthetic materials has been well documented *(1)*. Another common approach is to retain the cells or enzymes in the close proximity of the transducer surface using dialysis membrane *(1)*. Other techniques include entrapment in a variety of synthetic or natural

polymeric gels. Enzymes entrapped within reverse micelles have also shown promise in the fabrication of biosensors *(44)*. A major limitation of the entrapment technique is the additional diffusional barrier offered by the entrapment materials. This can often be minimized by increasing the porosity of the entrapment matrix. Techniques have been developed for obtaining such open-pore entrapment materials containing cells *(45)*. More gentle techniques and supports are required when viable cell systems are needed *(19–21)*. Immobilized viable yeast cells, which can metabolize glucose or lactose in food products *(46,47)*, and an immobilized viable cell consortium obtained by entrapment of activated sludge that can metabolize phenol *(48)*, have been reported from my laboratory.

Polyvinyl alcohol (PVA) is one of the most widely studied polymers, because it can form membranes, fibers, and so on. Enzymes have been immobilized in these membranes either by entrapment, covalent binding, crosslinking, freezing and thawing, γ-irradiation, photocrosslinking, or entrapment followed by crosslinking *(49)*. Recently, high-swelling membrane discs of PVA-glutaraldehyde containing free carbonyl groups have been prepared for the binding of biomaterials through their free amine groups *(50)*. Modified polyvinyl chloride membranes *(51)*, polyacrylonitrile membranes *(52)*, and albumin-poly(ethylene glycol) hydrogel *(53)* have also shown promise. Albumin-poly(ethylene glycol) hydrogels, in view of their biocompatibility, may gain importance in the fabrication of in vivo implantable biosensors *(53)*. Enzyme immobilization in electropolymerized conducting polymers has also attracted wide attention *(54)*. Biospecific reversible immobilization using lectins or hydrophobic surfaces can also be used for the introduction of biologic catalysts into analytical systems *(55)*. The basic advantage is that when the enzyme activity goes below the practical limits, the bound enzyme can be easily eluted, and the membranes or the transducer surface can be loaded with a fresh batch of the enzyme for reuse without significantly affecting the transducer or membrane characteristics. Studies from my laboratory have shown the possibility of using this approach for the simultaneous isolation and reversible immobilization of D-amino acid oxidase *(56)*. Recently, novel sol-gel synthetic techniques have been developed to immobilize biologically active molecules in stable, optically transparent, porous silica glass matrix. The resulting glass allows transport of small molecules while retaining the protein molecules within their pores. The prospects are excellent for use in optical biosensors *(57)*.

The development of molecular devices incorporating a sophisticated and highly organized biologic information-processing function is a long-term goal of bioelectronics. Biosensors have been miniaturized extensively in recent years. For this purpose, it is necessary to develop suitable methods for the microimmobilization of the proteins into an organized array on materials such as silicon wafers. In this direction, the application of peptides with photolabile protecting groups in conjunction with a photolithographic technique for microimmobilization of peptides on glass surface has been described *(58)*. Similarly, silane-coupling reagent and photolitho-

graphic techniques have also been investigated *(59)*. Microimmobilization will play an important role in the fabrication of DNA-based sensors such as the DNA chips. Protein Langmuir-Blodget (LB) films have raised considerable interest in bioelectronics *(39)*. The LB method has been shown to produce functional antibody coatings of high surface density when compared with conventional immobilization techniques such as adsorption and covalent binding *(60)*.

A number of new techniques and supports have been developed in my laboratory for immobilization of enzymes and cells *(19,21,40)*. Nonviable cell preparations have been immobilized in radiation polymerized acrylamide. The major advantage of γ-ray polymerization against chemical polymerization is that the polymerization can be carried out even under frozen conditions. This not only allows for the freezing of monomer and biocatalyst mixture in the required geometry (beads, membranes, tubes) but also reduces inactivation of the enzymes owing to the excess heat generated during the exothermic chemical polymerization reaction. Electron micrographs of such preparations indicate a highly spongy and porous nature of the matrix *(23–26)*. Whole cells containing alcohol dehydrogenase *(25,61)* and amino acid oxidase *(26)*, which have significance in biosensors, have been successfully immobilized using this technique. Another polymer that has been extensively used for the entrapment of cells is Ca-alginate *(62)*. However, one of the major limitations of Ca-alginate gels is their destabilization and subsequent solubilization by the Ca-chelators present in the processing solution. We have recently developed a novel technique for stabilizing the alginate membranes and beads by reinforcing them with polyacrylamide polymerized using γ-rays *(63)*.

Proteinic supports, in general, offer a natural environment for the immobilized enzymes and have been extensively investigated for the preparation of enzyme membranes for biosensor applications *(19,32)*. In this respect, studies carried out in our laboratory have shown the possibility of using hen egg white as a novel proteinic support. Enzymes or nonviable cells can be immobilized through crosslinking with glutaraldehyde to obtain them in bead, membrane, or block forms *(64,65)*. The unique characteristics of the egg white to form stable foam has also been made use of to obtain highly porous biologic foam matrix containing glucose oxidase *(66)*. The unique feature of this support is the large concentration of lysozyme naturally present in hen egg white, which gets coimmobilized, thus imparting bacteriolytic properties to the support *(67,68)*. Gelatin has been extensively used as a natural polymeric support for the preparation of enzyme membranes *(32)*. The method often followed is crosslinking using glutaraldehyde, which is known to inactivate some of the enzymes requiring amino groups for activity. The technique developed for the polymerization of gelatin using γ-irradiation can obviate the use of glutaraldehyde and can therefore be employed for the immobilization of glutaraldehyde-sensitive enzymes *(69)*. The technique has been used for the immobilization of D-amino acid oxidase by entrapment of the cells of

Trigonopsis variabilis (69). The presence of gelatin or acrylamide was found to protect the enzymes from radiation inactivation *(24,69)*.

One of the major contributions from my laboratory has been the use of PEI for the immobilization of enzymes and cells on a variety of natural and synthetic surfaces *(70)*. PEI is a weak, polybasic aliphatic amine with good anion-exchanger properties *(71)*. PEI coating has been shown, in my laboratory, to be a very simple and useful technique for imparting polycationic characteristics to polymeric surfaces ranging from cellulose and glass to various other synthetic surfaces such as polycarbonate and allied plastic materials *(70,72–74)*. The enzymes can be immobilized on PEI-coated surfaces through adsorption followed by crosslinking. We have shown that a number of enzymes with low p*I* such as invertase *(75)*, glucose oxidase *(76)*, catalase *(76)*, and urease *(77)* can be immobilized on PEI-coated polymeric surfaces using this technique. Glucose oxidase bound on cheesecloth using PEI has been used in conjunction with a platinum disc electrode for the fabrication of glucose sensor in my laboratory *(78,79)*. The cyclic voltammetric studies at the enzyme electrode showed that the cheesecloth as a support for the glucose oxidase enzyme offers minimal resistance to the transfer of electroactive species to the electrode surface *(78)*. In addition to its high porosity, other advantages of cheesecloth membrane are its mechanical stability and flexibility for use with any transducer geometry, longer life, as well as low cost. Potentiometric studies with the cheesecloth–glucose oxidase enzyme electrode showed good response slopes toward glucose in the range of 2–22 m*M*, making it suitable for the fabrication of a biosensor for glucose *(79)*.

My laboratory was the first to demonstrate the possibility of immobilizing viable or nonviable cells through adhesion on a variety of polymeric surfaces including glass, cotton fabric, and synthetic polymeric membranes using PEI *(28,70,72,73,80,81)*. The major advantage is that the cells immobilized through adhesion are in direct contact with the liquid phase containing the substrate, even though the cell and the liquid phase are distinctly separate, thus reducing or eliminating the mass transfer problems commonly associated with gel entrapment methods. Such biofilms have often been formed making use of the microbial cells' natural ability to adhere to surfaces *(82)*. However, a major limitation of such a natural adhesion process is the lengthy time required to build up a biofilm sufficient to carry out a bioconversion. Also, changes in the environmental conditions of growth or processing medium can often drastically alter the degree of cell adhesion. The novel techniques using PEI developed in my laboratory can obviate these problems. The adhesion is found to be rapid, and the cells adhere as a monolayer *(72,73)*. Adhesion is also very strong and high ionic concentrations and extreme pH conditions, which normally disrupt the ionic interactions, fail to desorb the cells. Cells can be adhered by coating either the cells, the supports, or both with PEI *(72,73)*. Viability of the cell was not affected by this treatment *(72)*. A variety of cells containing different enzymes including urease have been immobilized using this technique, and studies are

under way to test the use of such biofilms in the fabrication of microbial biosensors. The technique has also been extended to obtain enzyme-cell coimmobilizates *(33)*. The PEI technique has found application in the biosensor field *(83,84)*. Recently, Nandakumar and Mattiasson *(85)* applied the PEI technique developed in my laboratory for the fabrication of a microbial biosensor for the online monitoring of phenolic compounds.

Stabilization of Biomaterials

Any worthwhile biosensor must be robust and operationally stable. The enzyme must function reliably under varying environmental conditions over an extended time period. Often the intrinsic stability of the enzyme may not be sufficient to cope with these demands. Immobilization, in general, is known to stabilize the enzymes. Most of the stabilization efforts have involved either the limited intramolecular chemical crosslinking or protein engineering techniques. In this respect, useful strategies have been evolved for immobilization-stabilization of enzymes by multipoint covalent attachment to gels *(86)*. Such approaches stabilize enzymes toward the conformational changes induced by heat, organic solvents, an so forth. On the other hand, a very intensive enzyme-support multipoint attachment may lead to a loss of catalytic activity. Thus, very careful control of these enzyme-support multiinteraction processes is necessary to get derivatives with promising activity and stability characteristics. Amorphous enzyme aggregates prepared by chemical crosslinking with glutaraldehyde have shown enhanced stability under stress conditions such as temperature and exposure to organic solvents *(87)*. Formation of such aggregates is generally attributed to both intramolecular and intermolecular crosslinks introduced in the protein molecule. Horseradish peroxidase, which has applications in a variety of analytical systems including the construction of biosensors, has been extensively investigated in this respect for enhanced stability through either chemical modification or immobilization *(88,89)*.

Many bioprocesses in the future will be based on the use of organic solvents. In view of this, a great deal of research has centered on the behavior of enzyme reactions in organic solvents. From a biotechnologic perspective, there are numerous potential advantages in employing enzymes in organic as opposed to aqueous media. These include the increased solubility of nonpolar substrates and shifting of thermodynamic equilibrium to favor synthesis over hydrolysis *(90,91)*. The monitoring of such processes will require biosensors containing a biologic system that is active and stable under these conditions. In this direction, crosslinked enzyme crystals, and microcrystals grown from aqueous solution and crosslinked with a bifunctional reagent such as glutaraldehyde, have shown promise. Crosslinked enzyme crystals help in obtaining highly concentrated immobilized enzyme particles exhibiting better stability at elevated temperatures and in near-anhydrous organic solvents *(92)*.

In addition to protein stabilization, there is also an interest in the stabilization of cells and cellular organelles. The animal and plant cellular organelles such as mitochondria and chloroplast can exhibit specific biologic activity. However, the major drawback is their osmotic instability, thus limiting their applications for use only in isotonic solutions. Osmotic stabilization of cellular organelles such as the animal mitochondria using glutaraldehyde has shown promise *(93)*. Halophilic cells are gaining importance in biochemical conversions under high salt conditions in which the normal microbial cells fail to grow or function. The major limitation of these cells is their lysis with slight changes in the external salt concentration from the optimum. Crosslinking techniques developed in my laboratory can obviate these problems *(94)*. The crosslinking technique can also be used for the stabilization of halophilic enzymes toward denaturation under low salt concentration *(95)* and for the stabilization of microbial cells toward lysis by lytic enzymes *(96)*.

Design of Future Biosensors

A number of articles have appeared in the literature on biosensors. Most of these studies, however, deal with estimations under controlled laboratory conditions. As the biosensor technology starts moving from the proof-of-concept stage to field testing under realistic processes or waste-monitoring conditions, the need for the availability of stable biologic materials will become important. Recent developments in fiberoptics and microelectronics have expanded the scope and capability of signal transducers. Their applications in the biosensor field will also depend on simultaneous advances in obtaining appropriate biomass materials. Advances in biochemistry, molecular biology, and immunochemistry will result in a rapid expansion of wide-ranging biologic recognition elements, leading to more wider applications for the use of biosensors as alternative or newer analytical tools.

Genetic engineering (rDNA technology) can help in the overproduction of enzymes in microbes. Protein engineering, either through site-directed mutagenesis or through chemical modification, can help in designing enzymes to work under varied environmental conditions of temperature, pH, and salinity or to act on molecules slightly different in structure from their natural substrates *(9–11)*. In the future, computer-aided protein engineering will help in making a rational design of proteins with new analytical properties possible *(97)*. Another important development is "abzymes," the catalytic antibodies; it may be possible to produce "smart" enzymes that catalyze analytical reactions for which no known enzyme exists in nature *(98)*. rDNA technology may help in tailoring enzymes (fusion proteins) by introducing recognition sites (e.g., streptavidin/biotin) into a specific protein. This will not only be helpful in their easy purification using biospecific affinity techniques *(99)* but also in conjunction with lithographic techniques to obtain microimmobilized protein patterns on silicone chips *(100)*.

In the coming years, immobilized cell-based biosensors will gain importance. The major limitation has been the slow response of such sensors compared with the enzyme sensors. This has been attributed mainly to the mass transfer resistance offered by the cell membrane, especially for the intracellular enzymes. Efforts are under way to obviate these problems by genetically engineering the cells to transport the otherwise intracellular enzyme to the periplasmic place and naturally immobilize it between the cell membrane and the cell wall *(101)*. Application of this approach for the fabrication of a biosensor for the determination of organophosphate nerve agents has been reported *(102)*. The sensor uses a recombinant *Escherichia coli* with surface-expressed organophosphorous hydrolase *(102)*. Such approaches may lead, in the future, to the development of microbial sensors with faster response time and sensitivity. Development of biosensors involving enzyme reactions requiring diffusible cofactors such as adenosine triphosphate and nicotinamide adenine dinucleotide is presently limited owing to lack of efficient cofactor regeneration *(61)*. Efficient regeneration may be possible in the future using designer enzymes in which the cofactor is anchored and can swing between the oxidation and reduction sites *(40)*.

There are interesting possibilities within the field of biosensors. Given the existing advances in biologic sciences, coupled with advances in various other scientific and engineering disciplines, it is imminent that many analytical applications will be replaced by biosensors. A fruitful fusion between biologic sciences with others will help to realize the full potential of this technology in future applications.

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