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review

Immobilized Bioreagent–based Molecular Devices

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Summary

An overview of the current state of the art technology that is applied in the molecular biosensor development is presented. Science and technology behind these devices are introduced followed by some examples. The industrial needs, in particular of the food industry, environmental monitoring, clinical analysis, etc., that are to be addressed by such devices are discussed.

Keywords: molecular biosensor, immobilized bioreagent, food industry, environmental monitoring, clinical analysis, process control

Introduction

History

Historically, Clark and co-workers *(1)* and others *(2,3)*, were the first to explore rather simple biosensor formats such as physical entrapment of soluble enzymes or enzyme membranes fixed to an electrochemical transducer starting as early as 1962. These basic systems formed the foundation of sensors exploiting the bulk immobilization of biomolecules directly onto a transducer. The current stage of development for immobilization of biomolecules to transducers has produced a variety of approaches. These approaches include: covalent bonding of organized monolayers of biocompounds directly to the transducer, entrapment of biocompounds into sol-gel glasses, or immobilization of biocompounds into three--dimensional and multi-layer systems. These systems are immobilized directly onto transducers such as semiconductor devices, optical waveguides, and various electrode materials (e.g. gold, doped carbon, etc.). The technological advances in microelectronics and fibre-optics have dramatically increased the number of physical transducers which are available for use in biosensors *(4, 5)*. This is further augmented by advances in biochemistry, immunochemistry and biotechnology which have resulted in increased availability of well characterized enzymes, antibodies, tissues, living organisms and receptors for use in these devices.

The immobilized bioreagent-based devices, more commonly known as »biosensors«, have been historically regarded as a subgroup of chemical sensors in which a biologically-based mechanism is used for detection of the analyte. Because chemical sensors are regarded as miniaturized transducers which selectively and reversibly yield an electrical signal that is proportional to a specific analyte of interest, this definition would exclude many bioanalytical devices which employ irreversible binding. The scope of this review, however, will include all significant classes of molecular devices which use immobilized bioreagents.

Need

Advancements in the manufacturing processes, higher standards of living and increased quality conscience on part of the global population, coupled with the rapid analysis and user friendly instrumentation designs, require methods and devices that can meet these demands. These highly variable application or user needs are the major governing factors for the currently observed upsurge in the biosensor research activity and sensor activity in general. Particular analytes to be measured and the selectivity required will dictate the choice of a biocompound for molecular recognition. Other parameters such as sensitivity, sample environment, cost, life time, user skill, etc., play a vital role in the selection of a particular transduction technique. Regulatory requirements and the nature of the application may play a decisive role in the sensor design and development.

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Bioanalytical devices show the potential to meet monitoring needs in such areas as clinical diagnostics, process control, food, military and environmental monitoring. Nevertheless, of the numerous biosensor configurations reported in the literature, few have been commercialized *(6)*. Biosensors which have been commercially developed have been used in applications with well defined performance requirements such as matrix, concentration range, and reproducibility (i.e. blood glucose monitoring, single analyte monitoring for the food industry and determination of biological oxygen demand for waste water processing). Although biosensors have been able to meet the application criteria (in these specific cases), for the most part, biosensors have not been able to meet the performance standards for numerous applications for which they show potential.

The highest level of activity for biosensor development is currently in the clinical and diagnostics markets. Although in the clinical area, these bioanalytical devices show the potential for development as compact and low-cost devices, their most significant contributions will most likely be as components in the laboratory-based high volume multianalyte instruments currently offered by several manufacturers.

Analytical monitoring needs in the agricultural and food industry involve several areas, including process control and quality assurance monitoring for (i) specific compounds associated with the product, (ii) specific microorganisms (those endogenous to the product as well as foreign contaminants), and (iii) chemical indicators of spoilage. Currently available products for the agricultural and food industries are primarily related to the need to monitor single analytes found in the product. These molecular sensors consist of single analyte detectors for compounds such as glucose, lactose, galactose, starch, ethanol, methanol, urea, glutamate, lactate, oxalate, etc., as well as for compounds indicative of seafood freshness *(7)*.

Another area of application for bioanalytical devices is for the detection of analytes of military interest such as nerve agents, mustard gas, explosives, and a number of toxins and pathogens. One of the greatest challenges which these molecular monitoring devices face (for these applications) involves the variety of compounds and matrices encountered. In addition, these applications require that the device be durable (i.e. the ability to function in harsh environments such as dust, humidity and wide temperature variations); easy to operate (i.e. by minimally trained personnel); and function in potentially remote and continuous circumstances with high reliability and sensitivity.

The high-cost and slow turnaround times associated with the measurement of regulated pollutants clearly indicate a need for environmental field analytical methods. Biosensors show the potential to be developed as sensitive, selective, and potentially portable devices capable of continuous, *in situ*, and remote monitoring of environmental pollutants. As in potential military applications, one of the main challenges to the development of biosensors in this area is the requirement to monitor diverse matrices such as ground water, soil extracts, etc. In addition, these devices must show sensitivities commensurate with regulatory (national and international) action levels for specific analytes such as pesticides, PCBs, phenolics and polycyclic aromatic hydrocarbons.

Advantages/Features

Biosensors are typically composed of a biological recognition element, a physical transducer, an electrical amplifier and a data processing system (Fig. 1). Biological recognition elements which have been reported in biosensors include: enzymes, receptors, immunocompounds, DNA (total and sequence-specific recognition), microbes, transport proteins and tissues (both plant and animal).

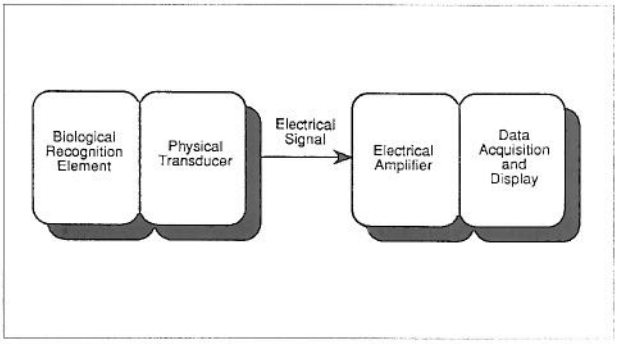

Fig. 1. Basic concept of a molecular device based on immobilized bioreagent [from Ref. 5]

Physical transducers which have been reported for use in biosensors include the following: electrochemical, optical, thermal and acoustic devices (Table 1). The biological recognition elements (i.e. enzymes, receptors, immunochemicals) can be interfaced to any of these transducers, given an appropriate signal transduction mechanism can be devised. Potential advantages offered by biosensor technology include minimal sample preparation, high speed of analysis and the potential for *in situ* and flow stream analysis for process control.

Table 1. Biosensor components

Biological recognition elements	Physical transducers
Enzymes	Electrochemical
Receptors	Optical
Antibodies	Optical-electronic
Cells	Acoustic
Tissues	Thermal
Sensing organs	

Biosensor characteristics are primarily determined by the choice of biological recognition element, the physical transducer, and the assay format. Consequently, it is critical that the operational characteristics be considered and reconciled against the potential application early in the development process. These issues include: (i) reagent requirements (i.e. need for reagents other than the analyte; substrates, cofactors, pH buffers, and oxidation reduction mediators); (ii) temperature requirements (i.e. enzyme-catalysed reactions approximately double in velocity with each increase in temperature of 10 °C); (iii) size and power requirements; (iv) environmental inter-

face requirements (i.e. aqueous, organic, sample size, etc.); (v) requirements for calibration.

Clearly, immobilized bioreagents offer a wide variety of features and hence choice to develop low cost molecular devices for a number of applications. Their adaptability to flexible formats and compatibility with a host of transduction techniques make these devices very attractive for practical exploitation. Such devices are expected to be non-toxic, non-carcinogenic, disposable or reusable and suitable for large scale manufacture.

Using Immobilized Reagents – Sensor Fundamentals

Molecular Recognition

Molecular recognition mechanisms used in biomolecular sensors are of three main types. These mechanisms include: (i) biocatalysis (e.g. the measurement of products or inhibitors of enzyme-catalysed reactions); (ii) stoichiometric binding (e.g. binding of an analyte to an antibody, receptor, or sequence of DNA); or (iii) combination of both (e.g. recognition of an analyte by a microorganism followed by enzymatic amplification of a measured product). In addition to recognition of an analyte at concentrations of interest, a variety of other issues must be considered. Such issues include: the specificity of the molecular recognition; the stability and immobilization options; and the characteristics of the assay format.

Enzymes have historically been used in biosensors primarily as a result of their ability to catalytically convert an analyte of interest to a product which is optically or electrochemically detectable. In addition, the availability of purified enzymes and extensive literature on immobilization techniques have made these proteins attractive for use in molecular recognition devices.

The general expression governing enzyme kinetics is typically presented in the following form:

$$v = V_{max}[S]/K_M + [S]$$

in which v is the reaction rate in (mol s^{-1}), $[S]$ is the substrate concentration, V_{max} is the maximum reaction rate and K_M is the Michaelis constant (i.e. the concentration of substrate resulting in one half the maximum rate for the enzyme catalysed reaction).

There are also certain limitations for use in molecular recognition devices imposed by enzyme kinetics. For example, the dynamic range (for substrate determination) using enzyme catalysed reactions is determined primarily by the K_M value. This is due to the fact that at low substrate concentrations (i.e. $[S] < K_M$) the reaction rate is linearly proportional to the substrate concentration *(8)*. Thus, for analytical enzyme applications, enzyme assays are typically configured such that the substrate concentrations are considerably lower than the K_M. In practical terms, however, substrate concentrations below 0.1 K_M are not typically useful due to the low rate of product formation. In the case of biosensors which measure enzyme inhibitors, the detection limits are primarily determined by the type of inhibition (i.e. competitive, non-competitive or irreversible) and the enzyme's affinity for these compounds.

Although enzymes are useful as biological recognition elements, the selection of substrates and inhibitors is limited to the finite number of biocatalysts which have been produced through evolution. By contrast, antibodies can be developed to practically any hapten that can be attached to a carrier protein (i.e. immunogen). Antibodies have been reported which are specific to a wide range of compounds. In addition, monoclonal antibodies can be selected which show a range of specificities from individual compounds to compound classes as well as a range of detection limits from 10^{-5} to 10^{-12} M.

Although the range of binding affinities between antibodies and antigens is broad, most analytical methods using immunochemicals including those reported for antibody-based biosensors, concentrate on the use of irreversible binding. This limitation has been overcome, in certain cases using several approaches. Antibody-based biosensors have been reported, which can be reactivated after a binding event by lowering the pH of the buffer solution to allow the dissociation of analyte from the immobilized antibody *(9–11)*. In addition, reversible binding of antigen to monoclonal antibody immobilized to a transducer surface has been demonstrated by using non-equilibrium kinetic measurements *(12)*.

Reagent Immobilization

Coupling of the biological recognition element to the transduction element is a critical aspect in the ultimate reliability and performance of the molecular recognition device. Due to the variability in both structure and function of the biological components as well as the variety of materials used for signal transducers, numerous coupling strategies have been reported. There are a number of issues, however, which should be considered in the choice of a particular coupling method.

With respect to the biological recognition element, chemical groups which are typically used for coupling include: carbohydrate, amine, carbonyl, and sulfhydryl groups. Immobilization of these biomolecules requires consideration of the number and accessibility of these groups as well as their location relative to the active (catalytic or hapten binding) site. Other important issues include the length, chemical characteristics, and »cleavability« of the linker chain used to attach the recognition element to the transducer. In addition, each type of recognition element (i.e. antibody, enzyme, or microbe) has certain functional and operational features, some of which are unique to each system. For example, in the case of antibodies, orientation of the binding domains F_{ab} and F'_{ab} can increase the stability, accessibility, and overall performance of these biosensors. Approaches which have been used to orient immobilized antibodies include: the use of proteins A, G, etc. (proteins that specifically bind to the F_c portion of the IgG); the use of the protein avidin which tightly binds biotin, a low molecular mass compound that can be covalently attached to an antibody); and the use of species specific anti-IgG antibodies which also bind to the F_c portion of the antibody allowing optimal orientation of the primary antibody.

In the case of enzymes, primary considerations include: the maintenance of catalytic activity and the access of the substrate to the active site. In addition to im-

mobilization strategies, various types of membranes have been incorporated which control substrate accessibility and prevent saturation kinetics as well as limit interferences from non-substrate compounds.

For receptors, many of which are integral membrane proteins, approaches which have been used to accommodate their need for a lipid bilayer environment include incorporation of these proteins into liposomes and surface-immobilized bilayer membranes. In some cases, however, receptors have been shown to retain their binding activity after immobilization on a solid surface. For example, transferrin-binding protein retains its activity after reconstitution onto Eupergit CB-6200 beads *(13)* and nicotinic acetylcholine receptor maintains a portion of its binding activity upon immobilization to both quartz fibres and nitrocellulose membranes.

Microorganisms typically require non-covalent immobilization methods such as entrapment behind a membrane or in a hydrophilic gel such as agarose.

Transducer surfaces to which biomolecules are typically immobilized include: quartz, silicon, metals, or polymers. In particular, the proposed operating format and mechanism of signal transduction are important issues in the choice of immobilization methods. For affinity-based devices, non-specific binding of the analyte, analyte-tracer, or affinity protein can cause significant interferences. For catalytic-based devices, which use electrochemical detection, co-immobilization of an oxidation-reduction mediator can dramatically improve biosensor performance. In addition, the recent use of Langmuir-Blodgett techniques have been useful in preparing monomolecular layers and organized structures.

Signal Transduction

Given the diversity in biological recognition elements in addition to variations in signal transducers, it is not surprising that a wide variety of signal transduction mechanisms have been reported for these devices. Most of these devices, however, fall into one of two main classes; those which use biocatalytic and those which use affinity-based transduction mechanisms.

The signal transduction mechanism for biocatalytic devices (enzyme-based) typically relies on the catalytic conversion of a non-detectable substrate into an optically or electrochemically detectable product. In the case of optically detected products, fluorescent compounds are typically used as indicators because of the high sensitivity afforded by their use. These indicators can either be part of the optical sensor (i.e. immobilized to the optical waveguide) or detected in solution at the end of the optical fibre. As mentioned previously, the range of analytes which can be detected by enzyme-based biosensors is limited to substrates or inhibitors of the enzyme, however, several strategies have been used to expand this range. The range of analytes which can be measured using enzyme-based devices can be extended by the use of a second enzyme to convert a non-detectable primary product into a detectable secondary product. Recycling cofactors and substrates which have limited diffusional constraints *(14)* is another strategy that has been employed. A second basic mechanism for signal transduction in biocatalytic devices detects inhibitors, cofactors, and modulators of enzyme activity. These compounds are detected as a result of their effect on the biosensor steady state signal established in the presence of the substrate.

In contrast to enzymes, the primary signal transduction mechanism for receptors and antibodies is through a stoichiometric binding event. In this mechanism, analytes are measured indirectly through binding of an optically or enzymatically labelled analyte-tracer which competes with the analyte for a limited number of binding sites. Where the analyte shows a measurable physical property [e.g. fluorescence of benzo[a]pyrene; *(15)*] the binding of analyte can be measured directly at the surface of the sensor. Signal transduction mechanisms which use an enzymatic label require a two step process. The first step involves competitive binding of the analyte and analyte-tracer to the immobilized antibody or receptor. In the second step, the catalytic conversion of substrate to product is used as a measure of the original binding event. Enzyme amplification affords considerable sensitivity for this format. However, free antibody-labelled analyte must be separated from labelled analyte which is bound to the antibody immobilized to the sensor surface which requires a multi-step procedure.

Electrochemical

Potentiometric — Potentiometry has been used as an analytical technique for over a century. Although this technique was limited primarily to the determination of pH, the development of ion-selective electrodes over the past 30 years has tremendously expanded the range of possible applications. The principle involved with potentiometric measurement is typically centred around a permeability-selective ion-conducting membrane that separates ions of interest (in the sample) from ions at a constant activity inside the electrode body. The unequal distribution of ions inside and outside the membrane results in a charge potential that is measured using a reference electrode. The measured potential (E) is related to the analyte activity (a_i) in the sample by the Nernst equation:

$$E = E^{\theta} \pm (RT/nF) \ln a_i$$

where E^{θ} is the standard potential for $a_i = 1$, R is the gas constant, F is the Faraday constant, T is the thermodynamic temperature, n is the total number of charges on ion i. In practice, decadic logarithm is used in place of the Napierian logarithm.

The selectivity of these devices to various ions (e.g. H^+, F^-, I^-, Cl^-) and gases (CO_2, NH_3) is derived from their perm-selective membranes *(16)*. The development of various membrane materials which will bind the ion of interest and reject other closely related ions has been and continues to be an area of active research. The interface of these devices with enzymes, however, has also recently become an active research area and shows the potential to greatly expand the possible application of these devices.

For example, enzymes which produce or consume protons as a result of catalysis (e.g. penicillinase, urease, glucose oxidase, and acetylcholinesterase) have been interfaced to pH electrodes *(17)*. Although these devices

are potentially useful, they are not without limitations. These limitations arise from the fact that in addition to the product inhibition effects typically observed with enzyme catalysed reactions, the product (i.e. hydrogen ions or hydroxide ions) indirectly affect the conformation and activity of the enzyme. In addition, the use of buffers to control this effect complicates the measurement of the reaction product. In response to these challenges, innovative solutions have been reported such as pH-stat configurations which electrochemically compensate for enzyme-catalysed pH changes. In this configuration, the current used to electrochemically compensate for the production or consumption of protons is also used as a measure of analyte concentration *(18)*.

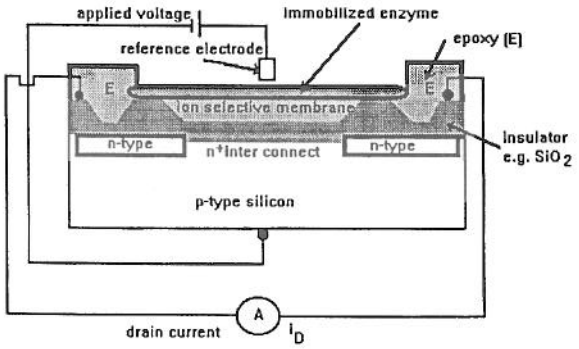

Fig. 2. An immobilized enzyme based potentiometric device [from Ref. 5]

Although the glass membrane pH electrode has become a standard laboratory device, characteristics such as durability, cost, and size have limited its use as a transducer for possible bioanalytical applications. A practical alternative which has been recently commercialized and extensively used in biosensors is the ion-selective field-effect transistor (ISFET) (Fig. 2). In this device, a gate voltage modulates the conductance between a source and a drain through the field-effect of a semiconductor. The ion sensitivity and selectivity of these devices arise from the characteristics of the membrane material used as the gate.

Silicon nitride (Si_3N_4) is one example of a gate membrane. Other materials are sensitive to ions such as Na^+, K^+, Ca^{2+}, NH_4^+, Ag^+ and Br^-. Although these devices have primarily been coupled to enzymes which produce or consume protons *(19)*, any biological recognition or catalytic event which modulates the concentration of ISFET-sensitive ions can potentially be used with these transducers. The primary limitations associated with ISFET-based biosensors involve the small surface area of the gate membranes and inconsistencies in protein immobilization.

The light addressable potentiometric sensor (LAPS) is another pH sensitive potentiometric transducer which has been recently introduced. This sensor, which also uses silicon nitride-coated surface, becomes highly sensitive to solution pH in discrete locations when illuminated by an LED array. This sensor has been used to measure the activity of various (proton producing or consuming) enzymes that are immobilized to a cellulose nitrate membrane and pressed against the sensor surface. The use of enzyme tracers has allowed the use of immunochemical assays which have been used to measure analytes such as DNA (total and specific sequences), human chorionic gonadotropin, pathogenic bacteria, acetylcholinesterase, drugs, and pesticides *(20)*.

Amperometric — Electroanalysis using controlled-potential techniques, basically involves the measurement of a current in response to an applied potential. The current is generated from an electroactive species which is either oxidised or reduced at the electrode surface.

The limiting current is given by the following equation:

$$I_L = (nFADc)/\delta$$

where n is the number of participating electrons, A is the area of the working electrode, F is the Faraday constant, D is the diffusion coefficient for the electrical species of concentration c in a given sample, and δ is the thickness of the diffusion layer.

The typical cell design includes working and reference electrodes. Reference electrodes are typically silver-silver chloride and working electrodes can be constructed from a variety of materials (e.g. gold, platinum, glassy carbon, carbon paste, etc.) in a variety of configurations (e.g. screen-printed planar, cylindrical, microfibre, etc.).

In certain circumstances, which require a potentiostat in place of a simple d.c. source, a three-electrode cell with an additional reference electrode can be used. In potentiostatic amperometry the voltage drop IR does not contribute significantly to the working electrode potential E.

For amperometric biosensors, a non-electrochemically active substrate is enzymatically converted to an electrochemically active product. This product is then oxidised or reduced at a working electrode which is maintained at a specific potential. The current is linearly proportional to the concentration of product which is (under certain conditions) proportional to the substrate concentration.

Amperometric biosensors typically rely on an enzyme system which catalytically converts electrochemically non-active analytes into products that can be oxidised or reduced at a working electrode which is maintained at a specific potential with respect to a reference electrode. The current is linearly proportional to the concentration of the electroactive product, which is proportional to the non-electroactive enzyme substrate. Enzymes typically used in amperometric biosensors are oxidases which catalyze the following class of reactions:

$$\text{Substrate (S)} + O_2 \xrightarrow{\text{oxidase}} \text{Product (P)} + H_2O_2$$

These reactions are electrochemically monitored by measuring the disappearance of O_2 or the increase of H_2O_2. For example, the ambient oxygen concentration can be monitored as it is depleted from solution. Because O_2 is continuously diffusing through the semi-permeable membrane, a steady-state current is established which is dependent on the substrate concentration. Alternatively, H_2O_2 can also be measured at the electrode surface.

Early configurations of this type of biosensor were limited by a small dynamic range and large overpotential required to oxidise H_2O_2. The overpotential resulted in the problem of oxidising non-substrate compounds present in the sample matrix (such as ascorbate in blood). Recent configurations, however, incorporate a number of innovations including: oxidation-reduction (redox) mediators, permeability selective membranes, and multienzyme systems that facilitate the use of cofactors and cofactor cycling systems. By transferring electrons from the enzyme to the electrode, the mediator substitutes for the natural electron acceptor (e.g. O_2). This allows the enzyme to be regenerated during the enzymatic cycle and effectively lowers the potential which must be applied to the electrode.

$$M_{ox} + E_{red} \longrightarrow M_{red} + E_{ox}$$

$$M_{red} \longrightarrow M_{ox} + 2H^+ + 2e^-$$

Although, the soluble redox mediators that have been used to reduce the effect of transient oxygen concentrations and reduce interferences from electroactive substances in the sample matrix, due to their small molecular masses, they must typically be supplied to the biosensor reaction mixture. Innovations such as the use of electron relay polymers, however, have facilitated the development of »reagent-less« amperometric electrodes which require only the substrate (analyte) of interest. Additional innovations include the use of multi-enzyme systems which can be used to couple an enzyme-catalysed reaction for which neither the substrate nor product are electroactive to a second reaction producing an electrically measurable product. Because advances involving the limitations of selectivity and sensitivity are continually being made, these devices are most likely to continue in their contribution to commercial applications in medical diagnostics, environmental, and food industries.

Conductance/Capacitance — The relationship of electrolyte conductivity $\varkappa$ to the conductance G is given by

$$\varkappa = K_{cell}\, G$$

where K_{cell} is the conductivity cell constant.

This relation makes it possible to monitor chemical reactions during which the overall conductivity of the solution is altered, by either production or consumption of ionic species. Clearly, any change in the electrolyte concentration, temperature or area of the electrode surface can affect the performance of the devices. For these reasons, conductometric/capacitance based sensors are considered to be relatively non-specific. Fortunately, a variety of molecules such as enzymes exist, that can be used to impart specificity to this type of transducer.

The ionic species producing or consuming enzymatic reactions, depending on the total ionic strength of the media, may alter the conductance/capacitance of the medium. This basic concept has been exploited in fabricating a number of planar interdigitated electrodes based on conductometric transducers for biosensors. These electrodes, together with a variety of enzyme systems, impart specificity to this type of transducer. One such example is the urease enzyme, which catalyses the production of ionic species, and which has been used as an immobilized coating form onto the planar interdigitated electrodes. However, the conductance being sensitive to temperature, Faradayic processes, double layer charging and concentration polarisation, it is desirable that differential methods using internal controls are used *(19)*.

Calorimetric

Temperature changes associated with molecular reactions has been exploited for bioanalytical applications because most biological reactions are thermogenic. In the case of enzyme-catalysed reactions, the enthalpy changes are quite high ranging from 20–100 kJ/mol *(21)*.

Relatively simple enzyme thermistors can be used to measure substrate-dependent biocatalytic reactions.

The temperature change due to enzyme catalysis is dependent on the molar enthalpy and heat capacity of the system, accordingly,

$$\Delta T = \frac{-\Delta H_m\, n_p}{C_S}$$

where ΔT is the temperature change, ΔH_m is the molar enthalpy, n_p is amount of the product and C_S is the heat capacity of the system, including the solvent.

A number of calorimetric methods such as »enzyme thermistors« and »immobilized enzyme flow enthalpimetric analysers« that have been reported exploit this behaviour *(21)*. A wide range of analytes, such as urea, glucose, ethanol, lactate, penicilin, oxalate, sucrose and urate, have been measured using calorimetry *(21, 22)*.

Recent developments in miniaturized devices have led to improved sensitivity in measuring changes in activities of metaloenzymes, due to the binding of apoenzymes to metals. The use of thermostated systems, heat exchangers and enzyme sequences which recycle a substrate or cofactor can be exploited to gain enhanced sensitivity. For example, co-immobilization of catalase with various H_2O_2 producing oxidases can more than double the enthalpy per mole of substrate, reduce the detrimental effects of hydrogen peroxide and reduce the net oxygen consumption *(21)*.

Optical

A vast number of optical transduction techniques are available for developing biosensors *(4,23)*. These may include linear optical phenomena such as absorption, fluorescence, phosphorescence, polarisation, rotation, interference, etc. or non-linear ones, such as second harmonic generation that have been exploited for biosensor development. Choice of a specific optical method depends on the nature of application and desired sensitivities. In principle, nearly all optical techniques can be coupled to fibre optics and integrated optic technology, thus increasing the versatility of these methods. In this review we shall discuss only a few development examples.

The construction of an optical waveguide is based on the principle of total internal reflection of light. As shown in Fig. 3, when the angle of incidence of light at the interface between the material of refractive index n_1 and the material of refractive index n_2, where $n_1 > n_2$, is greater than the critical angle θ_c

$$\theta_c = \sin^{-1}(n_2/n_1),$$

the light is reflected back into the medium of refractive index n_1. This phenomenon is known as total internal reflection (TIR). A waveguide is constructed from two optical materials of different refractive indices that allows the light entering at or above the critical angle to be repeatedly reflected (guided) down the lenght of the waveguide.

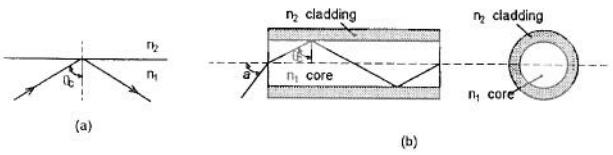

Fig. 3. Optical wave-guide principle and cross section of optical fibre [from Ref. *4*]

An example of TIR spectroscopy which utilises the phenomena of the evanescent field is termed total internal reflectance fluorescence (TIRF). TIRF has been used with planar and fibre-optic waveguides as the signal transducer in a number of reported biosensors *(23)*. These optical biosensor formats may involve direct detection of the analyte of interest or indirect detection through optically-labelled probes.

In these transducers, light is propagated down a waveguide which generates an electromagnetic wave (evanescent wave at the surface of the optically denser medium of the waveguide and the adjacent less optically dense medium (Fig. 4). The amplitude of the standing wave decreases exponentially with distance into the lower refractive index material. The fluorescence of a fluorophore (probe) excited within the evanescent field can be collected either outside the waveguide or by coupling the emission frequencies back into the waveguide. In configurations which use TIRF the biological sensing element is immobilized on the side rather than the end of the waveguide. This configuration is particulary useful for measuring binding events at a solid/liquid interface because the washing steps typically used to separate bound from unbound analyte probes are not required *(24)*.

Using a planar waveguide, a competitive immunoassay technique termed the fluorescence capillary fill device (FCFD) has been exploited (Fig. 3b). The FCFD allows a simple assay of a target protein by automatically measuring the correct volume of the analyte which is mixed with a predetermined amount of competitive immunochemical to produce a portion of labelled immunocomplex which is measured by TIRF. Light focused onto the waveguide base will generate an evanescent field that interacts with the fluorophore containing an immunocomplex. The resulting fluorescence is detected at the optical edge of the waveguide. The evanescent field ensures that only molecules within 10 nm will be detectable, thereby avoiding any of the wash or separation steps associated with an immunoassay. The measurement of human chorionic gonadotropin, a glycoprotein present in women during pregnancy and for the detection of the rubella antibody has been demonstrated using this method *(25,26)*.

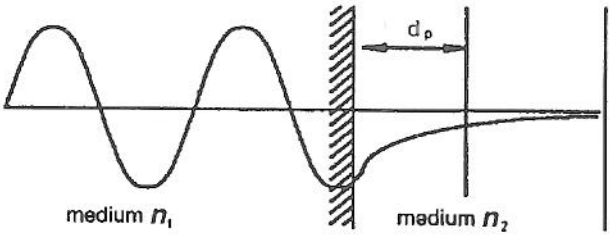

Fig. 4. Total internal reflection at a dielectric interface and generation of evanescence wave [from Ref. *4*]

The evanescent field can be enhanced by an order of magnitude by replacing the low refractive index medium with a thin layer (typically about 50 nm) of a metal, e.g. silver. Above a critical angle, all light is reflected except for a range at which the interfacial electron plasma undergoes surface plasmon resonance (SPR). The damping of the plasmons by the metal film at optical wavelengths results in a change of reflectivity at the SPR angle. A typical SPR device consists of a silver film deposited on a glass plate, prism, or optical fibre, and may potentially offer a sensitivity better than 5×10^{-2} degrees, although the science is providing difficult to implement. SPR has been applied to immunosensors *(27,28)* by depositing a thin metal film onto the immunosensor surface, resulting in an increase in the strength of the surface field.

Surface plasmon resonance has recently been used as the basis for signal transduction in biosensors. In a typical experimental set-up, incident light is reflected from the internal face of a prism in which the external face has been coated with a thin metal film (Fig. 5). At a critical angle, the intensity of the reflected light is lost to the creation of a resonant oscillation in the electrons at the surface of the metal film. Since the critical angle is dependant on the refractive index of material present on the metal surface, this method has been used to measure binding events such as the binding of antibodies to antigens immobilized at the sensor surface *(29)*.

The concept of integrated optics technology is analogous to that of integrated circuits, with the exception that photons – and not electrons – carry information. In-

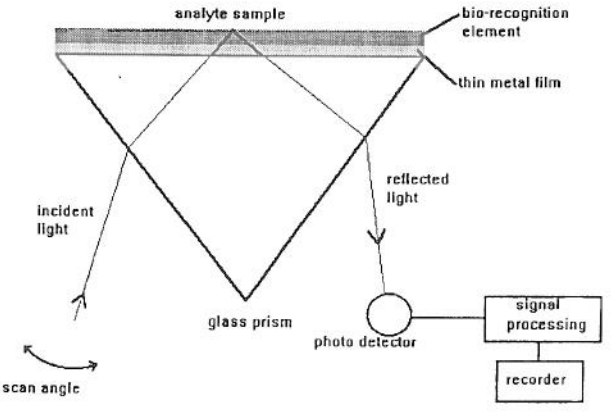

Fig. 5. Surface plasmon resonance (SPR) set-up [from Ref. *4*]

tegrated optics is based on the total internal reflectance properties of planar waveguide to produce two dimensional analogues of lenses, mirrors, diffraction gratings, etc. The main advantage of this technology is miniaturization, stability and raggedness of the optical structure, i.e. fewer set-up adjustments required, and the technique offers the promise of novel optical structures.

Heideman *et al. (30)* have used an experimental set-up wherein the light (incoming and outgoing) is coupled *via* etched gratings, and the complete set-up is fabricated on a silicon wafer using standard techniques. The changes in evanescent field due to immunoreactions are detected by an interferometer (Fig. 6). A sensitivity to chorionic gonadotropin of 25 nM was reported. This generic design is applicable to the construction of devices for other analytes as well.

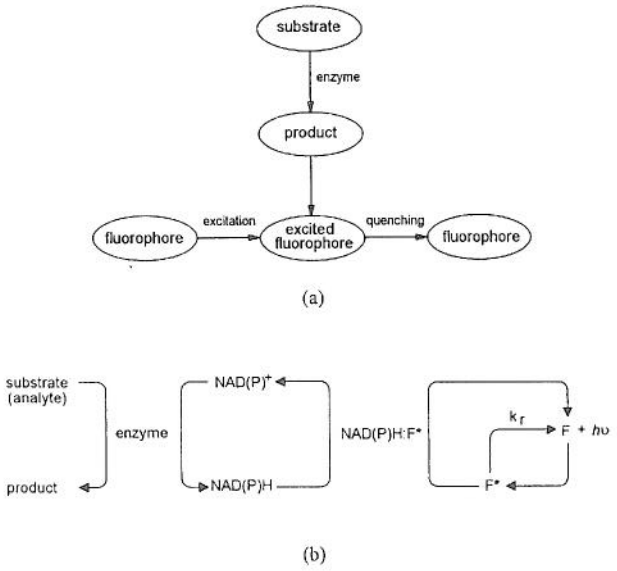

Fig. 6. Substrate induced quenching (SIQ) concept

Another interesting design of an integrated device for an immunochemical assay is that reported by Zhou *et al. (31)*. They constructed a differential measurement device using two wells in a buried monomode waveguide. One well contains the analyte specific immunochemical (immobilized), whilst the other well has an immobilized control immunochemical.

Acoustic

The linear relationship between the change in the mass at the crystal surface and the change in its oscillating frequency has been utilised in developing the acoustic devices as mass sensors. The vibrating piezoelectric crystal produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass. Therefore, the frequency of a crystal placed in an oscillating circuit, can be measured as a function of the mass. When the change in mass (Δm) is very small compared to the total mass of the crystal, the change in frequency (Δf) relates to Δm in the following expression:

$$\Delta f = Cf^2 \, (\Delta m / A)$$

where f is the vibrational frequency of the crystal in the circuit, A is the area of the electrode and C is a constant, determined in part by the crystal material and thickness.

Piezoelectric crystals, also known as quartz crystal microbalance (QCM), are typically made of quartz and operate at frequencies between 1 and 10 MHz. These devices offer a detection limit of mass bound to the electrode surface that is about 10^{-10} to 10^{-11} g in liquids, with a frequency discrimination limit of 0.1 Hz. Enzymes and antibodies based molecular devices for a number of analytes, including formaldehyde, cocaine and parathion, have been constructed using these transducers *(32)*.

Use of varying electrode configurations on the piezoelectric material results into generation of different types of electric waves. One such configuration results into surface acoustic wave (SAW) devices. Molecular devices based on SAW have been used to detect vapours which are absorbed by chemically selective coatings. One limitation of these devices is the excessive signal damping which prevents them from being used in liquids. These transducers can operate at higher frequencies (i.e. 250 MHz) than the QCMs and hence are capable of yielding higher sensitivities. Another type of piezoelectric transducer that is based on the generation of surface transverse wave (STW), and that has been recently reported operates at over 250 MHz in liquids *(33)*. This device type appears promising for development in clinical and environmental applications, since it is sensitive and stable, and is capable of functioning in liquid environments.

Miscellaneous

Development of a light addressable potentiometric sensor (LAPS) offers a novel transducer attractive for use in biosensors. In this device discrete locations on the silicon nitrite-coated sensor are made sensitive to pH, by illumination from an LED array. Various enzymes which produce or consume protons can be immobilized to a cellulose nitrate membrane which is pressed against the sensor, resulting in very small sampling volumes. Immunochemical schemes, using enzyme-probes, have also been demonstrated to measure such analytes as DNA (total and specific sequences), human chorionic gonadotropin, pathogenic bacteria, anticholinesterase drugs and pesticides *(20)*.

Recently, a novel concept involving use of a photochemical reaction between an indicator and reduced nicotinamide adenine dinucleotide (NADH) has been exploited for the development of biosensors *(34)*. One example of this concept has been demonstrated by Sharma and co-workers wherein excited thionine molecule has been employed as the indicator species. Several bioassays which have been successfully developed and incorporated into sensor formats include those for glucose, ethanol and glycerol, and pesticides. The important features of this sensing technique include possibilities of measurements in the desired region of the optical spectrum, continuous recycling of the NADH to NAD^+, improved sensitivities and use of low-cost solid state instrumentation.

Applications

Medical and Healthcare

Commercially available immobilized bioreagent based molecular devices in the medical and health care area include enzyme electrodes for the detection of glucose and lactate in blood and serum. These devices use enzyme electrodes *(7)* and are capable of continuous monitoring. Additionally, these devices are reliable, inexpensive to manufacture and demonstrate operational characteristics for simple and rapid clinical/diagnostics of target analytes. A number of other over-the-counter type disposable test devices are available in market. One of the most successful ones are those for glucose and for the pregnancy test. Biosensors appear well suited for other clinical diagnostic applications and detection of other clinically significant analytes, including i.e. metabolites, ions, drugs-therapeutics, pathogens, antibodies, hormones and blood proteins.

Considerable amount of effort has been directed towards the development of *in vivo* biosensor for continuous monitoring in intensive care medicine, surgery and life threatening situations *(35)*. The continuous monitoring of urea in dialyzate for use in artificial kidney during the hemodialysis procedure on patients with chronic kidney failure is another potential field of application. Another similar application is in the development of an artificial pancreas.

Undoubtedly, the development of biosensors for continuous and *in situ* operation is the greatest potential advantage over currently available diagnostic techniques. However, sensor fouling and clot formation pose major challenges in the development of implantable biosensors for long term monitoring.

Another potential area of biomolecular devices application is the semi-quantitative analysis of analytes in urine. Immunoassay tests for detection of a variety of clinical analytes have established themselves in the lucrative market of »over-the-counter« applications such as home pregnancy tests. In those cases where biosensors offer significant improvements or add new capabilities such as quantitative tests for lactate in blood or urea in urine (for athlete monitoring), these devices might find niche in this well developed market. Biomolecular devices that may also find market as part of portable (cart-top) clinical laboratory analysers continue to expand the range of clinical analytes which can be routinely measured.

Industrial Process Control

Immobilized biomolecular devices offer several potential advantages in industrial process control in the fermentation and biotechnology industries over conventional analytical methods, where a rapid turnaround on numerous measurements are required to maintain the system at maximum operating efficiency. Biosensor technology offers several potential advantages. Biosensor methods often require little or no sample preparation and can be used on-line (i.e. sample is removed and presented to the instrument in a continuous or discontinuous process) or in-line (i.e. the biosensor is placed in the flow stream). Although *in situ* biosensors may present a number of challenges in terms of sterilisation requirements to prevent product contamination, this configuration would have some distinct advantages in terms of system integration and automated feed-back control.

Because many industrial processes involve high temperature variations, vibration and harsh chemical environments, research efforts for development of process control devices have mainly been directed towards physical and chemical devices which are rugged, reliable and capable of automated calibration *(33)*. Of course, these processes are too rigorous for biological recognition elements. However, progress has been made towards solutions to many of these challenges, for example, by introducing biological reagents through controlled release polymers or specially designed fluidic systems *(36)*.

Environmental

With the exception of biosensors for biological oxygen demand (BOD), commercially available biosensors for environmental applications are virtually non-existent *(36)*. A number of research reports, however, have appeared in the literature, describing biosensors for detection of compounds such as phenolics, ammonia, chloroaromatics, benzo[a]pyrene and formaldehyde, as well as pesticides, including organophosphates and triazines *(37,38)*.

Opportunities for environmental field applications of biomolecular devices fall into two main areas: field screening and field monitoring. These areas differ primarily in their assay format requirements. Field screening applications typically require that single or composite samples be assayed at numerous locations for site characterisation purposes, whereas field monitoring requires that samples be continuously or semi-continuously analysed at fixed locations.

The environmental market (similar to the clinical market) is currently experiencing a proliferation of immunoassay test kits for detection of environmental pollutants. These kits are formated for single analysis-disposable use and have been targeted primarily towards field screening applications *(39)*. As a consequence, biosensors targeted for field screening applications must enter a very competitive market. Biosensors, however, can also be formated for continuous and *in situ* applications *(40)*. Although challenges concerning operational lifetimes, fouling and internal calibration have yet to be solved, biosensors developed for these applications may find a market niche occupied by few other competing technologies.

Food and Nutrition

Process and quality control procedures and requirements for the food industry are similar to those previously described for process industry in general, with the exception that the food industry must meet (in some cases) strict government requirements for the quality and composition of its products. Biosensors currently available for food analysis are exclusively of the enzyme electrode type and measure analytes such as glucose, lactate, ethanol, lactose, sucrose, galactose, methanol and starch *(7)*. Other biosensors with potential application to food analysis which have been demonstrated in the

laboratory include devices for detection of phenolics (in oils) and aspartame *(41)*.

Due to easily available backup from laboratory settings, most of the food analyses take place away from the process line. Hence, potential advantages for miniaturisation and portability do not seem to be as compelling for food industry applications. Nevertheless, due to the potentially high number of analyses required, the need for little or no sample preparation remains a distinct advantage for biosensor-based methods.

Miscellaneous

Immobilized bioreagents based devices are finding potential applications in the area of gas monitoring, warfare and other defence applications. The agriculture and marine sectors remain almost untouched and offer excellent opportunities for molecular devices. Among numerous applications, mapping of the movement of various ionic species and chemicals of interest such as hydrocarbons are attractive. Applications of these devices in the sedimentology and space sciences also remain underinvestigated.

Future Trends

With the recent and ongoing advances in biotechnology, transducer fabrication techniques and mass production methods have improved, and as a consequence, new biosensors for practical applications are bound to emerge. Further, due to their high specificity, sensitivity and miniaturisation potential, future applications for biosensors will most likely include their incorporation with separation techniques to form »hyphenated« methods. Compound or class specific detection could potentially improve chromatographic and electrophoretic methods.

Efforts will continue to improve the performance of enzyme based sensors, and mediators and indicators coupled with practical transducer designs will allow fabrication of improved enzyme-based sensors. On the biocompound side, chemical and genetic modification of enzymes offer exciting possibilities. Availability of antibodies which show high affinity and specificity for small molecular mass compounds, as well as being reversible while maintaining a high assay sensitivity, will create new horizons to develop biosensors. Another attractive and promising direction is to investigate the use of complex biological recognition elements, such as organelles, whole cells, tissues (plant and animal), etc. Use of direct chemoreception in biosensor design will allow a significant portion of the recognition, processing and transduction of signals to be accomplished by the biological system.

The electrochemical biosensors remain leaders to date, but increasing challenges from the other competing biosensor techniques such as optical, acoustic and calorimetric, are inevitable. The use of mass production technologies, for example, micro-fabrication of solid state devices, integrated electronics and optics, and deposition of biocompounds using techniques such as screen-printing and ink-jet printing will aid the commercialisation. Emerging trend is towards an all-integrated biomolecular device that is capable of performing separation, recognition and measurement at its surface thus avoiding any pretreatment of the complex samples. Another approach is to develop integrated devices that are capable of measuring a number of analytes. Coupled with the above techno-scientific advancements, there seems to be a need of rightly growing understanding of the commercial aspects of the immobilized biomolecular devices *(42)*.

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Molekularne (senzorske) naprave s imobiliziranim bioreagensima

Sažetak

Dan je pregled današnjeg stanja tehnologije koja se primjenjuje u razvoju i izvedbi molekularnih biosenzora. Opisana je znanstvena i tehnološka podloga funkcioniranja biosenzora navođenjem karakterističnih primjera. Raspravljena su potencijalna područja primjene biosenzora, kao što su medicinska i zdravstvena zaštita, vođenje industrijskih procesa, motrenje okoliša i kontrola kakvoće u prehrambenoj industriji i drugo.