Biosensors: Fundamentals, Applications and Trends*

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Abstract

Enzyme electrodes and optical immunosensors are at the leading edge of the biosensor field. The sensitivity of multienzyme electrodes is shifted to the subnanomolar concentration range. With monoenzyme sensors, several thousand samples per hour are measurable. Direct as well as enzyme-labelled immunosensors nowadays reach the sensitivity of immunoassays. The effect of non-specific binding is the biggest challenge for further improvement. Using multi-enzyme systems or intact cells, biologically related parameters, e.g., taste, odour, fatigue substances, mutagenicity, allergenicity and biological oxygen demand are quantifiable. Further progress is expected by applying tailor-made enzymes, antibodies and neuronal networks.

Introduction

Biosensors are in the forefront of current bioanalytical chemistry. They use immobilized biomacromolecules (e.g., enzymes, antibodies, receptors) and higher integrated systems (e.g., organelles, microbial cells, tissue sections) to recognize analyte molecules. Even though a definition by the IUPAC has not yet been established, the spatial unity of biomolecules with a signal transducer has been accepted to be the main feature of biosensors [1].

This introduction of biological recognition elements affects the performance of the total sensor as listed in Table 1.

TABLE 1. Special features of biosensors

Drawbacks	*Benefits*
Limited stability	chemical selectivity
Tedious preparation	provided by enzymes, receptors, antibodies (AB), DNA
	enzymatic sensitivity amplification: >1000/s
	AB affinity: $K = 10^4$–10^{12} M^{-1}
New concepts	
Synthetic analogues	
Protein engineering	

Appropriate Transducers and Immobilization of the Biocomponent

Chemical sensors (i.e., potentiometric, amperometric and impedimetric electrodes, various optical detectors using coloured indicators) as well as physical sensors (i.e., piezoelectric crystals, thermistors and reagent-free optical sensors) have been combined with appropriate biocomponents. Biospecific electrodes are at the leading edge of the biosensor field. This holds both for the number of measurable substances (almost 120 different analytes) and for the extent of routine application. Biospecific electrodes are expected to retain this leading position at least up to the end of the century, but with optical sensor systems being a serious contender [2].

Starting from biosensors based on conventional transducers, e.g., Clark-type oxygen electrodes, in the second sensor generation microsensors are used. The goals of miniaturizing biosensors are the reduction of the required sample volume, the multicomponent

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TABLE 2. Chip biosensors

Analyte	Enzymes	Transducer
Glucose	glucose oxidase (GOD) (+lactonase)	pH FET, TFME[a]
	GOD + peroxidase (POD)	pF^- FET
Maltose	glucoamylase + GOD + POD	pF^- FET
Lactate	lactate oxidase (LOD)	TFME
	LOD + POD	pF^- FET
Urea	urease	pH FET
		NH_3 MOSFET
Lipids	lipase	pH FET
Peptides	trypsin	pH FET
ATP	ATPase	pH FET
Acetylcholine	cholinesterase	pH FET
Creatinine	creatininase	NH_3 MOSFET
NADH	hydrogenase	H_2 MOSFET

[a]Thin-film metal electrode.

analysis of complex chemical substances by multiple microsensors, and cost reduction by mass production. Three basic types of microsensors have been used (Table 2):

(i) ion-sensitive field-effect transistors (ISFETs);

(ii) gas-sensitive metal–oxide–semiconductor (MOS) capacitors;

(iii) thin-film electrodes.

Recently, thermistors, integrated optical sensors and surface acoustic devices have also been included in this development [3, 4].

These sensors are fabricated by microelectronic production technology, such as thick- and thin-film deposition, photolithographic reduction and chemical and plasma etching, which permit well-delineated patterning of metallic, insulating and semiconducting surface layers to take place. Highly uniform, geometrically well-defined and identical metallic or other surface areas can be produced. In this way arrays of identical or different sensors can be produced, thus enhancing the reliability, repeatability and versatility of the sensor.

Most problems arise from the manufacturing technique for the formation of the immobilized biomembrane.

Using glow discharge in combination with the mask technique, plasma polymers can be deposited on the active sensor region. In a second procedure the enzymes or antibodies are covalently bound via bifunctional reagents [5]. Furthermore, layers of polymers, e.g., polypyrrole or polyaniline, are deposited on conducting areas by electropolymerization. The biomolecules are either entrapped in the polymer matrix during the layer formation [6] or coupled to the layer via typical chemical reactions [7]. Structuring of the uniformly deposited biocomponent-containing layer is also possible when photodeactivation of 'passive' regions is performed. For this purpose, illumination with UV light is applied [8]. Alternatively, enzymes are deposited only at the sensitive region by using an enzyme solution in a negative photoresist. In this manner ISFET biosensors for glucose, urea and lipids have been developed. When installing such enzyme FETs in a flow-through cell, which ensures both isolation and electric connection of the conductive fields to the measuring device, they can be used without any polymeric encapsulation and wire bonding.

Functional Stability

The protein nature of the biocomponents is the reason for a low functional stability of

biosensors as compared with that of the basic chemical or physical transducers. Continuous deactivation of the biomolecules may also occur during storage of the sensor.

Enzymes become inactivated during the course of their reaction. In addition to thermal denaturation, two kinds of operational inactivation can be distinguished: (i) Autoinactivation is due to transient reactive intermediates generated during catalysis. In this respect, activated species of oxygen are plausible candidates to react with sensitive parts of the enzyme molecules. (ii) The interaction of the enzyme with final reaction products may also lead to a decrease in activity. For glucose oxidase (GOD) sensors, inactivation by H_2O_2 has been reported, which is linked to the reaction with the reduced prosthetic group of the enzyme. Especially at elevated temperature, e.g., in *in vivo* application, the glucose oxidase activity restricts the lifetime to about 10 days [9]. Destruction of the reactive H_2O_2 by a second enzyme (catalase) resulted in a lifetime for an implanted glucose sensor of 105 days [10].

For enzyme electrodes, comparison of literature data is sometimes difficult because the experimental conditions employed to establish the biosensor stability vary over a wide range. The working stability, expressed as the remaining sensitivity after intermittent use, for various enzyme sensors using the enzymes entrapped in gelatine is surveyed in ref. 11.

The values demonstrate that peroxide-forming oxidases immobilized in a gelatine matrix are applicable at least for a period of 10–30 days. On the other hand, the lifetime of bienzyme electrodes containing an oxidase and another enzyme, e.g., glucoamylase, mutarotase or laccase, is of the order of 10 days. Obviously peroxide deactivates the coimmobilized enzyme more rapidly than the oxidase. Also, for tyrosinase a relatively low stability is found. In this enzyme reaction the formation of polymeric products leads to a rapid deactivation. An excellent stability is obtained for the lactate monooxygenase (LMO) electrode and even for the LMO/lactate dehydrogenase bienzyme sensor. This behaviour is probably related to the absence, or short lifetime, of reactive intermediates and products in the enzyme reactions.

TABLE 3. Substrate amplification systems

Analyte	Enzymes	Amplification factor
Lactate/Pyruvate	LOD/LDH[a]	48 000
	Cyt.b_2/LDH	15
Glucose	GOD/GDH	10
Glutamate	GPT/GLDH	60
	GLOD/GLDH	20
ADP/ATP	PK/HK	200
H_2Q/BQ	Lacc./Cyt.b_2	500

[a]Abbreviations: LOD, lactate oxidase; LDH, lactate dehydrogenase; Cyt.b_2, cytochrome b_2; GOD, glucose oxidase; GDH, glucose-dehydrogenase; GPT, glutamate pyruvate transaminase; GLDH, glutamate dehydrogenase; GLOD, glutamate oxidase; PK, pyruvate kinase; HK, hexokinase; Lacc., laccase, H_2Q, hydroquinone; BQ, benzoquinone.

The entrapment of enzymes in gelatine is a mild procedure which does not disturb the enzyme molecules. On the other hand, covalent fixation or crosslinking with bifunctional reagents (e.g., glutaraldehyde or cyanuric chloride) is accompanied by a drastic loss of enzyme activity; however, the remaining activity may be stable for a long period of application. Fixation of GOD by placing an acetonic solution of polyurethane containing the suspended enzyme particles on a thin-film metal electrode or on the pH-sensitive gate region of ISFETs results in a stability of more than 300 or 40 days, respectively [12]. The difference between both values may be explained by disturbances of the pH sensor function by the organic solvent during the enzyme fixation.

The enzyme loading to a major extent determines the stability of an enzyme sensor. An enzyme reserve is built up by employing more enzyme activity in front of the probe than the minimum required to achieve complete analyte conversion. Following this principle, several thousand blood samples can be determined by one membrane containing

GOD in a polyurethane layer [11]. As long as this reserve lasts, the sensitivity will remain essentially constant. This is, however, only significant for sensors for substrate determination. If inhibitors of the biocatalytic reactions are to be measured, kinetic control is desired. For this purpose the application of higher integrated biocatalytic systems, e.g., organelles or tissue slices, containing the enzyme in an environment optimized by evolution is most effective [13].

Unlike enzymes, antibodies do not catalyse the conversion of the analyte but remove it from the solution by complex formation. The gross molecular structure of antibodies is fairly constant, whereas differences in a small section of the molecule are responsible for the specific antigen binding. The splitting of the antigen–antibody complex requires extreme conditions, such as washing with solution of pH 2, if reuse of the immobilized immunoglobulin is desired. In this splitting process structural changes may occur which change both the affinity and specificity of the antibody. Therefore the applicability of immunosensors is restricted at maximum to several hundred samples [14].

Chemical Selectivity

The high selectivity of biosensors is well recognized in the literature. The prerequisites for this specific behaviour are: (i) a high specificity of the enzyme or the antibody; (ii) the interference-free indication of the reaction product as schematically represented in Fig. 1.

With regard to selectivity, nature offers two groups of enzymes as analytical reagents: (i) group-specific enzymes converting a family of analogous substances; (ii) enzymes possessing high chemical selectivity that permits them to discriminate even between stereoisomeric compounds, i.e., L- and D-amino acids.

Monoclonal antibodies, which recognize only a given surface area of the analyte, generally possess higher specificity than polyclonal antibodies, which are composed of an ensemble of different protein bodies.

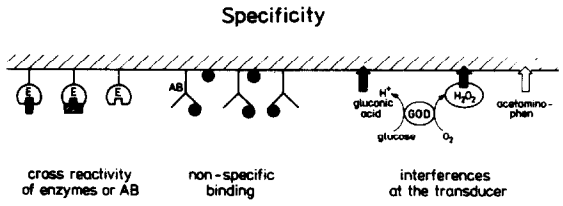

Fig. 1. Effects decreasing the specificity of biosensors.

Glucose oxidase is highly selective for β-D-glucose. Of the eight aldo-D-hexoses, only D-mannose and D-galactose are oxidized by GOD, but at extremely low rates. GOD is therefore well suited for application in biosensors for glucose assay.

On the other hand, L-amino acid oxidase (AAOD) catalyses the oxidation of a broad spectrum of substrates. L-leucine, L-valine and L-methionine are converted with comparable rates, whereas L-glycine and L-serine are 'poor' substrates. Therefore an AAOD electrode exhibits different sensitivities toward the respective amino acids. Similarly, the three L-lactate-converting enzymes, lactate monooxygenase (LMO), lactate oxidase (LOD) and cytochrome b_2, exhibit activity toward substances homologous to their natural substrate. Therefore all three enzyme electrodes respond to lactate, 2-hydroxybutyrate, malate and glyoxylate [11]. In amperometric indication, all substances with a conversion potential lower than the electrode potential contribute to the overall current. At the potentials usually applied for anodic H_2O_2 indication, an equimolar concentration of paracetamol causes between 30 and 300% of the glucose signal. This contribution may only be eliminated by decreasing the electrode potential to 300 mV [15].

Artificial electron acceptors with favourable enzyme kinetics and fast heterogeneous electron exchange improve the specificity and extend the linear range. Degani and Heller [16] bound the mediator to the enzyme protein in order to make it function as an electron transfer relay between the prosthetic group and the redox electrode. We modified

this approach by using benzoquinone (BQ), a common reactant of GOD and peroxidase (POD), to couple both enzymes [17]. Under the action of POD the H_2O_2 formed in the GOD reaction gives rise to a cathodic current at 0 V, a potential which is even lower than that for mediators or relays of the ferrocene type.

With all biosensors using physical transducers, non-specific binding of sample constituents is one of the major problems [18]. In order to suppress such disturbances, the sensor surface may be covered with hydrophilic material which exhibits a low interfacial energy.

Detectable Amount of Analyte

The minimum detectable amount is determined by the following parameters:

(i) sample volume;

(ii) affinity of the biocomponent for the analyte;

(iii) sensitivity of the transducer to the reaction effect indicated;

(iv) amplification of the chemical or electrical signal.

The sample volume necessary for quantitative determination has been decreased by several orders of magnitude during the last five years. Simon *et al.* [19] reported ion-selective electrodes which can be applied in a sample volume of 10 nl, thus measuring in the range of 10 ions. This device may also be applied in combination with biosensors. Keeping the sample volume as low as possible is also important for implanted sensor devices. Kissinger [20] indicated drugs or metabolites in the brain of animals in the femtogram region by using a microdialyser. Surface plasmon resonance (SPR) and piezo or surface acoustic crystals are examples of direct methods possessing an excellent sensitivity [21]. For antigens of sufficient size ($>100\,000$ D), such as immunoglobulins, the direct binding of the antigen to the immobilized antibody can be measured by SPR in the nanomolar range.

Evans [22] presented an immunosensor using SPR for thyroxine on the basis of a displacement assay working in the range 5 to 500 nM. A considerable increase of sensitivity is gained when a particle of large size and refractive index is attached to the immunobody being displaced. In a model assay, IgG has been measured in the range 10^{-9}–10^{-10} M. For DNA hybridization of probes consisting of 50 bases, as little as 1–10 attomoles have been indicated. The limit of detection for piezoelectric crystals is estimated to be about 10^{-12} g. This high sensitivity is characteristic of the transducer, whereas the selectivity is developed by the coating, using an antibody on the surface of the crystal as an adsorbent. Guilbault [18] has been able to indicate ppt amounts of cocaine in the gas phase. The author claims that relative humidity tolerance and immunochemical activity are retained for up to 13 days.

Similar sensitivities have been achieved with immunosensors based on fluorescence-labelled antibodies [21]. The disposable immunosensor of a capillary refill device consists of two glass slides separated by a capillary space of 100 μm. The lower plate functions as a waveguide and carries antibodies covalently fixed to its upper surfaces. Fluorescence-labelled antigens or, in the sandwich type, a second antibody, are retained on the lower surface of the upper plate in a soluble matrix. They are released into the solution upon addition of the sample and compete for the immobilized antibody sites or bind to the captured antigen on the waveguide. Owing to the very small diffusion distances, equilibrium data are obtained within 10 min. The emissions from the free and bound fluorophores can be separated because the latter couples into the guide via the evanescent field, whereas the solution fluorescence couples into the guide by refraction at angles below the critical angle. Therefore, free and bound fluorophores are separated without the need for washing or separation steps. The feasibility of this concept has been demonstrated for the determination of *Rubella* antibody in serum and

human chorionic gonadotrophin. A sensitivity of 10^{-11} M has been reached by using this technique.

With bare amperometric electrodes the lower limit of detection of low-molecular-weight substrates is around 10 nM. The introduction of the enzyme-containing matrix in front of the electrode decreases the sensitivity by one to two orders of magnitude due to the additional diffusion resistance (Fig. 2). The highest sensitivity is reached when large enzyme activity within a thin layer is used, and effective external mass transfer is provided. Under these conditions, substrate measurements can be managed down to 1 μM concentrations with imprecisions below 2%. This sensitivity is sufficient for the determination of metabolites like glucose, lactate and uric acid, using 5–20 μl samples in highly diluted solutions.

Recently the accurate determination of choline down to 10 nM has been performed using a membrane electrode in an optimized flow injection analysis (FIA) device [23]. The coefficient of variation for aqueous standard solutions was around 4%. The excellent sensitivity can be exploited for the determination of enzyme activities, e.g., in the inhibition of choline esterase or in enzyme immunosensors. In both cases the signal generation is carried out after the sample interacts with the sensor. Therefore no interfering substances have to be taken into consideration.

The determination of metabolites occurring in the lower micromolar concentration range, e.g., bilirubin, pyruvate or creatinine,

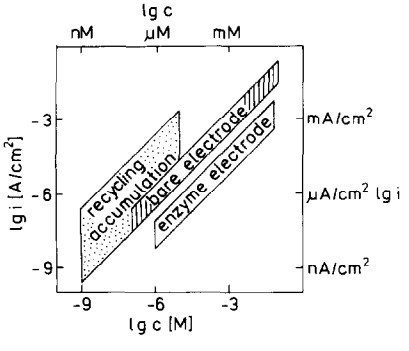

Fig. 2. Sensitivity of amperometric enzyme electrodes.

raises many problems because the electrochemical contribution of interfering substances exceeds the analyte signal. The sensitivity of enzyme electrodes can be enhanced by using substrate amplification. Operational conditions have to be adjusted in such a way that one enzyme catalyses the regeneration of the substrate of the second enzyme. This has been achieved by coupling the respective oxidase and dehydrogenase, and also by using kinases (Table 3). In this way, the limit of detection for lactate, pyruvate and hydroquinone has been shifted down to 1–10 nM. However, the application of these recycling systems to real samples is restricted by their sensitivity to both substrates of the enzymatic cycle, e.g., to lactate and pyruvate. Therefore the determination of pyruvate in plasma by this method could require removal of the endogeneous lactate first.

On the other hand, this type of amplification is very useful when only one cycling partner is present in the sample. This is true for glucose, since gluconolactone is not contained in physiological media. Furthermore, the sensitivity of enzyme immunosensors may be considerably increased by enzymatic recycling, too. Using a marker enzyme which generates the substance to be cycled with a turnover number of 1000/s, in a volume of 1 μl as few as 1000 enzyme molecules can be indicated after a one-minute preincubation. This value illustrates the potentials of enzymatic analyte recycling.

Enzymes are not only useful for the conversion of metabolites into measurable substances (the principle of enzyme electrodes) but they are also powerful labels for binding assays. Due to its nature as a biocatalyst, one enzyme molecule is able to generate typically 1000 molecules of product per second from the respective substrate.

Measuring Time

At present the maximum sample throughput of commercial enzyme-electrode-based

analysers is about 100 samples per hour. An electrode composed of a hydrogen peroxide sensing probe and GOD immobilized in a gelatine layer 200 μm thick sandwiched between two dialysis membranes reaches 95% of the steady-state current about 2 min after sample injection. However, the measuring time can be diminished to 60 s per sample by employing the kinetic measuring principle. Application of an analogous glucose electrode containing the enzyme in a polyurethane membrane with a characteristic diffusion time of 24 s and an enzyme loading factor of more than 100 (internal diffusion control) results in a response time for the steady-state current of 10–15 s both in a stirred measuring cell and in a FIA manifold. Therefore a sample frequency of 80 per hour can be realized in the steady-state mode. Reproducible peak-shaped signals are also obtained when the injected volume is not sufficient to reach the steady state. In this way the measuring time at only 1% carry over is diminished to 12 s by reducing the injected sample volume to 1.5 μl. Thus, a sample throughput as large as 300 per hour is possible [24].

The FIA system has been applied to the measurement of lactate. 200 lactate samples per hour can be analysed with good precision and negligible carry over. Such a high sample frequency can be achieved even with double-membrane type sensors. When the enzyme is fixed in polyurethane directly on the surface of a plane platinum electrode, a fast-responding glucose sensor is obtained. After addition of glucose the steady-state current is reached within 1–5 s. Using adsorption of GOD at a polymer-covered electrode containing redox active groups, the steady-state current is reached within less than 1 s [25].

Recently, further progress in increasing the measuring frequency of enzyme electrodes has been achieved. Wang reported that several thousand aqueous glucose samples have been processed per hour [26]. In these experiments a composite graphite–epoxy electrode containing the biological entity was integrated within the injector valve of an FIA device. For physiological solutions, however, a decreased measuring speed is anticipated, as may be deduced from experiments with film metal GOD electrodes.

Biologically Related Analytes

Combination of several enzymes or exploitation of metabolic chains within intact microorganisms offers a unique possibility to quantify biologically related parameters. Established examples are the measurement of freshness of meat or fish using four enzyme electrodes for hypoxanthine, inosine, isosine-5-phosphate and adenosine-5-phosphate [27], a bacterial mutagenicity sensor [28] and a microbial sensor for the 'biological oxygen demand' (BOD) [29]. Recently, new approaches resulted in the development of sensor systems for allergenic, fatigue and taste substances. The taste of consommé soup has been characterized by measuring the content of inosine monophosphate, L-glutamate, L-lactate and glucose, and the sum of volatile substances. Interestingly, the multiple regression analysis of these values gave a good correlation to the organoleptic value determined by experienced panellers [30].

Determination of fatigue substances in human sweat has been studied by non-invasively applied sensors of an arm-band type [31]. As a result, no relation between physical exercise and the concentration of Na^+, Cl^-, glucose and uric acid was observed. However, the concentration of lactate and NH_4^+ in sweat was remarkably increased after hard exercise. These results are very promising for a possible biocontrol of car drivers.

The release of histamine from IgE-sensitized rat basophilic leukaemia cells upon the addition of allergenic substances has been detected by a NH_4^+ electrode combined with diamine oxidase [32]. Egg white protein was used as a model allergen which was indicated with a response time of 9 min. Thus, a considerable reduction of the exposure time as compared with conventional tests is observed.

Analyser Concepts

Depending on the concept of sample pretreatment, in the analytical device different operational steps must be covered by the biosensor. This is schematically shown in Fig. 3.

The measurement of discrete samples may be carried out either in the laboratory using an analyser or in decentralized diagnostics, e.g., in the doctor's office or by the patient himself or herself applying a pocket device. Enzyme-electrode-based laboratory analysers for the determination of blood glucose, lactate or uric acid predominantly use stirred measuring cells. This is the case with the YSI model 23 (Yellow Springs Instrument Co., U.S.A.), Gluco 20 (Fujii Electric, Japan), GKM 01 (Academy of Sciences of the G.D.R.) and Exan 6 (Biochemical Institute Vilnius, Lithuania) analysers. Continuous flow with an air segment between carrier and sample is applied in the ECA 20/21 of PGW (Dresden, Germany), in the Glucoroder (AIC, Japan) and Diagluca (Toyobo, Japan). This principle is particularly well suited for diluted samples of blood or serum [33]. On the other hand, FIA is subject to disturbances if samples of changing viscosity or non-Newtonian fluids, such as blood, are applied [15]. However, in combination with dialysis or liquid chromatography, on-line analysis by FIA is an appropriate approach for process control and *in vivo* measurements [20].

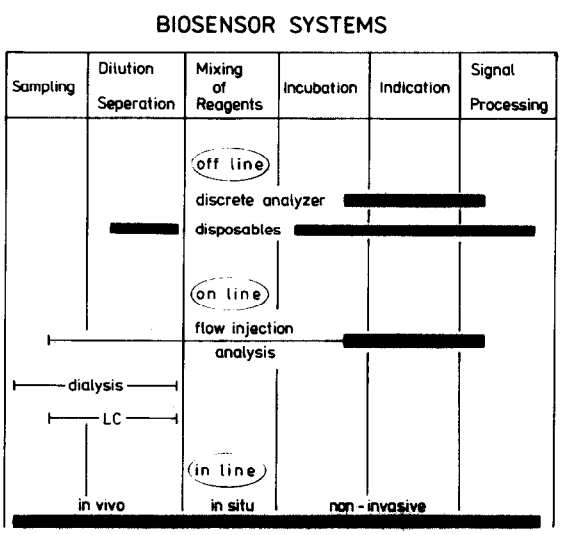

Fig. 3. Concepts of biosensor-based analysers.

The discrete measurement of undiluted samples has been realized by two principles. First, the one-use glucose electrode, e.g., ExacTech from Baxter (U.K.) [34] is based on the GOD–ferrocene system, thereby realizing an appropriate linear measuring range. In contrast, the pocket blood glucose analysers from Markwell [35] and from the Diabetes Institute, Karlsburg (Germany) use GOD membranes in combination with peroxide detecting electrodes. The appropriate enzyme membranes can be repeatedly used within one month.

Direct measurement of undiluted samples is also required for implantable biosensors, *in situ* application in reactors, and non-invasive measurements. Here, the requirements concerning the measuring range and the mechanical and biological stability are extreme. Very promising approaches are non-invasive measurements at the surface of the skin. In this manner, alcohol [36] and lactate [31] have been quantified, the latter correlating with the fatigue of the patient.

Trends

Although progress in biosensor development is expected to originate from contributions from both micromechanics and biotechnology, the latter will be more innovative. At present, the following developments can be envisaged to revolutionize the performance of biosensors:

The main analytical parameters, i.e., specificity, selectivity, and functional stability, will be improved by using chemically modified and genetically engineered enzymes. The direct communication between the biocomponent and the electronics will lead to substantial advancement. In this respect, direct electron transfer from redox enzymes to electrodes, or coupling of neuronal networks, will result in optimized signal transfer. The ability of neurons to convert binding events

into frequency-coded digital signals offers microprocessor-compatible responses. Furthermore, the high efficiency of neuronal receptors promises an extreme sensitivity to transmitters, e.g., amino acids or various pesticides.

Since the maximum signal is obtained when the transition moment of the absorbing particle is parallel with the exciting vector, it is advantageous to achieve a particular orientation of the biomolecules at the surface of the transducer. For immobilization of antibodies, the binding to a prefixed layer of protein A or complement C is effective to form a uniform orientation with the antigen binding fragment to the solution side. For interacting enzymes, 'site-to-site' fixation of the active sites results in a channelling of intermediates. Thus the overall reaction rate is accelerated, which is equivalent to a higher signal.

In addition to the new analytical potentials offered by monoclonal antibodies, the modification of the antibody molecule will open up new horizons. This is true for bispecific antibodies which react with two different species, catalytic antibodies, which are a functional hybrid of enzymes and immunoglobulins, and the application of genetically engineered fragments, which could result in a decreased effect of non-specific binding.

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