BIOTEC 2

Biosensors and Environmental Biotechnology

Edited by
C. P. Hollenberg and H. Sahm

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Foreword

Biological detection systems surpass in sensitivity and specificity most physical and chemical measuring devices. The sensitivity for certain stimuli as exhibited by the butterfly or the nose of a dog or even our eyes are startling. No wonder that such properties have been used in the past and have recently triggered the development of biosensors especially in many large companies in the USA. This development could culminate in the biochip. The task is difficult, but progress is already significant.

Biological waste water treatment is the largest biotechnological process currently in use. There is a tendency to abandon gradually the sludge-producing aerobic process in favour of the anaerobic treatment which produces much less sludge and moreover biogas as an additional benefit. The composting of solid organic waste could become the second largest biotechnological process. The city of Dresden for example has been composting the entire domestic waste of some 300,000 inhabitants for about 20 years. Biofilters for some odorous organic substances have been in satisfactory use for many years. A problem which remains largely unsolved is however the removal of toxic solvents from industrial waste air. This represents a challenge for microbiologists to produce special cultures which can also degrade these xenobiotic compounds. The development of heavy-metal-adsorbing microbial biomass opens up a new area in biotechnology with an extremely broad spectrum of potential applications; biosorbents can detect and absorb metals even at very low concentrations. Last not least the rehabilitation of dump sites and contaminated soils is certainly a prominent task. Methods range from the treatment of hazardous wastes in specially designed bioreactors to the in-situ-treatment, i.e. treatment by injecting into the soil microbes which are capable of degrading the contaminating substances.

In a series of up to date contributions BIOTEC 87 has experts discussing the current topics in biosensors and environmental biotechnology and speculating on future developments. Fifteen articles of leading specialists cover a broad application area. Devices for control of food quality, detection of explosives or drugs, cancer diagnostics as well as a number of more classical systems are discussed with regard to feasibility and future. Interesting biotechnological applications with respect to various waste water treatment systems, purification of waste air and decomposition of solid waste are presented. Exciting is the prospect of degrading toxic compounds in the environment by microorganisms tuned in the laboratory.

This book is written for the most part by biochemists, biochemical engineers, geneticists, and microbiologists. The chapters present an interdisciplinary synthesis of data which seem to us to have both theoretical and applied significance to anyone who is concerned with biotechnology.

We wish to thank the Nowea Düsseldorf for their generous support of this

meeting and all those who contributed to the success and who have helped in the production of this book. Our editorial task has been greatly eased by all the colleagues who supplemented their manuscripts with the most recent data making this book an up to date report.

Spring 1988

C.P. Hollenberg, Düsseldorf
H. Sahm, Jülich

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Development and Applications of Amperometric Biosensors

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Introduction

A biosensor is an analytical device in which biological material, capable of specific chemical recognition, is in intimate contact with a physico-chemical transducer to give an electrical signal. Most of the examples discussed in this contribution fall within this definition but others concern some novel approaches to sensing biological activity. Why is biosensor research important (1, 2)? The fact that we still use dogs to detect explosives and fish to monitor water quality emphasizes our technological inadequacy when there is a need to monitor specific chemicals on-line in the environment. Similarily, in our process industries, we have sophisticated electronic systems which allow us to control processes, control production, control planning, but we lack the sensors to supply the on-line information into these systems; hence a strong driving force for biosensor research. We are extremely dependent for analysis on sophisticated, complex, modern, analytical equipment and highly trained technicians. Dependence on specialist analytical laboratories is often unsatisfactory, especially where the information is required urgently, for example in a medical emergency.

The ultimate aim of a biosensor researcher must be to develop simple analytical devices which exhibit the properties of being reliable, specific, rapid, cheap, compact and user seductive. Of course, at this time there are very few such devices on the market, but they are beginning to appear. For example, a simple, pocket, pen-type biosensor has recently been developed from some technology which was jointly invented by workers at Cranfield Institute of Technology and Oxford University (3). This concerns new methods of connecting enzymes to electrodes using insoluble, low potential electrochemical mediators and it has enabled the company, Genetics International, to produce such a device, initially for the rapid determination of glucose on fresh blood primarily for diabetic self-monitoring. It contains memory functions and liquid crystal display (4). Its main advantages are rapidity, accuracy and simplicity of use. Such a system could not have existed prior to recent electrochemical biosensor research, because the nature of the connection between the enzyme and electrode permits reproducible manufacture of cheap, disposable electrodes that function rapidly on complex materials such as whole blood, even in the absence of oxygen.

Why are we resorting to biology to enable us to develop these new analytical devices? The reason is that biology offers a level of sophistication in the areas of

specificity, affinity, catalytic conversion and selective transport that we are, in general, currently unable to achieve by chemical methods.

Whilst there are considerable prospects for the application of biosensors in many areas, the driving force for the development of novel biosensor technology has come so far primarily from the clinical direction. There are a number of reasons for this, for example, the ready acceptance of disposables in medicine means that operational longevity may not be important and the diagnostic market is very large and rapidly expanding. Nevertheless, biosensors are finding applications in other areas, such as food and agriculture, fermentation and process control, environmental, military and security. Indeed, the largest existing commercial activity in biosensors is currently military, particularly in the form of nerve gas sensors.

The world analytical market is presently in the region of $20 billion per year. Of course with the introduction of more user-friendly, less technician dependent technology, this market is likely to expand over the next 10–20 years. These developments are largely dependent on current scientific advances in, for example, bioelectrochemistry and opto-electronics.

Development of Amperometric Enzyme Sensors

Conceptually, all biosensors have the same basic configuration comprising three components (1) – the biological element, which may be anything from an enzyme to an organelle; the transducer, which may be an electrode, a field effect transistor, optical device, a piezo-electric crystal or thermistor; finally, the transducer generates an electrical signal which is then processed, interpreted and displayed by the electronic component.

There are some specific requirements for a realistic device as opposed to an experimental curiosity. For example, the stability of the device must be appropriate for the particular application. There are two aspects to this, one is stability in use, the other is stability in storage. For many medical applications, for example, good, long-term storage stability is important, but functional stability is of little importance provided that a single test, disposable sensor is entirely dependable. Clearly, the sensor component should be non-toxic if it is for use in medicine or food testing. It may be required to work in non-aqueous systems for some applications. For simplicity, it is preferable that the sensor mechanism does not involve activators or cofactors. Finally, for a number of applications such as in vivo sensors for medicine or sensors for fermentation control, insensitivity to oxygen concentration may be important.

Biosensor development can be conceived as occurring in basically three generations. First generation devices in the form of more or less complex instruments such as laboratory glucose analysers have been available for some years. These systems are comprised of four elements; a dialyser, a receptor, a transducer and the electronic system. In second generation devices, for example, the pen device discussed above, there is a more intimate physico-chemical connection between the biological component and the transducer and the dialyser may be omitted. Finally, third generation sensors entail a fully integrated system with the biology, the transducer and the electronics associated intimately together in a microelectronic device, the «biochip».

Taking food industry applications as an example, the first generation devices allow us to analyse sugars, starch, lactate, glycerol, and alcohol. These expensive instruments, which are based on enzyme electrodes, have limitations in that they are off-line, require sample preprocessing, are slow, require a skilled operator, are labour intensive, involve high capital expense, and are applicable to only a limited range of analytes (5).

Many of the limitations of first generation sensors arise from the indirect link between the biological element and the transducer. Most commercially-successful biosensors to date invole the detection of substrates or products of oxidoreductase enzyme reactions using amperometric electrodes. In order to achieve improved, second generation electrochemical biosensors it is necessary to achieve some more direct link between the electronics and the enzyme redox centre. A substantial proportion of the biosensor research of the Cranfield Centre has been directed towards achieving close links between the electronic activities in biological elements and various transducers. Glucose sensing is an especially important example, currently being the largest market for analysis of one compound in the bio-medical area. The established method of measuring glucose using a biosensor configuration is to use the enzyme glucose oxidase, which converts glucose to gluconolactone and hydrogen peroxide, which in turn is detected at a high potential platinum electrode.

Much of our research on glucose sensors and some other biosensors has concerned developing systems in which electrons are transferred from the active site of the enzyme to some kind of electrode directly, thereby eliminating the peroxide intermediate step. It is possible to effect this process by direct electrochemistry at chemically-modified electrodes (promoted system) or using chemical mediators in solution or retained on or within the electrode (6). We have found the most successful approach to practical biosensors is the use of a few special mediator compounds, for example the organometallic substance, ferrocene, and some derivatives thereof. These compounds will replace oxygen, the natural acceptor for oxidase enzymes and transfer electrons to a carbon electrode, allowing the construction of the type of sensor described briefly above for glucose, which has excellent performance characteristics for practical use (3, 7). One particular advantage of these mediated sensors is that the electrodes have a much lower operating potential than classical amperometric electrodes based on hydrogen peroxide detection; this minimises interference from other electrochemically active substances in a sample.

These ferrocene glucose sensors have now been configured in many ways and have been extensively tested and proved for clinical use (7). They are also being developed for *in vivo* application where the non-toxicity of ferrocene is an important factor. It has been shown that the technology is applicable to an extensive range of enzymes and analytes, including pyridine nucleotide-linked systems (8) and also to amperometric ELISA-type immunoassay (9). Multi-enzyme ferrocene sensors have been developed, for example, a sensor employing both glucose oxidase and hexokinase can be used to measure creatine kinase activities in plasma (6).

More recently, we have been investigating alternative mediators and find that tetrathiafulvalene (TTF) in similar carbon electrode configurations is also an effective mediator (10). This compound has been used by other workers as a component of conducting salts which may also be employed as electron acceptors in enzyme electrode configurations (8).

Development of Amperometric Affinity Sensors

There are a variety of different approaches to the exploitation of the principles of mediated amperometric biosensors discussed above to ones incorporating antibodies or specific, single-stranded DNA sequences as the recognition elements. In the simplest case, it is possible to modify existing colourimetric enzyme-linked immunosorbent assay (ELISA) procedures to yield a current directly, by replacing the colour-forming reaction by a mediated, electrochemical enzyme link. This simple step alone offers the attractions of simpler, cheaper instrumentation and improved dynamic range.

It is also possible to devise an electrochemical immunoassay for a drug such as morphine, for example, by making a drug-conjugate with ferrocene (9). After use in a competitive immunoassay, it can then be detected eletrochemically, with or without enzyme amplification.

Now, most of the labels currently used for ELISA are not redox enzymes, but it is nevertheless possible to make these simple, cheap electrochemical sensors using enzymes such as alkaline phosphatase. In association with IQ Bio Ltd., we have developed a highly sensitive, broad range electrochemical immunoassay based on this enzyme. Phosphatase-labelled antibody acts on NADP to yield NAD. Electron generation and amplification is achieved using a combination of alcohol dehydrogenase and diaphorase. The final step involves electron transfer to a poised potential electrode via ferricyanide. This gives substantially improved performance compared to the alternative colourimetric system and requires far simpler instrumentation (11). Another approach involves use of ferrocene derivatives of phenylphosphate. Upon removal of the phosphate group by the phosphatase enzyme there is a substantial change in the cyclic voltamogram of the product compared to the substrate. This effect can be used as the basis for a simple, sensitive amperometric immunoassay (12).

Applications of Amperometric Biosensors to Process Monitoring and Control

Much conventional process monitoring involves sampling followed by laboratory analysis. Usually, an off-line analytical loop, probe, or ideally non-invasive system, would be preferable. We have developed a microprocessor-controlled, off-line, multiple ferrocene glucose sensor system for the control of laboratory fermenters (13). More recently, we have designed in collaboration with workers at Porton, a stainless steel membrane probe which will accommodate biosensors within a fermenter. This can be sterilized inside the fermenter prior to inserting a multiple, ferrocene glucose biosensor that can be automatically recalibrated *in situ* (13).

Another process application, again using ferrocene or TTF glucose sensors, concerns meat freshness testing. The profile of glucose concentration beneath the surface of a carcass is determined largely by the microflora density on the surface. We have demonstrated that this profile can be conveniently and rapidly determined using a multiple glucose sensor (14).

Rapid Amperometric Microbial Number Monitor

Most measurement of microbial numbers is done by slow Pasteur plating methods although some more rapid procedures have recently become available. These newer methods also have considerable disadvantages, such as high capital cost and being too slow for many applications. We have therefore developed a small, cheap, prototype device which will give a reasonably accurate assessment of microbial numbers in a wide range of samples such as process waters, milk and cutting fluids. Again we have used electrochemical methods (15, 16). The market for such a device could be considerable view of the fact that there are currently about a billion measurements per annum of microbial numbers in Europe alone. Our method involves the «short-circuiting» of the electrochemical activity of microorganisms using a poised potential electrode system and a proprietary cocktail of chemical mediators. The prototype has been developed in collaboration with De La Pena Biotechnology Ltd. and is called «Biocheck». A measurement is obtained within less than two minutes, the lower limit of sensitivity being about 100000 organisms although there are prospects for improving this, for example using a rapid pre-concentration step.

Amperometric Biosensors for Environmental Applications

A demonstration herbicide monitor to test the status of input waters into water treatment plants has recently been developed in collaboration with workers at Luton College of Higher Education. This employs microalgae immobilised on alumina or cellulose acetate, closely apposed to a poised potential electrode. Essentially the same principle to that of the microbial number sensor above is employed. The sensor is contained in a flow-cell and irradiated by a light emitting diode. Mediators are used to «short» the photoelectronic activity to an electrode poised at an appropriate potential. Herbicide contaminants inhibit the photorespiration, thereby reducing the current output of the sensor. The sensors are fairly stable (usable for up to one week), reusable and extremely sensitive (down to a few parts per billion) (17).

Concluding Remarks

This contribution briefly summarises some of our recent work on novel amperometric biosensors. Currently, most practical biosensors that have been on the market for some years, and most of those about to enter the market are electrochemical, but that is not to say that other approaches, particularly optical ones, will not eventually prove equally commercially promising.

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Biosensors Based on Thermistors and Semiconductors

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Introduction

Various biosensor concepts based on the combination of a biocatalyst held in close contact with a suitable transducer are attracting a considerable interest in bioanalysis. Today, there is a trend towards continuous processes in biotechnology and this increases the demand for suitable sensors for monitoring and control. Simple methods for continuous or rapid semicontinuous detection of hormones or other products of genetically engineered microorganisms have become increasingly important. The inherently high specificity of biosensors is important for detection of a certain compound present in crude fermentation broths or directly in the blood stream.

In many laboratories around the world attempts are made to develop implantable sensors, for instance for diabetes control. Special interest has been focussed on solid state sensors, such as FETs (field effect transistors) because of their small size, cheap production methods, possibilities to be integrated with electronics for signal evaluation and transmission and potential multifunctional capability.

Our work in this field mainly involves the development and applications of thermal biosensors, the enzyme thermistor (ET) (1) and «enzyme transistors» based on special semiconductors of MOS-type (2, 3). The ET is a simple flow calorimeter primarily intended for rapid metabolite assays, but it can be used as a simple, general biocalorimeter as well. The reaction heat in a small column containing immobilized enzyme or cells is measured as a temperature change of the effluent of the column. The ET assay can easily be automated and is well suited for monitoring and control of biotechnological processes due to its high operational stability. For monitoring of larger molecules than enzyme substrates, a thermometric enzyme immunoassay (TELISA) has been developed and automated.

Hydrogen- and ammonia-sensitive semiconductors of PdMOS- or IrMOS-type can be combined with enzymes or cells into highly sensitive biosensors. A common feature of these sensors is that the detection is made in gas phase, while the reaction is carried out in aqueous phase, which facilitates work with crude samples.