UDC 681.586.74:574.633
ISSN 1330-9862

review

Highly Sensitive Electrochemical Biosensors for Water Monitoring

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Received: November 21, 1995
Accepted: April 12, 1996

Summary

Environmental monitoring and prevention of pollution are becoming increasingly important especially in respect to media, e.g. water, coming into direct contact with living organisms. Many of these monitoring requirements can be accomplished by making use of biosensors. In comparison with classical analytical methods, biosensor devices play an increasing role, because they offer the possibility of rapid, highly sensitive and cost-effective measurements. Biosensors are based on a biological component which reacts with the analyte, e.g. an enzyme or antibody molecule, and a transducer that generates a signal. This report focuses on immunosensors, enzyme sensors and microbial sensors with electrochemical detection which offer the possibility both of single-analyte measurements and the estimation of sum-values.

Keywords: water monitoring, electrochemical biosensors, immuno-, enzyme- and microbial sensors

Introduction

The increasing pollution of the environment is a problem of enormous importance. This is particularly true for toxic compounds and substances with a potential human health risk and, consequently, environmental monitoring and the prevention of further pollution is becoming increasingly important. What is true for the environment in general is especially true for water. Many rivers are used as both, providers of raw water resources, and receivers of waste water. As the quality of ground water and surface water as well has direct effect on the quality of the drinking water much effort is spent on the development of methods for field monitoring of samples at certain locations such as ground water wells or aquifers.

Many of these monitoring requirements today can be accomplished by using biosensor technology. These kinds of sensors are based on a biological component which reacts with the analyte, e.g. an enzyme or antibody molecule, and a transducer that converts the biochemical signal into an electrical signal which then is electronically processed.

Among the transducers applied for water monitoring often optical biosensor techniques are used for signal generation. Minunni and Mascini *(1)* developed an atrazine sensor for water monitoring based on the commercially available BIAcore device (BIAcore, Pharmacia Biosensors, Sweden). This system uses surface plasmon resonance spectroscopy for signal generation as the interaction of an immobilized biomolecule with its ligand results in a change of the refractive index on the surface of the transducer. Reflectometric interference spectroscopy (RIFS) was used by Lang *et al. (2)* as optical transduction principle for the determination of low molecular weight analytes in water. The surface of the sensor chip was modified with a dextrane layer, carrying the pesticide (simazine or atrazine). In a competitive assay format the pesticides on the chip compete with the corresponding pesticide in the sample for the binding to the antibodies. The amount of antibodies bound to the sensor surface was therefore inversely proportional to the amount of analyte in the sample. Atrazine and simazine could be determined with a detection limit below 0.1 μg/mL, the limit set by the European Union.

Besides optical transducers solid phase sensor techniques have also been applied in biosensors for water monitoring. Using piezoelectric crystals Guilbault *et al. (3)* developed an atrazine assay. Whereas up to now optical transducers have mainly been used in combination with antibodies, electrochemical transducers offer the opportunity to be combined with a variety of biological components. Out of the electrochemical techniques, amperometry and potentiometry have successfully been employed for the detection of relevant analytes. With

this in mind we focus in this report on immunosensors, enzyme sensors and microbial sensors that make use of these electrochemical detection and transduction principles.

Electrochemical Immunosensors

One of the major advantages of immunosensors is the versatility of these devices. As the specificity of the sensor is almost entirely governed by the antibody molecule the system can be easily adapted to another analyte just by changing the corresponding antibody. By selecting an antibody molecule with almost no crossreactivities towards structurally similar compounds the sensor can be optimized for highly selective single-compound measurements. If a polyclonal antibody is applied as the recognition element, however, the sensor can be tailored for the estimation of an overall sum-value below or above a certain threshold concentration. Such a sensor would be the ideal tool for screening purposes. Due to these reasons immunoassays are widely applied for measurements of pesticide residues in food and crops, as screening methods for contaminants in field samples and for water monitoring *(4)*.

A very attractive technique for automated water monitoring is the flow-injection analysis (FIA) in combination with biosensors as the recognition element. As biosensors show the potential for continuous operation these devices are particularly well suited for this task *(5)*. The major advantages of these systems are a short contact time between the analyte and the biosensor, a high sample rate and small sample volume, often without any sample pre-treatment. The immuno-FIA-system described here was developed for the determination of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), a weed killer widely applied in agriculture. In European Union countries the maximum admissible concentration of 2,4-D is 0.1 μg/L.

The FIA-system consists of a sampler, a selector, a peristaltic pump, the immunoreactor, an amperometric detector and a potentiostat (Fig. 1). The whole system is computer-controlled and almost entirely automated. The most important part in the FIA-arrangement is the immunoreactor, where the biological detection of the herbicide takes place. The reactor itself consists of a glassy capillary usually used in gas chromatography. On the surface of the reactor the hapten 2,4-D was covalently

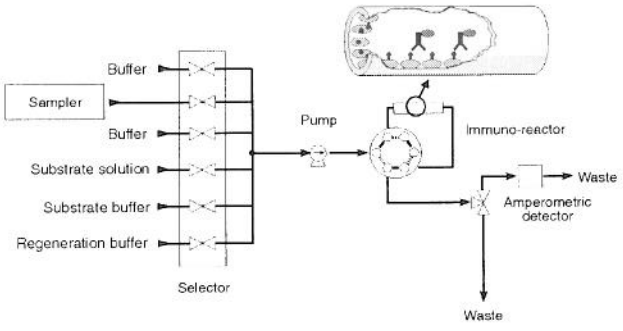

Fig. 1. Schematic set-up of amperometric immuno-FIA arrangement

immobilized via poly-(L-lysine). Several other immobilization procedures were investigated, however, best reproducibility and stability of the sensor was achieved by using poly-(L-lysine).

The detection principle is depicted in Fig. 2. For 2,4-D determination a sequential titration scheme was chosen. First, 2 mL of the sample were mixed with 500 μL of a so-called »cocktail«, containing the monoclonal anti-2,4-D

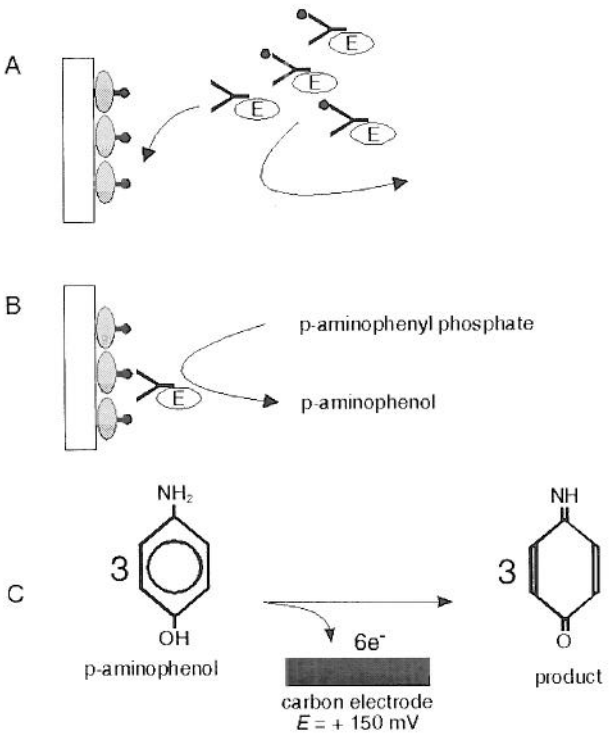

Fig. 2. Detection principle. A, Application of antibody-enzyme-conjugate/analyte cocktail; B, enzymatic conversion of the substrate; C, electrochemical detection of p-aminophenol; D, regeneration of the sensor

antibody labelled with alkaline phosphatase, a concentrated buffer system and additional ions and salts. Due to the addition of this cocktail each sample is preconditioned and differences in ionic strength or pH-value do not effect the subsequent measurement.

When this mixture is applied to the immunoreactor only antibodies that have not previously bound to 2,4-D can bind to the hapten on the reactor surface and unbound species of the antibody-enzyme-conjugate are washed away. Consequently, the amount of bound antibodies is inversely proportional to the amount of analyte in the sample. In 1988 Tang *et al. (6)* proposed *p*-aminophenol phosphate as a substrate for the electrochemical determination of alkaline phosphatase activity. Enzymatically generated *p*-aminophenol can be easily oxidized at an electrode at low potentials of about +100 mV. The substrate has a lot of advantages in electrochemical assays, i.e. the low potential to avoid interferences and

the lack of electropolymerization, however, it is not commercially available and therefore was synthesized according to DeRiemer and Meares *(7)*. In the FIA-system *p*-aminophenol was detected at a carbon working electrode at +150 mV *vs.* Ag/AgCl.

Before the measurement of another sample the immunoreactor must be regenerated after signal generation. A decrease in the pH-value is most commonly used to weaken the specific antibody-antigen binding and to remove bound immuno-complexes. In the present immuno-FIA-system a glycine/HCl buffer system with a pH = 2.7 had the best regenerating properties among several buffers tested, nevertheless, the lifetime of the immunoreactor was limited to about 30 regeneration cycles. The time required for one measurement was 12 min, including all washing steps, electrochemical signal generation and regeneration of the immunoreactor.

A calibration curve for 2,4-D determination is shown in Fig. 3. Although the lowest detectable 2,4-D concentration is 0.1 μg/L, the maximum admissible concentration, present work focuses on the improvement of the detection limit. In comparison with enzyme-linked immunosorbent assay (ELISA) measurements *(8)* the sensitivity in the concentration range between 0.1 and 1 μg/L is rather low, however, as the ELISA uses the same immunoreagents and a similar assay format, it is likely that the detection limit of the sensor can be further improved. Krämer and Schmid *(9)*, e.g., developed a FIA-system for triazine determination in water. The sensitivity of the sensor was equivalent to that of the ELISA. Although the ELISA in general is more sensitive than the corresponding sensor, the ELISA principle is time-consuming, labour-intensive and too slow for process control or on-line monitoring.

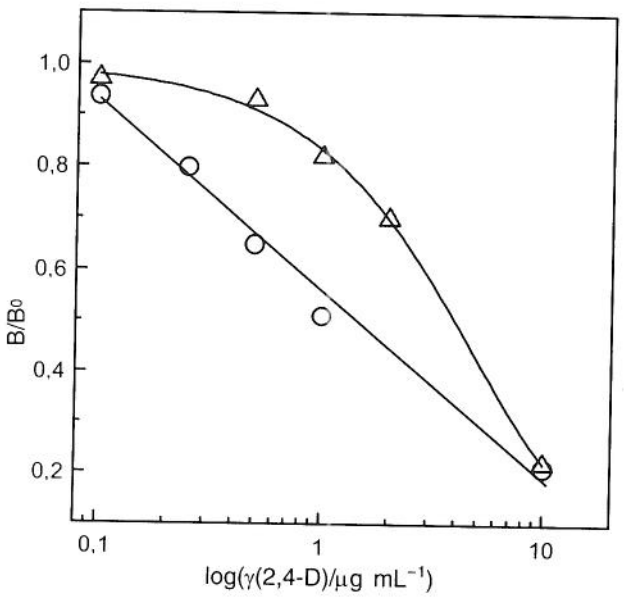

Fig. 3. Calibration curve for 2,4-D determination. Circles: ELISA; triangles: immunosensor.

The low detection limits of immuno-FIA-systems are even more impressive when taking into consideration that small sample volumes are required and that these methods work without any sample pre-treatment. Our 2,4-D-FIA-system requires a sample volume of 2 mL only. In contrast to this very simple and rapid method, HPLC measurements require sample volumes of 2 L with subsequent 2,4-D pre-concentration, using e.g. solid-phase extraction methods.

First experiments with spiked water samples and various samples from rivers indicate an excellent correlation between the sensor data and concentrations determined with HPLC, used as a standard routine method for 2,4-D determination. Further efforts are made to implement automatization of the FIA-system and to apply it, e.g. for on-line monitoring of water supply stations.

Electrochemical Enzyme Sensors

Two specific recognition principles in sensor formats may be used to develop electrochemical enzyme sensors for water monitoring. Firstly, inhibitory effects of hazardous compounds on enzyme activity can sensitively be detected using kinetically controlled enzyme sensors. Secondly, direct enzymatic conversion of water pollutants can be employed using a diffusion limited enzyme sensor.

Enzyme inhibition can be investigated by comparing the sensor signal prior to and after exposure to an analyte containing sample. Butyryl- and acetylcholinesterases derived from different sources have proven to be well suited for the detection of organophosphorus and carbamate pesticides. Low and well defined amounts of enzyme are applied for inhibition experiments so that sensor signals are kinetically controlled to allow sensitive detection of a change in enzyme activity.

Activity of cholinesterases has been determined using different approaches based on the reactions 1a, 2 and 1b.

$$\text{choline ester} \rightarrow \text{choline} + \text{organic acid} \qquad /1a/$$

$$\text{choline} + 2O_2 + H_2O \rightarrow \text{betaine} + 2H_2O_2 \qquad /2/$$

$$\text{thiocholine ester} \rightarrow \text{thiocholine} + \text{organic acid} \qquad /1b/$$

Reaction 1a depicts the hydrolytic cleavage of choline esters by cholinesterase which have been detected by two types of potentiometric sensors: the resulting pH-shift was detected by Durand *et al. (10)* with a flat glass pH electrode and by Colapicchioni *et al. (11)* with an ion-selective field-effect transistor (ISFET). Imato and Ishibashi *(12)* used a potentiometric ionselective electrode for the detection of butyrylcholine employing a PVC membrane modified by a cation exchanger. Reaction 2 may be used to couple reaction 1a to any system sensitive for hydrogen peroxide. Reaction 1a and 2 may be carried out separately in two steps applying soluble cholinesterase *(12–14)* or cholinesterase separately immobilized on e.g. a Nylon net *(15)* for the enzyme inhibition reaction and in a subsequent step an amperometric choline oxidase biosensor. Wollenberger *et al. (16)* described a kinetically controlled amperometric bienzyme sensor comprising coimmobilized cholinesterase and choline oxidase. Reaction 1b alone is needed for specific pesticide determination when thiocholine esters are used as substrates. Screen printed platinum electrodes have been used for the determination of thiocholine at a po-

tential of +410 mV *vs.* Ag/AgI *(16)* and screen printed tetracyanoquinodimethane (TCNQ) modified graphite electrodes at a potential of +150 mV *vs.* Ag/AgCl *(17)*.

If irreversible inhibition took place, enzyme inhibitor complexes could not be hydrolyzed and cholinesterase must be replaced *(18)*. Taking advantage of this fact, the inhibition process can be monitored either by flow injection analysis systems for continuous sampling or by disposable sensors for point of testing measurements. A FIA system with an automatically exchangeable immobilized enzyme reactor has been described *(19)*. Single-use sensors have been fabricated by screen printing *(14,16,17,19)*.

Sensitive detection of analytes important for environmental monitoring can be accomplished by enzymatic conversion involving cyclic regeneration of one of the intermediate reaction products. Phenols, whose maximum admissible concentration in drinking water should not exceed 0.5 μg/L, and inorganic phosphate which mainly contributes to eutrophication of rivers and lakes readily serve as model analytes.

Tyrosinase catalyzes two different reactions: the hydroxylation of phenols to *o*-phenols and their subsequent oxidation to *o*-quinones. Not only phenol may serve as a substrate but also various substituted phenols are converted by tyrosinase. Therefore amperometric tyrosinase sensors provide a sum parameter for detection of phenols called »phenol index« as proposed elsewhere *(20, 21)*. Amperometric sensing is based on the reduction of the quinoid products of the tyrosinase catalyzed reactions *(22)* leading to regeneration of *o*-phenol. To increase catalytic selectivity reduction potentials applied should be low enough to avoid falsification of the sensor signal by simultaneous detection of oxygen consumption. Thus, modification of the electrode surface has been investigated. Önnerfjord *et al. (23)* used tyrosinase graphite-epoxy biocomposite materials as working electrodes and found an optimal detection potential of –100 mV *vs.* Ag/AgCl and reported a detection limit of 1 μM phenol. Much more sensitive sensors may be obtained by employing mediated recyclization of enzymatically formed *o*-quinones and efficient reduction of the mediator at the working electrode according to Fig. 4. Kulys and Schmid *(24)* immobilized tyrosinase onto graphite electrodes modified with TCNQ and applied potentials up to +180 mV *vs.* Ag/AgCl with a detection limit of 230 nM phenol. A detection limit as low as 25 nM phenol has been described by Kotte *et al. (25)* using methylphenazonium-modified polymer thickfilm sensors. For the development of miniaturized sensor systems for water monitoring miniaturized enzyme sensors fabricated in silicon technology are favoured. Hence, spatially controllable immobilization procedures like electropolymerization are desirable. The next step is covalent binding of a mediator to a monomer suitable for enzyme entrapment by electropolymerization *(26)* to obtain miniaturized, long term stable phenol detecting enzyme sensors.

Enzyme sensors for phosphate determination have been realized by immobilizing different enzyme sequences onto amperometric oxygen and hydrogen peroxide sensors. One of the phosphate detecting enzyme sequences was established by the coimmobilization of nucleoside phosphorylase and xanthine oxidase. In the presence of phosphate nucleoside phosphorylase catalyzes the cleavage of inosine to ribose-1-phosphate and hypoxanthine. The later can be detected by oxidation catalyzed by xanthine oxidase to give hydrogen peroxide and uric acid which both are amperometrically detectable at a Pt-electrode at a potential of +650 mV *vs.* Ag/AgCl. Keeping the level of the cosubstrate inosine constant, the current increase is directly proportional to the phosphate concentration added *(27)*. Signal amplification has been achieved by coimmobilization of a third, a phosphate recycling enzyme, the alkaline phosphatase. Wollenberger *et al. (28)* described a 20-fold signal increase on addition of alkaline phosphatase resulting in a detection limit of 25 nM phosphate when using an oxygen sensor as transducer. In our lab the patented *(29)* four-enzyme sequence shown in Fig. 5 was used for even more sensitive phosphate determination. Phosphate is recycled by phosphatase as in the above mentioned enzyme sensor. Maltose phosphorylase was purified in-house from *Lactobacillus brevis (30)*. The concentration of the cosubstrate maltose is kept constant during phosphate measurements. Due to this amplification system we found a detection limit for phosphate of 10 nM *(31)*. Thus, it has been demonstrated that enzyme sensors can be used for evaluation of toxic effects as well as for highly sensitive single-analyte measurements.

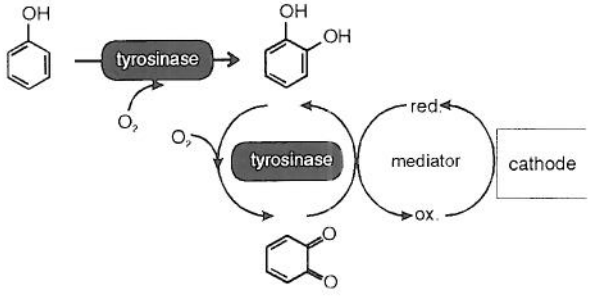

Fig. 4. Principle of the phenol sensor coupling tyrosinase and mediator to regenerate intermediate reaction products and to generate sensor signal

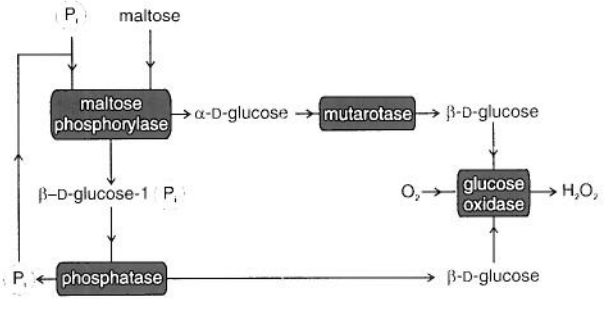

Fig. 5. Four-enzyme sequence for the detection of inorganic phosphate involving signal amplification by recycling of phosphate and by enhancing β-D-glucose concentration for signal generation

Microbial Sensors

In general, microbial sensors are based on a combination of immobilized whole cells with a physical transducer. The choice of the transducer depends on the metabolic properties of the microorganisms.

The transducer which is applied in most of the cases, is the amperometric oxygen electrode. About 80% of all described microbial sensors are based on the increasing respiration in the presence of organic components. Under aerobic conditions oxygen is consumed while substrates are transported into the cells and subsequently assimilated. The immobilized cells are fixed behind a dialysis membrane in front of a Clark oxygen electrode, which is covered by a gas permeable membrane. In absence of a substrate the cells are in a steady state representing endogeneous respiration. In presence of a substrate the microorganisms immediately consume additional oxygen. Thus, a decrease in current can be observed which is proportional to substrate, respectively analyte, concentration.

Microbial sensors exhibit some principal characteristic features, which in certain applications offer advantages in comparison to enzyme sensors: (i) Microbial sensors often show an increased stability, because they use highly integrated systems under quasi-physiological conditions. (ii) They are easy to prepare as enzyme extraction and purification steps are not necessary. (iii) They are able to catalyze complex reaction sequences and because of that the addition of cofactors is not required. (iv) The multireceptor behaviour of intact cells enables microbial sensors to recognize groups of compounds yielding complex parameter signals, however, at the cost of less selectivity.

Microbial sensors for the determination of toxic compounds applicable for an environmental water monitoring were developed for example for the determination of chlorophenols *(32)*, benzene *(33)* and naphthalene *(34)*. The sensor with most widespread application in controlling waste water pollution is the one for the detection of biochemical oxygen demand (BOD). The BOD is a widely used parameter that indicates the content of biodegradable organic compounds in waste water. The conventional BOD_5 method takes five days and is therefore not practicable for monitoring and process control of waste water. A more rapid method is the estimation of the BOD by using a microbial sensor that contains immobilized whole cells in front of an oxygen electrode *(35,36)*. Meanwhile four BOD sensor systems are commercially available on the market, three of them produced by German companies and one by a Japanese.

A general problem of all commercial BOD sensors consists in measuring samples containing heavy metal ions which are known to cause inhibitory or toxic effects on microorganisms. Commercial BOD sensor analyzers using normal microorganisms are significantly influenced by these ions present in some contaminated waste water. To avoid heavy metal interference in the BOD estimation we used for our measurements *Alcaligenes eutrophus* KT02 *(37)* which was isolated from a sewage plant near Göttingen in Germany *(38)*. This organism carries plasmids encoding resistance to 40 mM $NiCl_2$, 20 mM $CoCl_2$, 10 mM $ZnCl_2$ and 1 mM $CdCl_2$ *(39)*.

The cells were immobilized via entrapment in a poly(urethane)hydrogel *(40)*. All measurements have been carried out at 31 °C with the microbial sensor integrated in a flow through system (BSB-Modul, PGW Medingen, Dresden, Germany). Air saturated buffer or sample is sprayed directly onto the capillary pore membrane (Fig. 6). The analyte solution has contact with the sensor for 30 s, whereas the sensor shows a response time of 80 s allowing a sample throughput of 7.5 per hour.

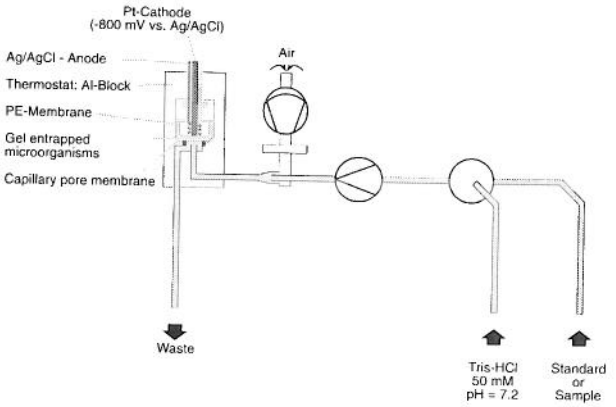

Fig. 6. Diagram of the flow through system of the microbial sensor system

To study the influence of heavy metal ions the microbial sensor based on *A. eutrophus* KT02 was fitted into the BSB-Modul and repeatedly exposed for 30 s first to the standard solution of 2 mg/L glycerol in order to get a standard response. Afterwards, the sensor was exposed in the same way to a standard of equal concentration but containing additionally 4 mM heavy metal ions. After this treatment the recovery of the sensor in absence of heavy metal ions was examined. The normalized sensor responses against process time is shown in Fig. 7.

In absence of heavy metal ions the sensor was stable. Nickel was tolerated at 4 mM over 10 h with only about 5% loss in signal intensity. Copper and zinc were also tolerated at 4 mM over the same time, with regard to zinc an irreversible loss of 30% in sensor activity was found. Measurements with a sample containing 4 mM cadmium showed a stable sensor signal for about four hours. After that the sensor suffered an increasing inhi-

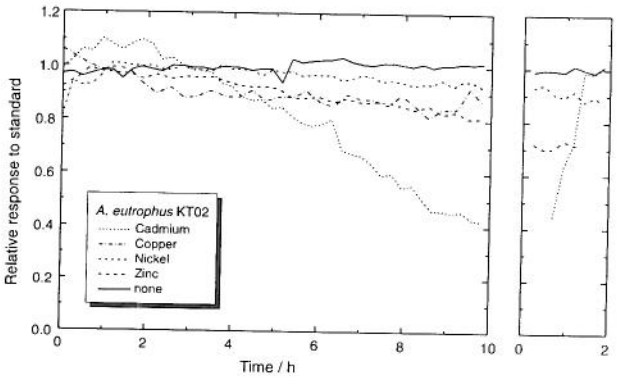

Fig. 7. Influence of heavy metal ions on sensor performance using *A. eutrophus* KT02. Left graph: treatment with 4 mM heavy metal ions; right graph: recovery without heavy metal ions

bition of up to 60%, which was fully reversible within 90 min after contact with cadmium had stopped.

The same experiments were done with *A. eutrophus* H16, a strain which does not contain any heavy metal resistances, in order to show the influence of such ions on sensors containing normal cells. Nickel immediately decreased the sensor response by about 35%, this inhibition was found to be largely reversible. Cadmium, copper and zinc showed a nearly complete inhibition after only three measurements – that means after 90 s contact with the samples. The inhibition was not reversible on the part of cadmium and zinc. After 90 min treatment with 4 mM copper, however, the sensor was damaged to 30% of the starting response.

The sensor containing immobilized *Alcaligenes eutrophus* KT02 had an operational lifetime of more than four weeks. After 30 days 60% of the sensor response were preserved.

The result of our experiments clearly demonstrates the need to use heavy metal resistant strains while measuring the BOD of waste water contaminated by high concentrations of heavy metal ions.

Conclusions

In the environmental field a strong desire for rapid and reliable analytical methods has evolved. As water is the most important foodstuff, the maitenance of its quality is essential for human health. Water monitoring either by on-line measurements or by frequent sampling is thus required. Among the analytical systems which are feasible for this purpose biosensors may be the devices of choice, because they offer rapid, highly sensitive and cost-effective measurements.

One of the major advantages of biosensors is the high degree of versatility. As the specificity of the sensor is almost entirely governed by the biological component, the sensor can easily be adapted to another analyte just by changing the biological recognition element. Biosensors are suitable for both single-analyte measurements and the estimation of complex parameters, e.g. overall sum-values or groups of components. If a single-analyte measurement is the ultimate aim enzyme sensors or immunosensors with specific monoclonal antibodies are best suited, for the estimation of a sum-value, however, sensors based on polyclonal antisera or microbial sensors have proven to be superior.

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Vrlo osjetljivi elektrokemijski biosenzori za nadzor voda

Sažetak

Kontrola okoliša i sprječavanje onečišćenja postaju sve važniji, osobito za vodu koja dolazi u izravni doticaj sa živim organizmima. Vode mnogih rijeka upotrebljavaju se istodobno kao sirovina u procesu i kao odlagalište za prihvat raznih onečišćenja. Djelotvoran nadzor stupnja onečišćenja voda može se postići primjenom biosenzora. U usporedbi s klasičnim analitičkim postupcima, biosenzorski uređaji imaju sve veće značenje jer omogućavaju brza, vrlo osjetljiva i jeftina mjerenja. Biosenzori se zasnivaju na biološkom sastojku koji reagira s nekim agensom, npr. enzimom ili molekulom antitijela, a povezani su s transduktorom koji proizvodi signal. Ovaj je prikaz usredotočen na imunosenzore, enzimske i mikrobne senzore s elektrokemijskom detekcijom koji omogućavaju mjerenje pojedinačnih agensa i procjenu ukupnih vrijednosti.