Environmental biosensor technology

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1. Introduction

The increasing endangering of the environment and the grown environmental awareness of the society leads to a new responsibility in the field of analysis. The environment consists of the three media water, air and soil. Water, air and soil pollution are interdependent and pollution in one media generally affect pollution in the other two media depending on the pollutant in question (1). Conventional analytical techniques, although highly precise, suffer from the disadvantages of high cost, the need of trained personnel and the fact that they are mostly laboratory bound (1). Biosensors present distinct advantages in certain applications, due to their good selectivity, high sensitivity, fast response times, reasonable price, easy handling and portability. They can be described as an analytical device using biological recognition components associated with a physicochemical transducer. As recognition elements enzymes, antibodies, biological receptors, microorganisms, organelles or nucleic acids can be used. The principal transducers used are electrochemical, optical, thermometric, piezoelectric or acoustic devices.

An increasing number of papers concerning the application of biosensors to environmental control has been published over the last few years. These biosensors are developed for a fairly broad range of analytes, including for example pesticides (2,3), phenol (4,5), heavy metals (6), polluting gases (7) and formaldehyde (8). This area was recently reviewed by Rogers (9) and by Dennison and Turner (1). Up to now most of the biosensor developments are in the state of laboratory model or sometimes of prototype. Only a few have already been commercialized, for example analysers for the biochemical oxygen demand (BOD). Nevertheless these can still be developed further to multifunctional on-line and handy monitors.

As biosensors combine biology and engineering, there is a need for interdisciplinarity to develop them. Teamwork in the fields of analytical chemistry,

biochemistry, physics, microsystem-technology and computer sciences should lead to biosensor developments up to prototypes and eventually to commercialization. Such an approach is taken in our Institute and in the following some newest developments will be presented.

2. Enzyme sensors

2.1. Amperometric multi-enzyme sensor for the determination of inorganic phosphate

The role of phosphate in eutrophication of lakes, as well as the influence of excess intake of phosphate in food products upon human health is well known. Thus, knowledge of inorganic phosphate concentration is especially important in the field of environmental and food analysis. Classical phosphate determinations are complicated and time consuming, whereas enzyme sensors should offer a rapid and simple alternative taking advantage of direct measuring the analyte substrate in the sample. Recently we proposed such a sensor for the determination of inorganic phosphate (10).

The enzyme sequence used in this determination is shown in Fig. 1. The combination of maltose phosphorylase and acid phosphatase generates two glucose molecules per reaction cycle and recycles one molecule of phosphate. Finally, the oxidation of glucose is catalysed by muratorase and glucose oxidase. In essence the two amplification steps in this sequence dramatically lower the detection limit. The four enzymes were coimmobilized onto a re-

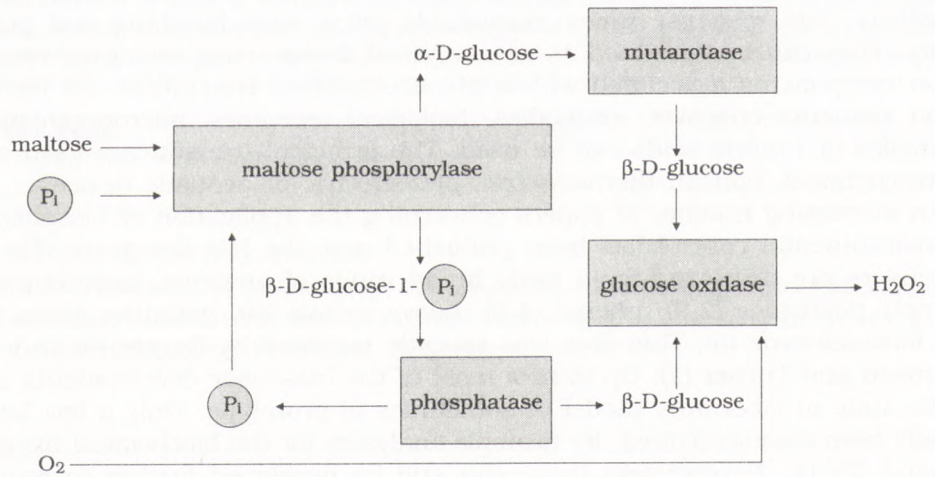

Fig. 1. Enzyme sequence for the detection of inorganic phosphate.

generated cellulose membrane which was mounted on the tip of a platinum electrode for the amperometric detection of enzymatically formed hydrogen peroxide. A detection limit of 10^{-8} M was obtained and the sensor response was linear in the range of 0.1-1 μM, which is relevant for the monitoring of water pollution (Fig. 2).

The measuring device could be improved by using a flow injection analysis system (FIA), thus reducing the drift which results from the glucose accumulation in the batch analysis. The adoption of such a system will be necessary in any case for the determination of phosphate in natural or polluted water. Due to the high phosphate concentration usually found in most European waters (0.05 — 0.1 mM) on the one hand and the sensor's good sensitivity on the other hand, potential inhibitors like metal ions or competitive enzyme substrates can be diluted to minimal concentrations. For monitoring waste water, interfering concentrations of glucose or hydrogen peroxide can be removed by arranging cartridges with immobilized glucose oxidase and catalase in the sampling part of the FIA system.

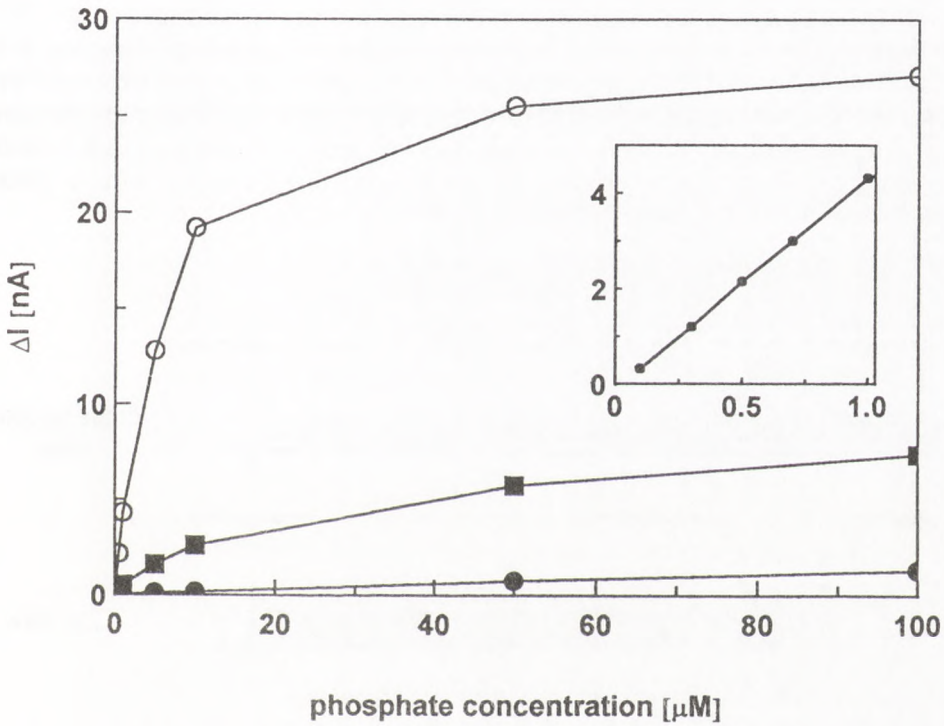

Fig. 2. Signal amplification as affected by the multi-enzyme sequence of the phosphate sensor. ● – ● maltosephosphorylase + glucose oxidase; O – O maltose phosphorylase + mutarotase + glucose oxidase; ■ – ■ maltose phosphorylase + alk. phosphatase + mutarotase + glucose oxidase.

2.2. Potentiometric biosensor in double matrix membrane technology

Many biosensors are designed for repeated use in e.g. flow system set-ups. For certain applications, for example in field testing and medicine or for biosensors using enzymes with low stability, disposable biosensors would be advantageous. As a basic, low cost solution for this problem a potentiometric biosensor with electrodes in double matrix membrane technology was developed. This technology describes a process where an ion-sensitive polymer matrix membrane is formed in the presence of an additional inert matrix which improves the reproducibility of the production process. The suitability of this technology could be demonstrated by monitoring ions (11) and urea (12). In the latter case a one-side silver-coated filter paper was used as the inert matrix which was encapsulated by a heat sealing film leaving room for the ion-selective electrode (ISE) contact and a small circle (diameter 4 mm) for the ion-sensitive membrane. A pH-sensitive membrane was casted into this paper circle and on the surface of this membrane a urease containing layer was deposited (Fig. 3). Up to now this sensor electrode was used for medical applications, but experiments are under way to determine heavy metals by exploiting the sensitivity of urease for these metals.

The simplicity of the double matrix membrane technology enables it for economical mass production of these electrodes in a batch process. This allows the production of low cost potentiometric biosensors for different analytes. The advantage of mass production of sensor electrodes with nearly identical response characteristics is realistic as first results with a potassium-sensitive sensor have shown (11).

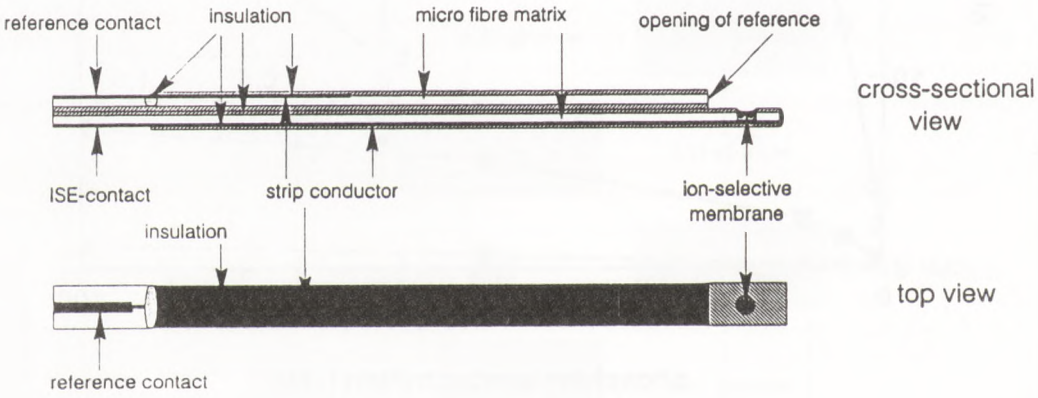

Fig. 3. Schematic view of the potentiometric biosensor electrode in double matrix membrane technology.

3. Immunosensors

3.1. Immuno FIA for the determination of herbicides in drinking water

An immunosensor system for the measurement of haptens based on an immunoreactor with immobilized haptens on a silica surface was constructed (13), as a model analyte 2,4-dichlorophenoxyacetic acid (2,4-D) was chosen. Such herbicides of the phenoxyacidic acid-type play an important role in agriculture and consequently the determination of 2,4-D is of special interest with respect to monitoring drinking water quality. Usual methods are based on chromatographic methods (GC or HPLC) requiring sample pretreatment and expensive laboratory equipment. According to EU guidelines for drinking water quality 0.1 µg/l 2,4-D is the highest permissible concentration.

The principle of the developed highly sensitive immunosensor system is shown in Fig. 4. The analytical system contains a fused silica capillary tube in which the hapten is immobilized via bovine serum albumin on the inner side of the capillary as sensing element. This immunoreactor is combined with an amperometric carbon electrode. In the competitive assay formate enzyme labeled antibodies (conjugate) bind to 2,4-D of the injected sample during a preincubation step and are then applied to the reactor, where excess conjugate binds to immobilized hapten (Fig. 4). Alkaline phosphatase (AP) is used as the enzyme label with p-aminophenyl phosphate as substrate. The signal is then generated after the addition of the substrate to the reactor and

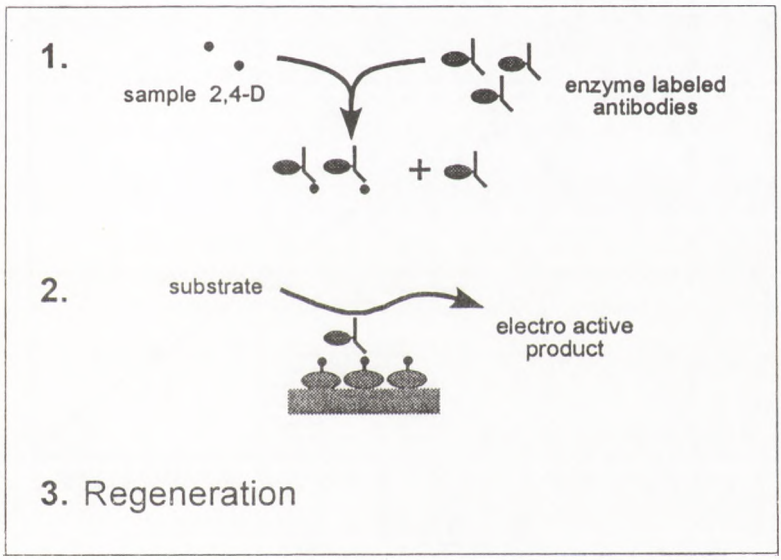

Fig. 4. Principle of the immunosensor for the detection of 2,4-D.

the enzymatically generated p-aminophenole is oxidized at the electrode surface at a potential of +150 mV vs Ag/AgCl. After measurement a regeneration of the immunoreactor is performed by treatment with glycine/HCl pH 2.0.

With this immunosensing system the automatic detection of 2,4-D in the range of 0.1 µg/l up to 5 µg/l is possible (Fig. 5). The detection limit of 0.1 µg/l meets the demands of the EU standards for pesticide detection in drinking water. One measurement requires about 12 min. Due to the high functional stability of the immunoreactor up to 30 measuring cycles could be performed.

3.2. Immuno FIA for the determination of polycyclic aromatic hydrocarbons in soil

A number of expensive and time-consuming techniques have been reported for monitoring the cancerogenic polycyclic aromatic hydrocarbons (PAH) at contaminated industrial waste sites.

For this determination a FIA principle similar to the one described above (Fig. 4) was used. The hapten (carboxylated phenanthrene derivative) is immobilized on the fused silica capillary reactor via poly-L-lysine. In a competitive reaction, anti phenanthrene antibodies, which also show some cross-reactions to EPA (Environmental Protection Agency) standard-PAH, react with the analyte phenanthrene during a preincubation step. Applied to the im-

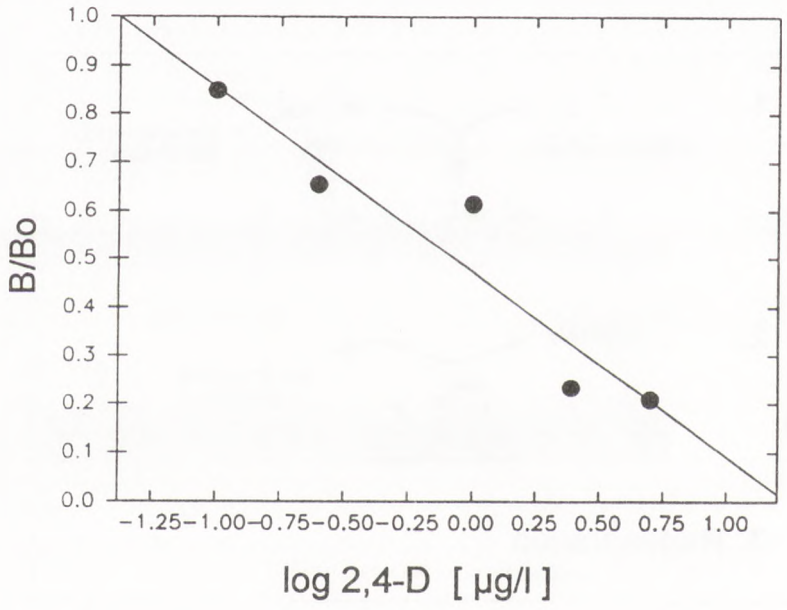

Fig. 5. Calibration curve for 2,4-D.

muno reactor only the excess antibodies in the reaction mixture can bind to the immobilized phenanthrene. These antibodies were then detected with secondary antibodies conjugated with alkaline phosphatase allowing a signal generation as described above.

A calibration for phenanthrene is possible in the range of 10 ppb — 5 ppm (Fig. 6). With this sensor system the determination of the sum of PAH concentrations became possible. For example seven methanol extracts from soil of a German manufactured-gas plant site were measured using this immuno FIA and compared to data obtained with the reference HPLC method. In the latter case soils were extracted by supercritical fluid extraction. The correlation shown in Fig. 7 is impressive and recommends immunosensing as a fast and cheap method for screening and mapping PAH of industrial areas.

4. Microbial sensors

4.1. Microbial sensing of naphthalene

In another approach of PAH determination, e.g. of naphthalene, an amperometric microbial biosensor was developed using either immobilized *Sphingomonas* sp. B1 or *Pseudomonas fluorescens* WW4 cells that are able to use PAH as sole carbon source consuming oxygen. The microorganisms were immobilized in a polyurethane based hydrogel which was mounted onto an

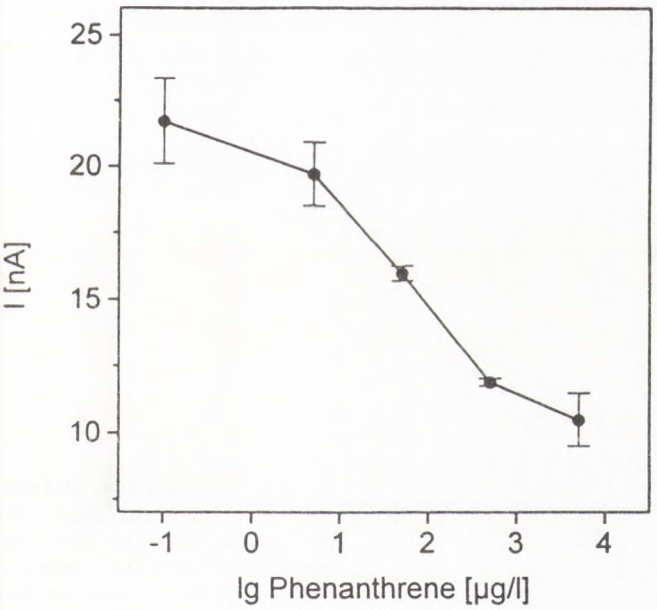

Fig. 6. Calibration curve for phenanthrene.

oxygen electrode. Both strains were shown to be equally suited for the quantification of naphthalene in aqueous solutions. The biosensors were tested in a flow through system and a stirred cell (batch method). In both systems a linear response down to the detection limit was obtained. Measurements in the flow through system gave sensitivities of up to 1.2 nA/(mg/ml) and a linear range from 0.03 to 2.0 mg/l (Fig. 8). The repeatability was within ±5%. Using the batch method, sensitivities of between 3 and 4 nA/(mg/ml) and a linear range from 0.01 to 3.0 mg/l were obtained (Fig. 9). The sensors reached an operational lifetime of up to 20 days. The sensitivity of both sensors for naphthalene was more than four times higher than for most other substrates measured (14).

4.2. Sensor for rapid estimation of the biochemical oxygen demand (BOD) employing heavy metal resistant microorganisms

The biochemical oxygen demand (BOD) is a widely used parameter in environmental water control. It indicates the content of biodegradable organic compounds in waste water. The conventional BOD_5-method takes five days and therefore is not practicable for monitoring waste water. The BOD can be estimated more rapidly by using a microbial sensor containing immobilized whole cells in front of an oxygen electrode.

In the last years some BOD-analysers based on microorganisms have been commercialized in Germany and Japan. One of the disadvantages is that heavy metal ions usually are present in waste water and cause inhibitory

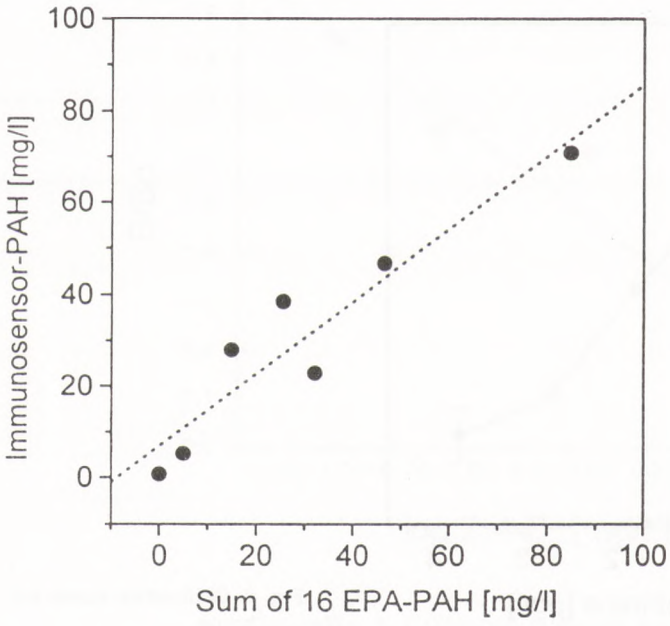

Fig. 7. Correlation of PAH concentration determined in soil extract by HPLC and the immunosensor, the immunosensor signal is corrected by factor 3.3.

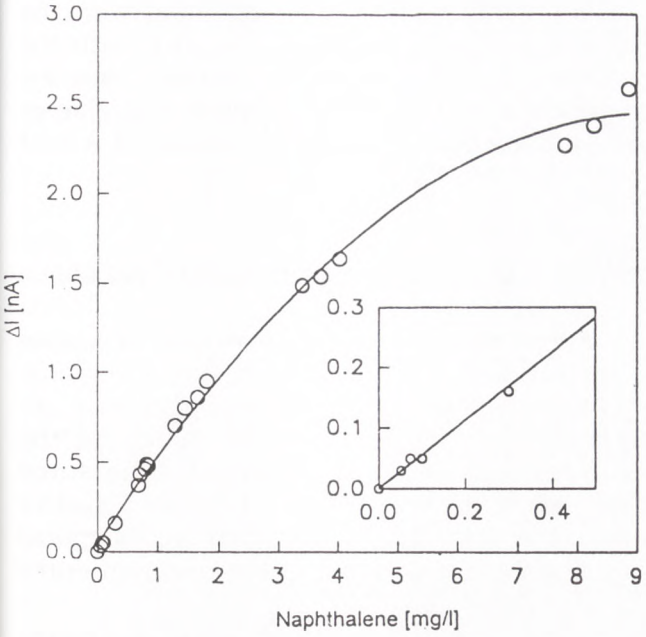

Fig. 8. Calibration curve for the determination of naphthalene with *Sphingomonas* sp. B1 in the flow through system.

effects on microorganisms. This problem can be avoided by using heavy metal resistent strains.

We developed such a sensor using *Alcaligenes eutrophus* KT02 which carries plasmids encoding resistance to 40 mM $NiCl_2$, 20 mM $CoCl_2$, 10 mM

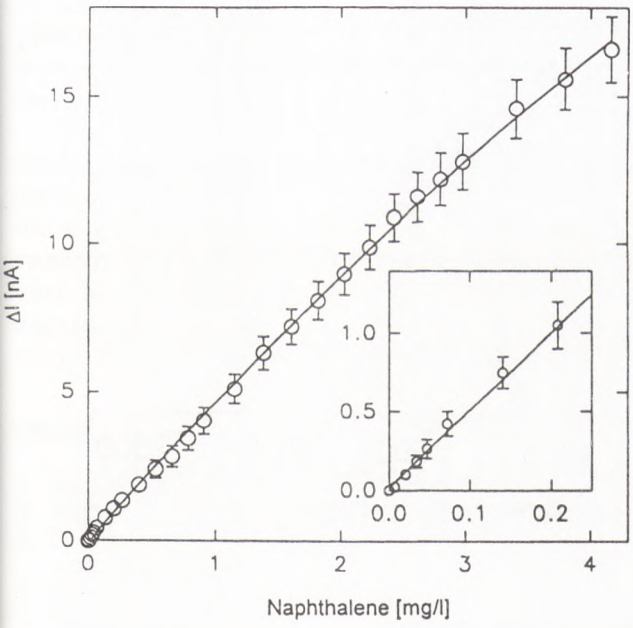

Fig. 9. Calibration curve for the determion of naphthalene with *Pseudomonas fluorescens* WW4 in the batch system.

$ZnCl_2$ and 1 mM $CdCl_2$. The cells were immobilized via entrapment in the polyurethane hydrogel. All measurements were carried out at 31°C with the microbial sensor integrated in a flow through system and showed that the estimation of BOD even in presence of 4 mM nickel, copper and zinc is possible. Cadmium is tolerated by this strain in a concentration of 4 mM for a period of 4 h.

5. Fiber optical surface plasmon resonance spectroscopy sensor

A miniaturized fibre optical sensor based on surface plasmon resonance spectroscopy (SPRS) was investigated with the aim for sensing of organic solvent vapours, particularly tetrachloroethene (15). Surface plasmons are excited on a silver coated multimode fibre by polychromatic light, and the resonant excitation is detected as a resonant absorption band in the measured output spectrum. When tetrachloroethene is absorbed in a thin gas sensitive polysiloxane film deposited on the silver layer the polymer changes its optical properties. These changes result in a wavelength shift of the resonant curve depending on the analyt gas concentration.

The sensor exhibits excellent response to tetrachloroethene, whereas acetone, ethanol and water show cross sensitivities below 1%. The sensor response

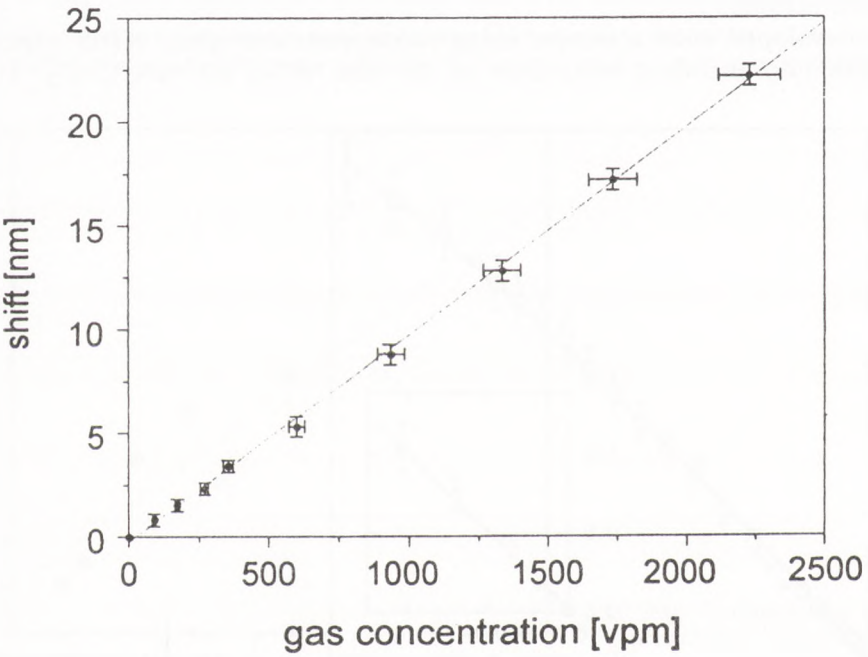

Fig. 10. Calibration plot for a polydimethylsiloxane coated fibre for tetrachloroethene.

is linear in the range between 20 to 2200 ppm tetrachloroethene (Fig. 10). With a detection limit of 20 ppm tetrachloroethene it is possible to have reliable measurements below the level required by the German law (50 ppm). Remarkable is the very short sensor response time of less than 2.5 s.

Beneficial for a practical application of this optical sensor is the possibility for remote sensing, e.g. in dry cleaners. In contrast to semiconductor gas sensors this sensor works at room temperature and measurements in explosion endangered areas are possible. A further advantage of the miniaturized sensor is the insensitivity to strong electromagnetic fields. Because of its small size, flexibility and simple set-up this approach offers exciting new opportunities in new application fields.

6. Conclusions and outlook

In the field of environmental control currently exists a clear and increasing need for fast, portable and cheap analytical devices. Due to their versatility biosensors can be of advantage to fill specific niche applications in this area. Providing good selectivity and high sensitivity biosensors are easy to handle with no or simple sample preparation and, in terms of cost, should be competitive to present devices. The measurement is fast and not tied to the laboratory. Important for industrial production is that one successful transducer configuration can be used for different analytes only by changing the sensing layer.

Biosensors still show some disadvantages. Due to problems in the stability of the sensing layer the life-time and the reproducibility of the sensor can decrease. Some transducers may show interferences, or the sensing layer is susceptible to toxic substances. Due to these problems only few biosensors have been commercialized up to now.

Nevertheless, after appropriate development, testing and commercialization with regard to their practical use and the demands of possible users, biosensors will be accepted on a wider market as new, sophisticated tools for environmental monitoring. The application of microtechnology and eventually nanotechnology allows considerable miniatiurization of sensors. Then they can be produced in large numbers at low price and therefore will have a significant effect on reducing costs and increasing the efficiency of applications in the environmental area.

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Summary

The evolution of biosensor research corresponds with the increasing need for fast, sensitive and selective measuring devices. This paper will focus on recent developments that have been advanced in our Institute. (I) As an electrochemical device a highly sensitive multi-enzyme sensor for the determination of inorganic phosphate was developed using 4 enzymes for signal amplification. (II) Immunosensing of low molecular mass analytes is demonstrated by a flow injection system for the determination of herbicides in drinking water, such as 2,4-D and for the determination of polycyclic aromatic hydrocarbons in soil. (III) Microbial sensors for the determination of polycyclic aromatic hydrocarbons were developed as well as new approaches to the determination of the biochemical oxygen demand (BOD). (IV) The "double matrix membrane" technology is an innovative possibility for the production of cheap disposable electrodes for sensors, for example for the detection of urea. (V) Based on surface plasmon resonance spectroscopy (SPRS) a miniaturized fibre optical sensor was developed for sensing environmental hazards in liquids as well as in air.

Key words:
biosensor, environmental monitoring, microbial sensor, immuno flow injection analysis system.

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