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Electrochemical Biosensors – Ways to Improve Sensor Performance*

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Introduction

The selective and sensitive detection of a large number of substances is of paramount importance in analytical chemistry. The combination of the biomolecular recognition capacity of enzymes with the simplicity of amperometric detectors is an attractive approach to creating low-cost sensors for the fields of analytical chemistry, such as biomedical science, health care, biotechnology, and environmental monitoring.

Traditionally, enzymes have been used as analytical reagents for the determination of minute amounts of a substance in complex samples (Bergmeyer, 1983). In addition to their substrates, compounds modifying the rate of the enzymatic reaction, such as activators, prosthetic groups, inhibitors, and also enzymes themselves, are accessible to measurement. Immobilization of the enzyme makes the reagent reusable, hence the amount of enzyme required per analysis is reduced to an extremely low level (a few milliunits per sample). A biosensor is created when the immobilized biological material is in close contact with a physical transducer. Here, the biological component translates the recognition of the analyte into a change of an easily measurable physico-chemical parameter. This change is converted into an electrical signal by the transducer, amplified, processed, and displayed in a suitable form. *Figure 1* illustrates the general structure.

The specific recognition is not only provided by enzymes. Other immobilized biological material can be employed, such as cells, organelles, and tissues, making quantifiable biologically related parameters, such as taste, odour, fatigue substances, mutagenicity and nutritive value. Furthermore, binding assays can be performed with receptors, nucleic acids, antibodies, and antigens.

The most frequently used transducers are electrochemical, optical, and thermal detectors, but piezoelectric and surface acoustic wave methods may also be used (the reader is referred to a number of excellent books and reviews: Guilbault, 1976;

* dedicated to my father

Biotechnology and Genetic Engineering Reviews – Vol. 13, December 1995
0264–8725/94/12/1–38 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

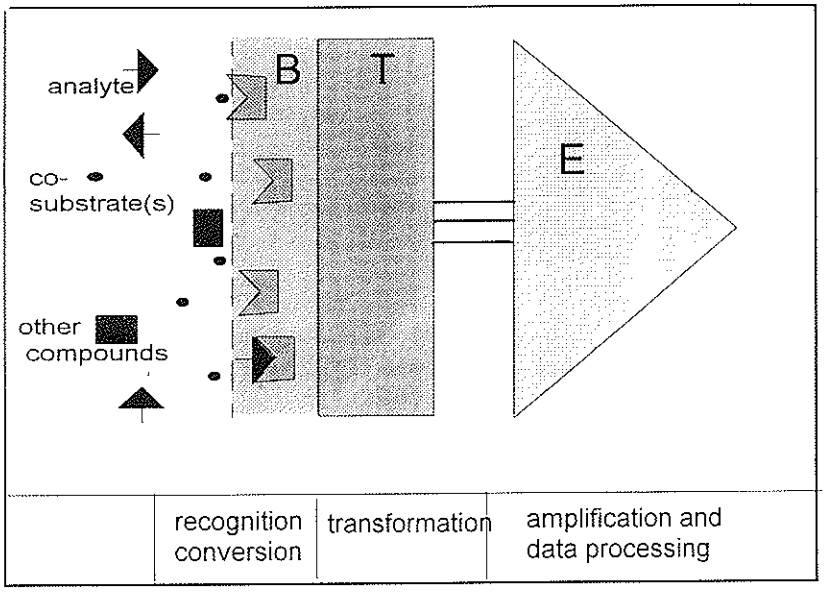

Figure 1. Schematic illustration of a biosensor. **B** represents the bioactive layer, **T** the transducer, and **E** the electronic unit.

Turner, Karube and Wilson, 1987; Scheller and Schubert, 1989; Cass, 1990; Hall, 1990; Buck *et al.*, 1990; Schmidt *et al.*, 1993; Sethi, 1994; Byfield and Abuknesha, 1994).

Enzyme electrodes are the first type of biosensors to have been proposed (Clark and Lyons, 1962) and work with these electrodes is at present the best established branch of biosensor research. In amperometric enzyme electrodes, the function of the enzyme is to generate or consume a redox-active compound in the specific catalytic conversion of the target analyte (substrate). The redox-active compound is oxidized or reduced at the electrode poised at an appropriate potential. The generated current can be correlated to the analyte concentration. In the simplest approach the biocatalyst is entrapped between or bound to membranes and this arrangement is fixed at the surface of the transducer (Mosbach, 1976). The direct adsorptive or covalent fixation at the electrode surface, sometimes together with reagents necessary for signal transfer, allows for the elimination of semipermeable membranes. The direct binding of the biocatalyst to an electronic device transducing and amplifying the signal is the basis for a further miniaturization. The miniaturization of the transducer leads to novel measurement characteristics. Electropolymerization is one of the favoured procedures for the spatial distinct application of enzymes to those sensor surfaces (e.g., Foulds and Lowe, 1986; Schuhmann *et al.*, 1990; Bartlett and Cooper, 1993).

In amperometric enzyme electrodes artificial redox mediators have been included in addition to the natural electron acceptors (Cass *et al.*, 1984; Green and Hill, 1986). When the redox potential of the artificial mediator is low, electrochemical measurements can be performed with diminished interference. Organic conducting charge-transfer salts and ferrocenes provide the best mediator characteristics with respect to oxygen independence and low cross-sensitivity. Integration of auxiliary

enzymes or cofactors and mediators in the enzyme layer improves the analytical performance and simplifies the handling (Albery *et al.*, 1987; Hale *et al.*, 1989; Bremle, Persson and Gorton, 1991; Schuhmann, 1993).

An alternative to the use of mediators is the direct electron transfer between the prosthetic group of an enzyme and the electrode (Armstrong, Hill and Walton, 1988; Guo *et al.*, 1989; Wollenberger *et al.*, 1990; Ikeda, Matsushita and Senda, 1991; Gorton *et al.*, 1992). A symbiosis of mediated and direct electron transfer is obtained by covalent bonding of electron relay molecules to the enzyme protein close to the active centre of oxidoreductases (Degani and Heller, 1988; Gregg and Heller, 1990; Ye *et al.*,1993). Furthermore, the development of redox molecules carrying polymers such as poly(vinyl pyridine) $Os(bpy)_2Cl$ for binding to and immobilization of GOD provides a dense network of such polymeric wires facilitating a very fast electron shuttling without the necessity for real diffusion of mediator molecules. With this approach significant improvement of sensor performance has been achieved.

The number of substances that can be measured by single enzymatic approaches is limited, because in the majority of biocatalytic reactions electrochemically active compounds are not involved. To form readily detectable species, different enzymatic reactions have to be coupled, as is already routine in wet biochemical analysis. This coupling can be accomplished in ways analogous to those present in a living cell. Here, nature provides us a variety of ways of regulating metabolic pathways (Stryer, 1988). Exploiting the principles of metabolically controlled 'switches' and enzyme cascades, whereby both inactivation and activation of enzymes might occur when a threshold value of a particular metabolite is reached, is of paramount interest in designing analytical procedures. In addition to switches, pathways may also be regulated by the availability of intermediates of continuously cycling reactions. Thus, as in nature, catalytic activities of different enzymes can be combined in biosensors either in sequence, in competing pathways, or in cycles (*Figure 2*). In conjunction with appropriate measuring regimes not only does a much wider range of analyte species

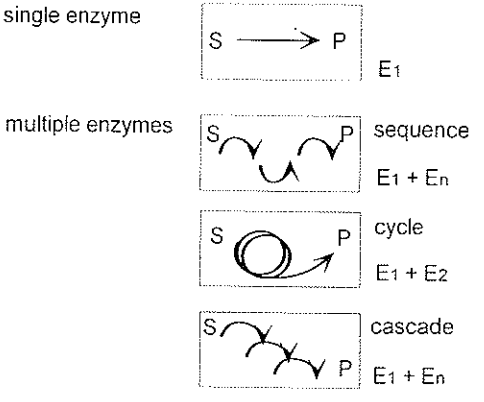

Figure 2. Illustration of enzyme coupling principles. S and P represent the target analyte and the detectable product. E_1 is the initial analyte converting enzyme followed by further enzymatic reactions that are numbered 2 to n.

become in this way accessible to measurement by the bioelectroanalytical approach but, in addition, the selectivity and sensitivity of the biosensor may be enhanced through the appropriate choice of the coupling strategy (Scheller and Schubert, 1989; Wollenberger *et al.*, 1993).

This chapter provides a survey of principles involved in coupling enzymatic reactions for the purpose of improving the analytical performance (with respect to sensitivity and selectivity) of biospecific sensors. A number of examples are presented for medical and biotechnological important substrates. Special attention is paid to biochemical amplification by cycling analyte conversion in comparison with chemical, electrocatalytic, and electrochemical regeneration strategies.

New analytes

SEQUENTIALLY AND COMPETITIVE ENZYME REACTIONS

The term enzyme sequence electrode has been introduced for a biosensor, where two enzymes metabolize a substrate in consecutive reactions with the formation of a measurable secondary product. Obviously, the number of enzymes (E_1–En) in such a reaction chain can be increased as long as recognition of the primary substrate S ends up in a detectable metabolite P* (*Figure 3*). For example, choline liberated from acetylcholine by acetylcholine esterase is subsequently manifested in the choline oxidase reaction under formation of hydrogen peroxide. The measurement of the secondary product allows for determination of acetylcholine (Wollenberger *et al.*, 1991). On the same basis, families of electrodes have been developed, which combine for example glucose-, lactate- or glutamate-generating primary enzyme reactions with the respective oxidases (e.g. Scheller *et al.*, 1989). Sensors for oxidase substrates have also been studied using co-immobilized peroxidase for hydrogen peroxide transformation. In most cases soluble and mediator-modified electrodes have been used. The approach of 'electrically wired' enzymes has also been published (Ohara *et al.*, 1993). In addition, a number of investigations have been reported that dealt also with the application of the apparent direct electron transfer between electrode and peroxidase that occurs at a favourable low electrode potential. Thus the influence of interfering electrochemical reactions is minimized (e.g. Kulys and Schmid, 1990; Tatsuma and Watanabe, 1991; Gorton *et al.*, 1992).

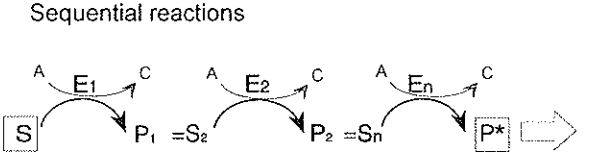

Figure 3. Scheme of a sequential enzyme sensor. A and C represent co-reactants. Up to n enzymes can be used. P* is electrochemically indicated.

Recently, an enzyme sensor for the determination of citrate has been developed based on a linear reaction sequence of three enzymes (Gajovic, Warsinke and Scheller, 1995):

$$\text{Citrate} \xrightarrow{\textit{citrate lyase}} \text{oxaloacetate} + \text{acetate} \qquad \text{Eq. (1)}$$

$$\text{Oxaloacetate} \xrightarrow{\textit{oxaloacetate decarboxylase}} \text{pyruvate} + CO_2 \qquad \text{Eq. (2)}$$

$$\text{Pyruvate} + \text{phosphate} + O_2 \xrightarrow{\textit{pyruvate oxidase}} \text{acetyl phosphate} + CO_2 + H_2O_2 \qquad \text{Eq. (3)}$$

Citrate conversion by citrate lyase does not yield a directly detectable species. Therefore the enzymatic decarboxylation of its product oxaloacetate has been appended and the product of that secondary reaction is easily measurable by pyruvate oxidase. Hence, the concentration of citrate is related to oxygen consumption or hydrogen peroxide formation in the pyruvate oxidase indicator reaction.

Best results were obtained with co-immobilisation of 50 units of citrate lyase/ cm^2 with the other two enzymes in a gelatin matrix. When the multi-enzyme layer was sandwiched between two dialysis membranes onto a modified Clark-type oxygen electrode, functional stability for 5 days was achieved. The sensor covers a linear measuring range of up to 1 mmol/l citrate with a lower detection limit of 0.5 µmol/l (*Figure 4*). The response time of 5 min shows one of the disadvantages of such a multi-enzyme assembly that is related to the large thickness of the enzyme membrane. Nevertheless, the concentration values obtained for citrate in real samples correlate well with an enzymatic photometric reference method.

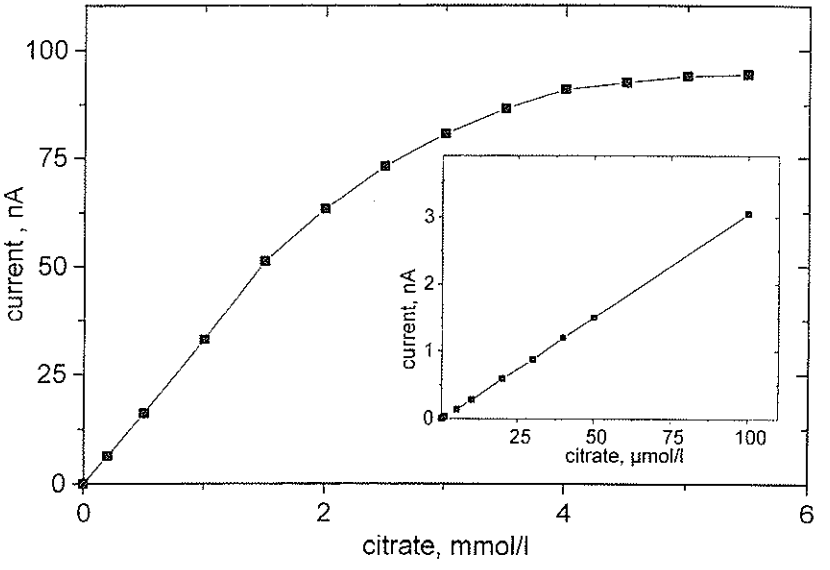

Figure 4. Citrate concentration dependence of a three-enzyme sequence electrode comprising citrate lyase, oxalacetate decarboxylase, and pyruvate oxidase in a gelatin membrane.

The main hindrance for practical application of those multi-enzyme sensors is that they respond to all substrates of the sequence. However, this may be advantageous if not only the initial substrate is to be measured. In favourable cases, e.g. for a sensor using gelatin-entrapped lactate dehydrogenase (Equation (4)) and lactate monooxygenase (Equation (5)) for pyruvate and lactate assay, the sensitivity for the two substrates is virtually the same, so that the calibration is simplified to one step (Weigelt, Schubert and Scheller, 1987). The oxygen consumption of the sequential reaction:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\textit{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+ \qquad \text{Eq. (4)}$$

and

$$\text{Lactate} + \text{O}_2 \xrightarrow{\textit{lactate monooxygenase}} \text{acetate} + \text{CO}_2 + \text{H}_2\text{O} \qquad \text{Eq. (5)}$$

is indicated. With this assembly linearity is obtained up to a sum of lactate and pyruvate of 0.7 mmol/l. The sensor is applicable to lactate and pyruvate forming enzymes such as alanine aminotransferase and pyruvate kinase. An additional co-immobilization of pyruvate kinase permits the measurement of creatinine kinase. These are examples of the lactate monooxygenase-based sensor 'family' (Schubert *et al.*, 1990). Glucose sensor-based assemblies have been designed with a similar coupling strategy (Pfeiffer *et al.*, 1980).

Another coupling principle uses the competitive action of two enzymes on the same substrate, whereas one of these enzymes produces the electrochemical signal. Examples are the ATP-sensor with co-immobilized hexokinase and glucose oxidase (Pfeiffer *et al.*, 1980) and the sensor for aminopyrine with peroxidase and catalase immobilized on an oxygen electrode (Renneberg *et al.* 1982).

Design of sensor performance

Important problems in analytical chemistry are connected with measuring range, selectivity, and stability. A typical problem, for example, is the determination of trace concentrations of adrenaline in the presence of higher amounts of other catecholamines that might interfere with the analysis. On the other hand, for in situ and in vivo application, analyte measurements must proceed at concentrations that exceed the measuring range of the sensor (this aspect is not discussed in the present contribution). Several approaches have been created to tailor biosensors to practical applications. In order to achieve the desired (bio)sensor performance permselective membranes and modifier introducing permselectivity have been employed; the transducer design itself has been varied and enzymes have been coupled.

The latter approach involves the introduction of an additional enzyme into the biocatalytic layer in order to filter chemical signals by eliminating constituents of the sample, disturbing either the enzymatic or the electrochemical reaction, thus leading to improved selectivity. Furthermore, preconcentration and stripping of intermediates of the enzyme reaction results in amplification of the response and, at the same time, in better selectivity. Sequential activation of enzymes that leads directly to a cascade-like increase in reaction rate is another powerful principle for signal amplification. The best studied way, however, to amplify the response to very low concentrations is that

of analyte recycling between two enzymes (Lowry and Passonneau, 1972). Therefore, special attention is paid to this particular arrangement.

Selectivity

BIOCHEMICAL FILTER

Coupled enzyme reactions can be used also to eliminate disturbances of the enzyme or electrode reaction caused by constituents of the sample. This is important in particular for the development of sensors well suited for measurement of real samples. *Figure 5* illustrates the basic set-up. The enzyme E_2 is immobilized in the layer to prevent the interfering compound I from reaching the detector surface or the indicator enzyme E_1 that is responsible for analyte conversion. For example, ascorbic acid and acetaminophen (4-hydroxyacetanilide), which interfere with the anodic hydrogen peroxide oxidation, can be transformed into an inert product by reaction with an eliminator enzyme.

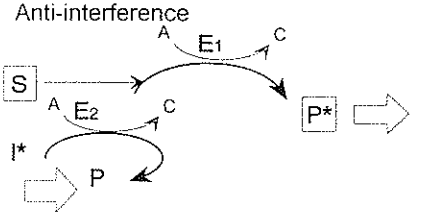

Figure 5. Schematic illustration of enzymatic elimination of interfering constituents in biosensors. A and C are co-reactants to the indicator enzyme E_1 and the eliminator enzyme E_2. I* represents the interfering compound.

Several approaches have been reported for removal of these particular compounds during glucose measurement. One probe uses the combination of the chemical reaction between ascorbic acid and hexacyanoferrate (III) in the external sample solution and a glucose electrode consisting of glucose oxidase with co-immobilized laccase for catalytic oxidation of hexacyanoferrate (II), which has been generated in the reaction and is also oxidizable at the working potential of + 600 mV. In this way oxygen consumption is the only result of the removal of the interfering compound. With this method ascorbic acid in the urine was completely eliminated up to 20 mmol/l (Wollenberger *et al.*, 1986). Tyrosinase, which has phenol oxidizing capacity, has been also employed for the elimination of acetaminophen interference (Wang, Naser and Wollenberger, 1993). A layer comprising gelatin-entrapped tyrosinase is capable of preventing 0.3 mmol/l acetaminophen from reaching the electrode (*Figure 6*). Above that concentration a breakthrough is observed. The diffusion-controlled behaviour becomes subject to control by enzyme kinetics. Maidan and Heller (1991) recommend horseradish peroxidase for the catalytic peroxidation of interfering substances by H_2O_2.

For the determination of di- and polysaccharides in real samples by enzyme sequence sensors based on saccharidases and glucose oxidase as indicator enzymes,

the effective elimination of glucose is critically important. The use of GOD and catalase (Scheller and Renneberg, 1983), but also hexokinase with ATP addition have been described (Renneberg *et al.*, 1983). Other anti-interference systems have been devised to eliminate lactate (with LMO in pyruvate measurements (Schubert *et al.*, 1990)), ascorbic acid (with ascorbate oxidase for catecholamine detection at a graphite electrode (Nagy, Rice and Adams, 1982)), ammonia (with glutamate dehydrogenase in creatinine measurements (Kihara and Yasukawa, 1986)), and oxygen (with GOD/catalase for NAD^+ reduction (Scheller and Schubert, 1989)).

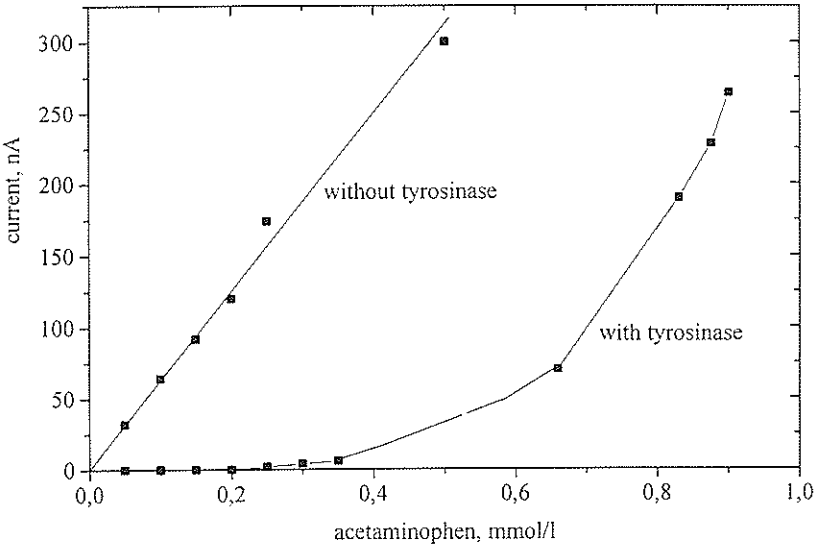

Figure 6. Repression of electrochemical acetaminophen oxidation by tyrosinase. Tyrosinase has been co-immobilized with glucose oxidase in a gelatin layer on top of a hydrogen peroxide indicating electrode.

Selectivity and enhanced sensitivity

ACCUMULATION OF INTERMEDIATE

The sensitivity of electroanalytical measurements can be enhanced by accumulation of the electrochemically active analyte at the electrode before measurement (stripping analysis) (Wang, 1985).

Already in 1982 Kulys and coworkers illustrated that it is possible to increase the sensitivity of biosensors by controlled accumulation of charge in the presence of the corresponding substrate. Furthermore, biocatalytic accumulation, followed by chronoamperometric detection is shown to greatly enhance the sensitivity of mediator-free biosensing of peroxides at peroxidase modified carbon paste elctrodes (Wang, Ciszewski and Naser, 1992). The principle of stripping analysis is adapted to enzyme membrane electrodes based on sequentially acting enzymes with the aim to develop sensitive and selective sensors.

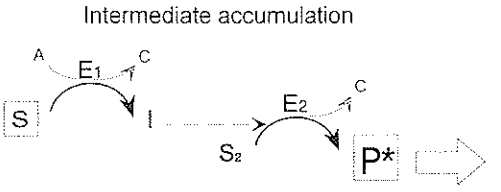

Figure 7. Scheme of an intermediate accumulating biosensor. A and C represent co-reactants; I is the accumulated intermediate, that is stripped after the substrate S_2 is added to initiate enzyme reaction E_2.

The sensors combine preconcentration of an intermediary product with a biocatalytic indicator system (Schubert, Lutter and Scheller, 1991). Oxygen probes as well as chemically modified electrodes have been used as base sensors. The principle of the measurement is illustrated in *Figure 7*. In the first step of the measurement the addition of the analyte S and a saturating concentration of appropriate cosubstrate A leads to the formation of an intermediate product I by the generator enzyme E_1. The intermediate is accumulated in the enzyme membrane, due to its slow diffusion. When this reaction approaches equilibrium, the second step, the actual measurement, is triggered by injection of an excess of substrate (S_2) of the second indicator, namely the enzyme E_2, which converts the accumulated intermediate under formation of an electroactive product P^*. For the determination of glucose, for example, NADH dependent glucose dehydrogenase (Equation (6)) is used for oxidation of the analyte with formation of NADH.

The indicator sequence lactate dehydrogenase/lactate oxidase senses NADH with resulting oxygen consumption:

$$\text{Glucose} + \text{NAD}^+ \xrightarrow{\textit{glucose dehydrogenase}} \text{gluconolactone} + \text{NADH} \qquad \text{Eq. (6)}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\textit{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+ \qquad \text{Eq. (7)}$$

$$\text{Lactate} + \text{O}_2 \xrightarrow{\textit{lactate oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2 \qquad \text{Eq. (8)}$$

Pyruvate acts as initiator substance (*Figure 8*). The rapid current increase is indicative of the fast diffusion of pyruvate into the membrane and its rapid reaction with the preconcentrated NADH. Finally, the steady state concentration of the conventional measuring regime of NADH is reached. The evaluation of the peak in the current-time curve provides an enhanced signal for glucose. When the derivative of the current-time curve is recorded, the sensitivity is increased by a factor of 40. The method has also been applied to sensors for the determination of glycerol, glucose-6-phosphate, hypoxanthine, formate, and $NADP^+$.With the exception of the latter, fast diffusing pyruvate is initiating the indicator reaction (*Table 1*).

Table 1. Enzyme sensors using the accumulation principle (Schubert and Scheller, 1992)

Analyte S	Generator E_1	Intermediate I	Detector E_2	Initiator S_2	Amplification factor
Formate	formate DH	NADH	lactate DH/ lactate monooxygenase	pyruvate	6
Glucose	glucose DH	NADH	lactate DH/ lactate monooxygenase	pyruvate	40
Glucose-6-phosphate	glucose-6-phosphate DH	NADH	lactate DH/ lactate monooxygenase	pyruvate	17
Glycerol	glycerol DH	NADH	lactate DH/ lactate oxidase	pyruvate	64
Hypoxanthine	xanthine DH	NADH-PEG	lactate DH/ lactate monooxygenase	pyruvate	1.7
$NADP^+$	alkaline phosphatase	NAD^+	lactate DH	lactate	2

DH- dehydrogenase, PEG- polyethylene glycol

A limit of accumulation is set by the equilibrium of the reaction. Differences in the amplification factors are mainly attributed to the equilibrium constants of the generator enzyme and the diffusion behaviour of intermediate and initiator substrate.

The largest enhancement due to accumulation is obtained when the intermediate is a large molecule and the initiator a small molecule. This principle is less effective in terms of sensitivity enhancement (as compared with the cycling amplification described later), but bears the inherent capability to increase the selectivity for a given analyte in a complex sample. It is applicable to all analytes converted in sequential enzyme reactions, provided the terminal enzyme is cofactor-dependent and leads to a signal at the transducer.

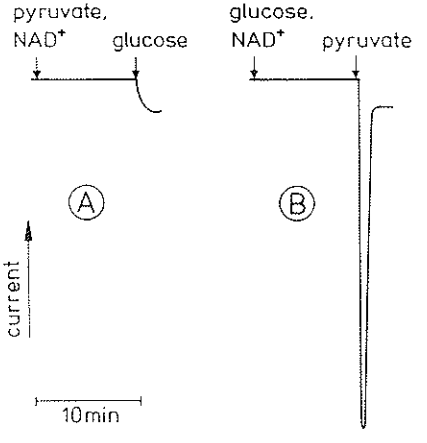

Figure 8. Current-time curves for a sensor operated without (A) and with (B) intermediate accumulation. Glucose measurements are performed with an enzyme electrode containing immobilized glucose dehydrogenase, lactate dehydrogenase, and lactate monooxygenase. NADH is stripped by pyruvate addition after 6 min of accumulation.

As mentioned above, an important aspect in the practical application of this approach is the possibility to eliminate any effects on the signal that are due to interfering molecular species. For this purpose, the sample is allowed to equilibrate with the sensor to establish a background signal before initiating the reaction that measures the analyte. This suppresses any contribution from interfering species to the signal and improves the selectivity of the analysis.

Sensitivity

AMPLIFICATION BY ANALYTE RECYCLING

Electroanalytical techniques are fairly sensitive and currents as low as 10^{-10} A can be recorded with commercial devices. Thus with bare electrodes the lower detection limit of amperometric electrodes is about 10^{-7} mol/l (for a desired redox reaction the charge exchange rate differs with the electrode material, for example, carbonaceous material reveals the fastest electron transfer for a number of quinonoid molecules).

The introduction of a layer incorporating the enzyme over the surface of the electrode decreases the sensitivity of the electrode by one to two orders of magnitude, due to the additional diffusion resistance. Therefore, for the measurement of analyte concentrations in the nanomolar range an increase of sensitivity of the enzyme electrode is required. One way to solve this problem is the continuous regeneration of the analyte in cyclic reactions.

The combination of the electrochemical detection principle and the recycling of the analyte can be performed in a number of ways (*Figure 9*), each having its own merits, but the enzymatic system possesses the potential for the highest amplification rate.

A detailed description of the different approaches is given in the following section.

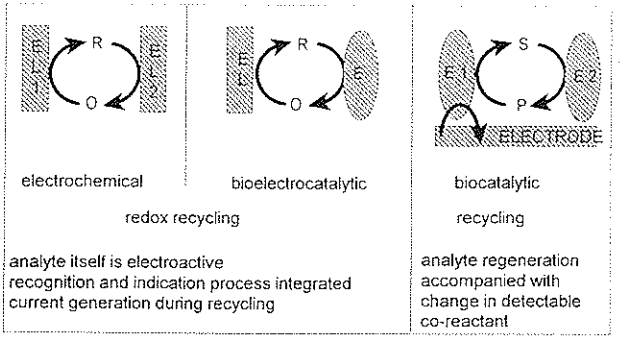

Figure 9. Illustration of analyte recycling schemes. EL represent an electrode and E an enzyme.

Electrochemical recycling

Modern fabrication technology opens new dimensions in structuring transducer elements for microsize sensor probes. With photolithography and electron beam lithography high structural resolution of the electrode pattern can be realized. With these electrodes novel measurement methods can be created.

Recently, the potential of an interdigital arrangement of electrodes with submicrometer spacing for the enhancement of analytical performance has been demonstrated with respect to selectivity and sensitivity (Niwa, Morita and Tabei, 1990, 1991, 1993, Wollenberger, Paeschke and Hintsche, 1994).

The increase in sensitivity is based on redox recycling of the analyte between adjacent microband electrodes of an interdigitated electrode array (IDA) (*Figure 10*). Hence, this principle is necessarily limited to reversible redox species.

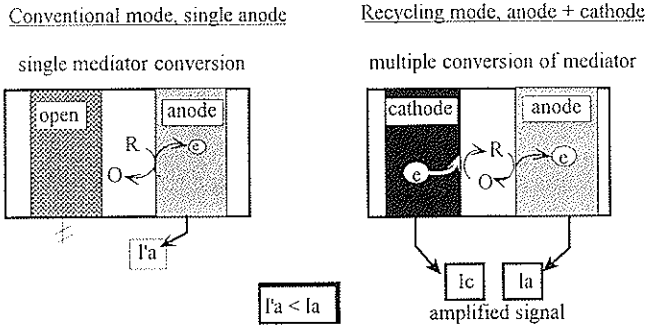

Figure 10. Schematic diagram of the redox recycling of oxidized (O) and reduced (R) redox species with closely spaced microband electrodes. i_a and i_c represent anodic and cathodic currents, respectively, i'_a is the unamplified current response of the single anode. (Reproduced from Wollenberger, Paeschke and Hintsche, 1994, with permission of the Royal Society of Chemistry.)

The idea was derived from rotating ring-disk experiments, where the intermediate generated on the disk is detected (collected) at the ring. In contrast to that technique at the microband electrodes diffusion, not convection, is the primary means of mass transport. Differing from rotating ring-disk electrodes, the product of the collector electrode diffuses back to the generator where it is again electrochemically transformed. When a number of band electrodes are placed close to each other and are alternatingly polarized to an oxidation and reduction potential of the electroactive analyte, electrolytically steady-state concentration gradients of oxidized and reduced redox molecules are established in the interelectrode space. Thus multiple oxidation and reduction can be observed. This redox recycling leads to both a steady-state response and a high current.

Theoretical considerations and experimental studies revealed that the limiting current is not only dependent on the concentration and the diffusion coefficient of the redox species, but is also related to the lateral dimension of the gap (Bard and Falkner, 1980; Aoki *et al.*, 1988; Nishihara, Dalton and Murray, 1991; Paeschke *et al.*, 1995a). Short average diffusion length and the large number of alternating electrodes are necessary for effective current amplification by recycling of reversible redox species (Bard *et al.*, 1986; Aoki *et al.*, 1988). At a sufficiently close spacing the collection efficiency approaches 1. Bard and co-workers (1986) defined the collection efficiency as the ratio of the currents at the generator and collector electrodes. The ratio between the steady-state currents of the generator electrode with and without connecting the collector electrode is the amplification factor. A further reduction of the lateral structures to nanometer extensions would have the effect of exponentially increasing this amplification factor.

Consequently multielectrode arrays were fabricated where pairs of interdigitated thin-film metal electrodes in micrometer and submicrometer geometry are arranged on a silicon substrate. Each of the microband electrode consists of 70 finger electrodes arranged in nanometer to micrometer distance to the neighbouring finger (Schnakenberg, Benecke and Lange, 1991; Wollenberger, Paeschke and Hintsche, 1994; Hintsche *et al.* 1994). This array has been fabricated using photolithography, electron beam lithography and lift-off technique. A custom made multipotentiostat was used to control the potential of the electrodes independently (Paeschke, unpublished device). The current response of the individual electrodes were separately preamplified after current to voltage conversion and drawn into a data acquisition board.

At an interelectrode spacing of 1 μm, each electrode finger lies within the diffusion layer of the closest neighbouring electrode band. Redox recycling has been accomplished by applying both the oxidation and the reduction potential of the reversible redox species to pairs of the interdigitated electrodes. The species is detected at one microband electrode, while the generated product is collected at the other electrode, where it is converted to its initial state. Hence, repeated forward and backward reaction occurs with the result of increased anodic and cathodic currents.

The signal enhancement by redox recycling has been studied for a number of reversible species at interdigitated gold and platinum electrodes with 1 μm spacing (*Table 2*). Obviously collection efficiencies around 0.90 have been achieved.

Table 2. Indication of redox mediators using the electrochemical recycling between closely spaced interdigitated electrodes

Mediator	Potential, anode Ea, mV	Potential, cathode Ec, mV	Sensitivity nA l/μmol	Amplification factor	Collection efficiency (Ic/Ia)
$Fe(CN)_6^{4-}$	*350*	*–120*	2.8	8	0.98
Catechol	*600*	*–200*	7.2	9	0.92
p-Hydroquinone	*400*	*–75*	5.7	14	0.94
p-Aminophenol	*250*	*–50*	16.4	12	0.92
Dimethylferrocene*	*600*	*0*	4	n.d.	0.96
Ferrocene dicarboxylic acid*	*600*	*0*	2.5	8	0.93
Ferrocene lysine*	*600*	*200*	4	15	0.98
Dopamine*	*800*	*100*	7	3.5	0.82
PMS	*0*	*–200*	18.6	12	0.95
Cytochrome c**	*50*	*–150*	1	8	0.98

*1: Au IDA; **Au modified with 4,4'-dithiodipyridine
average values, amplification factor in quiescent solution

These data compare well with theoretical predictions and experimental studies of IDA with large number of alternating microband electrodes of 2 μm width and 1 μm gap published by other authors (Aoki *et al.*, 1988; Niwa, Morita and Tabei, 1990, 1993).

An extremely high sensitivity has been reported by Niwa and co-worker, who covered the metal surface with a carbonaceous film. As regards the film property, catecholamines could be detected at nanomolar concentrations (Niwa and Tabei, 1994). The modification of the surface of a gold IDA with electron transfer promoters

provided access to the study of redoxprotein cytochrome c (Paeschke *et al.*, 1995b).

Figure 11 illustrates a concentration dependence of the analytically interesting redox carrier p-aminophenol. The interest results from the fact that it can be used to trace enzymatic activities (Razumas, Kulys and Malinauskas, 1980; Tang *et al.*, 1988; Rosen and Rishpon, 1989; Hilditch and Green, 1991) such as alkaline phosphatase and ß-galactosidase. These enzymes are often used in enzyme immunoassays.

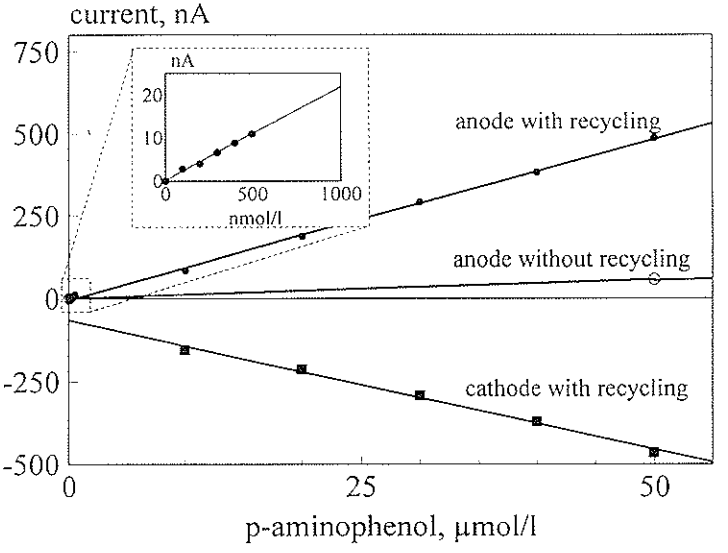

Figure 11. Comparison of the p- aminophenol concentration dependence of the IDA with and without redox recycling. (Reproduced from Wollenberger, Paeschke and Hintsche, 1994, with permission of the Royal Society of Chemistry.)

For the detection of p-aminophenol the anode, which serves as generator electrode, is potentiostated to +250 mV and the cathode (collector) to –50 mV. The addition of 10 µmol/l p-aminophenol results in an anodic current of 160 nA within 1 s. A response to the same extent is indicated simultaneously at the cathode. P-aminophenol diffuses to the anode, where it is detected, while the oxidation product is collected at the adjacent cathode. Here it is converted back to its initial state.

In comparison, the single anode shows a poor response at this low substrate concentration.

Efforts have been made to improve the performance of the interdigitated electrodes by technological means. Thus the detection limit for p-aminophenol could be reduced to 10 nmol/l when either the surface of a 1 µm spaced IDA was modified with carbon (Niwa and Tabei, 1994); this enhances the rate of electron transfer between phenolic compounds and the surface, or an interelectrode distance of 500 nm was produced (Paeschke *et al.*, 1994).

A field of application is not only the assay of the compounds themselves. A promising application is the measurement of enzyme activities (Wollenberger, Paeschke and Hintsche, 1994) and their indication as label in enzyme immunoassays (Niwa *et al.*, 1993).

The determination of alkaline phosphatase and ß-galactosidase activity is based on

quantitation of enzymatically generated p-aminophenol from p-aminophenyl phosphate or aminophenylated pyranose derivatives that are electrochemically inactive at the working potential of the interdigitated electrodes. Thus, a highly sensitive electrochemical enzyme immunoassay can be created with this technique.

BIOELECTROCHEMICAL RECYCLING

In the bioelectrocatalytic approach the target analyte is recycled between the electrode and the redox centre of the enzyme, thus mediating the charge transfer to the electrode. Therefore the enzyme product has to be essentially electroactive (*Figure 12*). In ideal cases sufficient (co)substrate of the enzyme is present, the overpotential required for regeneration is low, and the analyte is stable in both redox states.

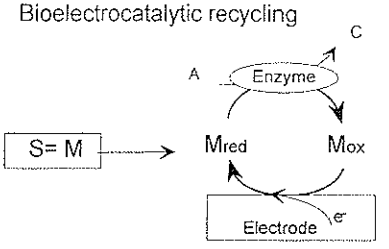

Figure 12. Scheme of bioelectrocatalytic analyte regeneration.

Vital for a rapid heterogeneous electron transfer is the close contact of enzyme and electrode material. Starting from surface immobilisation using adsorption, covalent binding, and entrapment of the redox enzyme, bulk modification procedures have been established, the latter appearing to be the most effective way. Improvement results from the additional integration of modifier and promoter, which binds the protein to the electrode surface and, while not itself taking part in the electron transfer process, encourages electron transfer with the protein to proceed. The influence of an optimal orientation of the redox molecule has been extensively studied in recent years with regard to direct electron transfer beween enzyme and electrode (for example, see Hill and Hunt, 1992).

Already in 1984 Wasa and coworkers reported on the enhancement by laccase of sensor response for, e.g., adrenaline, hydroquinone, and o-phenylene diamine. Since then a number of other enzymes have been investigated, which belong in the great majorityof cases to the family of copper oxidases. Very recently bioelectrocatalysis has been accomplished also with PQQ-dependent dehydrogenases (*Table 3*).

Obviously, with one exception in the reported cases carbonaceous electrode material has been employed. This material facilitates fast redox conversion of quinones and related compounds. Therefore, these substances could be detected in the nanomolar concentration range, while the amplification compared to a simple electrochemical detection was moderate. For example the immobilisation of PQQ-dependent glucose dehydrogenase in polyvinyl alcohol onto the surface of a glassy carbon electrode

results in a very sensitive detector for various quinonoid compounds. For p-benzoquinone (BQ) the enzymatic reaction is :

$$\text{Glucose} + \text{BQ} \xrightarrow{\textit{glucose dehydrogenase}} \text{gluconolactone} + \text{p-hydroquinon} \quad \text{Eq.(9)}$$

The recycling works most effectively when the electrode is polarized at a fairly high overvoltage of +0.5 V (vs. SCE) in order to permit diffusion limitation of the electrochemical reaction and glucose is present in excess. Hence p-aminophenol, hydroquinone, and adrenaline were detected in nanomolar concentration with the lower detection limit being 0.5 nmol/l (Eremenko *et al.*, 1995). The integration of the enzyme to a polyethylenediimine-modified graphite stabilizes the enzyme and at the same time permits an effective electron shuttle between the electrode and PQQ (Wollenberger, unpublished observations). The restricted shelf-life can be dramatically increased by covalent binding of the enzyme to self-assembling (chemisorbed) bifunctional reagents on gold surfaces (Jin *et al.*, 1995).

Table 3. List of bioelectrocatalytic recycling electrodes for di(hydroxy)phenols such as aminophenol, catechol, dopamine, adrenaline, noradrenaline, and hydroquinone

Enzyme	Electrode material	Detection limit, nmol/l (amplification rate)	Comment	Reference
Laccase	RVC	70–200 (17)	glutardialdehyde	Wasa *et al.*, 1984
	GC	600	AQ, measurements in organic solvents	Wang *et al.*, 1993
	CPE	2		Wollenberger, unpublished
Tyrosinase	GC	5		Skladal, 1991
	CPE	10–40	bulk modification with PEI promoter	Gorton, 1994
				Ortega *et al.*, 1993
Glucose (PQQ) dehydrogenase	GC	0.5–20 (<300)	PVA	Eremenko *et al.*, 1995
	CPE	0.5	bulk modification with PEI promoter	Wollenberger, unpublished
	Au	5.0	covalent multi-layer assembly	Jin *et al.*, 1995
Fructose dehydrogenase	GC			Ikeda, Matsuchita and Senda, 1991
Glucose oxidase	GC	1.0 (60–120)		Mizutani, Yabuki and Asai, 1991
	GC			Ikeda, 1984
Cytochrome b_2	GC		gelatin membrane	Scheller and Schubert, 1989

RVC-reticulated vitreous carbon, GC-glassy carbon, CPE-carbon paste electrode

The interaction of tyrosinase with a carbon electrode has been extensively studied by Gorton and co-worker (Ortega *et al.*, 1993; Gorton, 1995; Lutz *et al.*, 1995). Tyrosinase oxidizes phenolic substances in the presence of oxygen, forming in a first step a quinone, which can be very effectively reduced at a potential near 0 V (vs SCE). At this potential the sensor is essentially free of interfering reactions. Due to the bioelectrochemical recycling the electrochemical response is amplified and detection as low as 40 nmol/l is possible (Ortega *et al.*, 1993). Tyrosinase-based sensors have been used for monitoring a spectrum of mono- and diphenolic compounds which are

of particular interest for environmental control in aqueous (Gorton, 1995), nonaqueous, and gas phases (Karayannis *et al.*, 1994; Kaisheva *et al.*, 1995).

The bioelectrocatalytic sensing principle is not restricted to enzyme substrates. Smit and Rechnitz (1993a) demonstrated the application of a tyrosinase-based sensor for cyanide detection. The inhibition of tyrosinase results in a diminished rate of ferrocyanide oxidation and therefore a decrease of the sensor signal. Furthermore, benzoic acid has been determined; the inhibition, however, depends on the nature of the redox mediator used (Smit and Rechnitz, 1993b).

ENZYMATIC RECYCLING

In the bienzymatic approach, the sensitivity enhancement is provided by shuttling the analyte between enzymes acting in a cyclic series of reactions accompanied by cosubstrate consumption and accumulation of by-products (*Figure 13*).

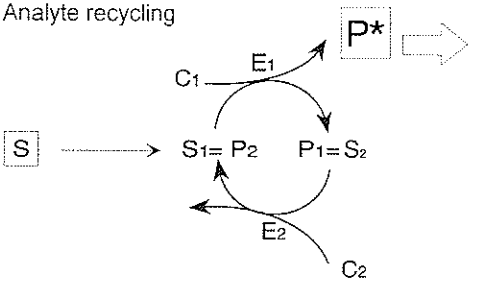

Figure 13. Scheme of enzymatic analyte recycling. C represent cosubstrate of the enzymatic reactions.

The target analyte S can be a substrate or coenzyme of the appropriate enzyme. Assuming a sufficiently high activity of enzyme E_1 in the presence of its cosubstrate C_1 and an analyte at a concentration far below its Michaelis constant, an amplification is achieved by turning-on the second enzyme (E_2) by addition of its cosubstrate C_2.

By measuring the concentration change of one of these coreactants directly or in an additional analytical step, the recycling system is used as a biochemical amplifier for the analyte ($S = S_1$ or S_2). Theoretical considerations have been made for the case of linear recycling by a pair of enzymes. According to theory (Kulys, Sorochinsky and Vidziunaite, 1986) the amplification factor, G, is under steady state conditions:

$$G = k_1 k_2 L^2 / 2D(k_1 + k_2) \qquad \text{Eq. (10)}$$

where k_i is the first order rate constant, L the membrane thickness, and D the diffusion coefficient. At high activities of both enzymes immobilized into the enzyme layer with high characteristic diffusion time (L^2/D), the possible amplification is very large.

LINEAR RECYCLING WITH OXIDASE AND DEHYDROGENASE

When one molecule of product is formed per substrate molecule the total concentration of intermediate substrates (S1 + S2) remains constant and the concentrations of

Table 4. Substrate recycling in biosensors

Analyte	Enzyme couple	Transducer	Amplification factor	Reference
Glucose	glucose oxidase/ glucose DH	oxygen electrode	10	Schubert *et al.*, 1985
Lactate/pyruvate	lactate oxidase/ lactate DH	oxygen electrode	48 000	Scheller *et al.*, 1992
			4100	Wollenberger *et al.*, 1987a
			250	Mizutani *et al.*, 1985
		Pt-ring electrode	10^9	Raba and Mottola, 1995
Lactate/pyruvate	cytochrome b2/ lactate DH	Pt-electrode	10	Schubert *et al.*, 1985
NADH/NAD$^+$	peroxidase/glucose DH	oxygen electrode	60	Schubert *et al.*, 1985
Glutamate	glutamate DH/ alanine aminotransferase	modified carbon electrode	15	Schubert *et al.*, 1986
	glutamate oxidase/ glutamate DH	oxygen electrode	20	Wollenberger *et al.*, 1989
	glutamate oxidase/ alanine aminotransferase	hydrogen peroxide electrode	500	Yao *et al.*, 1989
ADP/ATP	hexokinase/pyruvate kinase	oxygen electrode with lactate DH/ lactate monooxygenase	220	Wollenberger *et al.*, 1987b
		modified carbon electrode with glucose-6-phosphate DH	1200*	Yang *et al.*, 1991
ADP	myokinase/pyruvate kinase	oxygen electrode with pyruvate oxidase	800	Pfeiffer *et al.*, 1994
Ethanol	alcohol oxidase/ alcohol DH	oxygen electrode		Hopkins, 1985
Adrenaline	(PQQ)glucose	oxygen electrode	10 000	Ghindilis, Makower
p-aminophenol	DH/laccase		5000	and Scheller, 1995
ferrocene deriv.		antimony pH electrode	1000	Eremenko Makower and Scheller, 1995
Phenol derivatives	(PQQ)glucose	oxygen electrode	100–1000	Eremenko *et al.*, 1995
adrenaline	DH/tyrosinase			
peptides (containing tyrosine)			150–450	Makower *et al.*, 1995
Adrenaline p-aminophenol	oligosaccharide DH/ Laccase	oxygen electrode	3000	Bier *et al.*, 1995
Benzoquinone/ hydroquinone	fructose DH/laccase	oxygen electrode	700	Wollenberger unpublished
Benzoquinone/ hydroquinone	cytochrome b2/ laccase	oxygen electrode	500	Scheller *et al.*, 1992
Malate/oxalacetate	malate DH/ lactate monooxygenase	oxygen electrode	3	Scheller *et al.*, 1988
L-Leucine	leucine DH/ amino acid oxidase	oxygen electrode	40	Scheller *et al.*, 1990
Phosphate	nucleoside phosphorylase/alkaline phosphatase	oxygen electrode with xanthine oxidase	20	Wollenberger, Schubert and Scheller, 1992
Phosphate	maltose phosphorylase/acid phosphatase	hydrogen peroxide electrode with glucose oxidase	n.d.	Warsinke and Gründig, 1994

DH- dehydrogenase. *- in combination with intermediate accumulation

the coreactants increase or decrease linearly with time. Then the number of cycles in which the substrate is turned over in a given time is a function of the substrate concentration. This concept of linear enzymatic signal amplification has been realized by coupling dehydrogenases with oxidases or transaminases, or by coupling kinases with each other. When oxidases are coupled with their respective dehydrogenases, electrode detectable species are included in the reaction system. Therefore, the change of coreactant concentration can be measured directly at the electrode onto which the recycling enzyme pair is immobilized. In most cases oxygen consumption has been followed (*Table 4*).

Depending on the measurement arrangement, enzymes, and membrane materials used, amplification by several orders of magnitude has been realized. Detection limits as low as 10^{-15} mol/l have been achieved. When dealing with extremely high amplification one has to bear in mind, however, that the sensor signal becomes highly susceptible to minute amounts of contaminants affecting the enzyme reactions or the diffusion of the reactants. For example laccase, which oxidizes a wide range of substances including catecholamines, phenols, and ferrocyanide by dissolved oxygen has been used in combination with haem and pyrroloquinoline quinone (PQQ) containing (NADH independent) dehydrogenases.

$$\text{Mred} + O_2 \xrightarrow{\textit{laccase}} \text{Mox} + H_2O \qquad \text{Eq. (11)}$$

$$\text{Glucose} + \text{Mox} \xrightarrow{\textit{glucose (PQQ) dehydrogenase}} \text{gluconolactone} + \text{Mred} \qquad \text{Eq.(12)}$$

An ultrasensitive biosensor has been created in which PQQ dependent glucose dehydrogenase from *Acinetobacter calcoaceticus* and laccase from *Coriolus hirsutus* were co-entrapped in a 10 μm layer of polyvinyl alcohol in front of a Clark-type oxygen electrode (Ghindilis, Makower and Scheller, 1995). Owing to the broad spectrum of cosubstrates for both enzymes the sensor responds to various catecholamines, phenols, and ferrocene derivatives. The highest sensitivity was obtained for aminophenol and adrenaline, where the lower limit of detection (S/N 3:1) is 70 p mol/l and 1 nmol/l, respectively (*Figure 14*). The extraordinary efficiency of the amplification sensor is based on the excess of enzyme molecules compared with the concentration of the analyte molecule within the reaction layer. The current density of the membrane-covered sensor is almost three orders of magnitude higher than is the bare electrode. The electrode can also be used for the detection of low amounts of ferrocene derivatives. This is of particular interest for the development of immunoassays.

As an alternative to the oxygen sensor the enzyme couple has been combined with an antimony electrode that indicated the pH shift during the recycling process (Eremenko, Makower and Scheller, 1995). The accumulation of H^+ is not as pronounced as the diminution of the dissolved oxygen concentration. Therefore, the response of the potentiometric sensor is somewhat smaller than that of the amperometric system.

Oligosaccharide:acceptor oxidase (oligosaccharide dehydrogenase) has been employed in place of the above mentioned glucose dehydrogenase. With this enzyme couple a rather broad spectrum of substances, such as aminophenols, diamines, and

catecholamines is accessible to sensitive detection, but with different sensitivities as compared with the laccase-GDH couple.

$$\text{Maltose} + \text{Mox} \xrightarrow{\textit{oligosaccharide dehydrogenase}} \text{maltose lactone} + \text{Mred} \quad \text{Eq. (13)}$$

The highest sensitivity has been achieved for p-aminophenol, achieving a response of 35 nA l/µmol, while adrenaline was half as effectively recycled. This compares well with results obtained with other dehydrogenase -laccase pairs. Obviously laccase specificity determines the relation.

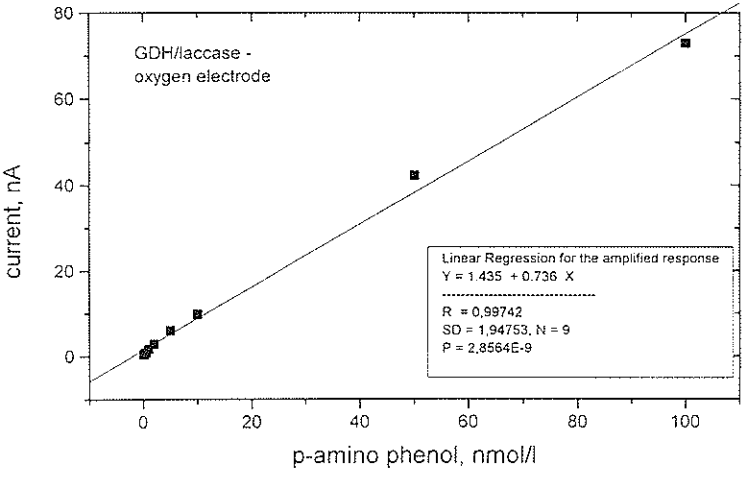

Figure 14. Dependence of the oxygen consumption of a glucose dehydrogenase/laccase electrode on p-aminophenol in presence of 25 mmol/l glucose, i.e. with recycling.

The potential of the amplification of the sensor response by enzymatic substrate recycling has been demonstrated for the determination of catecholamine secretion in cell cultures, of goat IgG and human thyroid stimulating hormone in a sandwich type immunoassay (Bier *et al.*, 1995) and in cocaine displacement assay (Makower *et al.*, 1994). Both alkaline phosphatase and ß-galactosidase label were indicated by the rate of release of p-aminophenol from aminophenylated substrates.

The cocaine assay uses the well-studied enzymatic recycling pair lactate oxidase/ lactate dehydrogenase (Equations (4) and (8)).

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\textit{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+ \quad \text{Eq. (4)}$$

$$\text{Lactate} + \text{O}_2 \xrightarrow{\textit{lactate oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2 \quad \text{Eq. (8)}$$

The oxygen consumption in the gelatin membrane bearing both enzymes is enhanced, yielding an increase in the sensitivity to lactate by a factor of up to 4100 (Wollenberger *et al.*, 1987a). The experimental results have found to be in line with theory (Equation 10). Thus, the study of the influence of the enzyme loading, i.e. the amount of enzyme activity in the membrane, on the amplification of the lactate signal

showed, in agreement with the theory, that a 4100-fold amplification was reasonable using the gelatin matrix with the characteristic diffusion time of about 90 s.

With this amplification sensor lactate concentrations as low as 1 nmol/l could be determined with reasonable precision. On the other hand, for the lactate response signal an up to 48000-fold enhancement was achieved when the immobilisation was performed with polyurethane, which permits higher enzyme loading (Scheller *et al.*, 1992).

However, the reproducibility and stability of this large amplification is poor.

The amplification of the lactate response decreases with progressive enzyme inactivation during sensor operation, while the unamplified lactate signal (using only lactate oxidase) remains stable. This behaviour is due to the fact that the enzyme excess maintains diffusion control of the sensor for the simple process.

LINEAR RECYCLING WITH KINASES

Recycling systems are not necessarily limited to reactions in which electrochemically active compounds are produced. In these other cases the recycling enzyme pair is combined with an indicator enzyme (or sequence) transforming one of the cycle coreactants (mostly a product) into a measurable species. Owing to their usually favourable equilibrium constants kinase reactions are well applicable to recycling experiments.

For the sensitive measurement of ADP and ATP a biosensor has been constructed by combining an oxygen electrode and a gelatin layer comprising the enzyme pair pyruvate kinase and hexokinase catalyzing:

$$\text{Glucose} + \text{ATP} \xrightarrow{\textit{hexokinase}} \text{glucose 6-phosphate} + \text{ADP} \qquad \text{Eq. (14)}$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\textit{pyruvate kinase}} \text{pyruvate} + \text{ATP} \qquad \text{Eq. (15)}$$

and a lactate dehydrogenase and lactate monooxygenase sequence (Equations (4) and (5)). The latter, which was also used in intermediate accumulation experiments, transforms the very large amount of pyruvate formed in the recycling reaction into oxygen consumption (Wollenberger *et al.*, 1987b). In contrast to lactate/pyruvate recycling sensors ADP and ATP were indicated with different sensitivities, which were mainly attributed to their deviating diffusion coefficients. The sensitivity in the linear measuring range for ADP was amplified by a factor of 220, while the ATP response was slightly higher (*Figure 15*).

The lactate dehydrogenase/lactate monooxygenase indicator sequence has been substituted in addition by pyruvate oxidase or glucose-6-phosphate dehydrogenase (Yang *et al.*, 1991). Two different cycling enzyme arrangements have been proposed for the determination of inorganic phosphate. Interestingly, both set-ups include the production of two indicator molecules for each cycle of phosphate conversion. The first approach comprises nucleoside phosphorylase and alkaline phosphatase for phosphate recycling, and xanthine oxidase for detection of the liberated hypoxanthine (Wollenberger, Schubert and Scheller, 1992). Hypoxanthine is oxidized in a two step reaction; thus per molecule of phosphate cycled or hypoxanthine formed two molecules of oxygen are consumed. Only 5 % of the response to phosphate addition could

be observed when the alkaline phosphatase was omitted. However, in the recycling sensor a slight inactivation of enzyme leads to decrease in recycling rate after a few measurements. In order to prevent hydrogen peroxide from inactivating the enzymes catalase has been co-immobilized. The resulting sensor has full activity over a period of eight days with a measuring range of 0.5–50 µmol/l. Very recently the combination of maltose phosphorylase and acid phosphatase has been claimed (Warsinke and Gründig, 1994). The phosphate dependent phosphorylation of maltose proceeds with formation of one molecule of glucose and glucose-1-phosphate. The hydrolysis of the latter liberates a second glucose molecule and phosphate, which enters again the cycle. A glucose oxidase membrane has been employed additionally in order to indicate glucose formation.

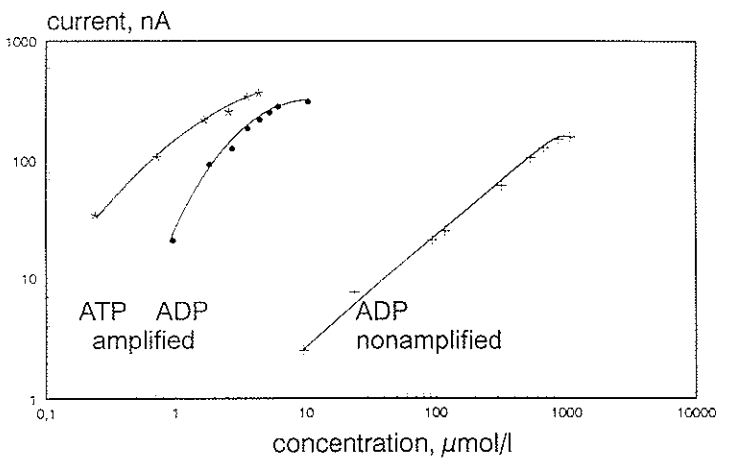

Figure 15. ADP and ATP concentration dependence of a sensor comprising pyruvate kinase/hexokinase for analyte recycling and lactate dehydrogenase/lactate monooxygenase for indication of pyruvate accompanied by oxygen consumption.

EXPONENTIAL RECYCLING WITH KINASES

Enormous signal amplification is to be expected if in the cycling reaction more than one analyte molecule is regenerated. Here the total amount of intermediates and by-products is increasing exponentially with time. Theoretical considerations show that the concentration of any of the cycling intermediates or byproducts at any given time is a linear function of the initial substrate concentration (Kopp and Miech, 1972). An example illustrating this principle is the ADP/ATP cycling system myokinase/pyruvate kinase (Pfeiffer *et al.*, 1994). These enzymes have been co-immobilized on top of a pyruvate oxidase modified oxygen sensor. In a single cycle two molecules of ADP are formed by myokinase per molecule ATP derived from the phosphorylation of ADP by pyruvate kinase.

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\textit{pyruvate kinase}} \text{pyruvate} + \text{ATP} \qquad \text{Eq. (15)}$$

$$\text{ATP} + \text{AMP} \xrightarrow{\textit{myokinase}} 2\ \text{ADP} \qquad \text{Eq. (16)}$$

The amount of ATP and ADP initially present in a very low concentration is increasing exponentially with cycling time, when AMP and phosphoenolpyruvate concentrations are high enough to ensure no cosubstrate limitation. The abundance of pyruvate formed in the cycle is manifested by the oxygen consumption in the pyruvate oxidase layer. With an optimized sensor configuration an increase of sensitivity for ADP by a factor of 800 was obtained, resulting in a measuring range between 50 nmol/l and 2 μmol/l.

MONOENZYMATIC SUBSTRATE RECYCLING

Lactate dehydrogenase exhibits the unique property of catalyzing forward and backward reactions when the respective reagents are present. On the basis of this characteristic the recycling of NADH is performed by LDH alone (Schubert, Scheller and Krasteva, 1991). In the presence of NAD^+, LDH catalyzes the oxidation of glyoxylate to oxalate with concomitant formation of NADH. In the presence of pyruvate the cofactor is cyclically regenerated. Necessarily, this effect can be observed only at limiting amounts of pyridine nucleotide. Thus a 170-fold increase in lactate formation results in a detection limit for NADH of 50 nmol/l. This kind of monoenzymatic cycle might facilitate the design of novel, simple enzymatic amplification schemes. On the other hand, the multiplication of the amplification by a second recycling enzyme pair in a double amplification system may yield a further increase in sensitivity.

Sequential activation of enzymes

An enzymatic reaction cascade can be defined as sequential activation of a series of enzymes triggered, for example, by ligand binding to a receptor and resulting in a large scale amplification of the initial step or signal.

Figure 16 illustrates the application of this principle for signal generation in biosensors.

The binding of the activator (initiator) transforms the inactive enzyme E_{1i} to its active form E_{1a}. This enzyme activates a second inactive enzyme and so forth. At the end of the cascade an enzyme is activated which produces the product P' indicated by the transducer.

In the case of glycogenolysis binding of adrenaline to the ß-adrenoceptor activates adenylate cyclase as starting enzyme followed by a sequence of phosphorylation reactions, the last of which is the phosphorylation (activation) of glycogen phosphorylase (phosphorylase *b* → *a* transformation). The signal amplification provided by this cascade is of six orders of magnitude (Fischer *et al.*, 1976). Glycogen phosphorylase is a key enzyme in glycogenolysis. This enzyme catalyzes the sequential phosphorolytic removal of terminal glycosyl residues from the non-reducing end of glycogen. The products of the reaction are –glucose–1–phosphate and a cleaved glycogen (glycogen$_n$ with n glucose residues → glycogen$_{n-1}$ with (n–1) glucose residues).

$$\text{Glycogen}_n + P_i \xrightarrow{\textit{phosphorylase}_{active}} \text{glycogen}_{n-1} + \text{a–glucose–1–phosphate} \quad \text{Eq. (17)}$$

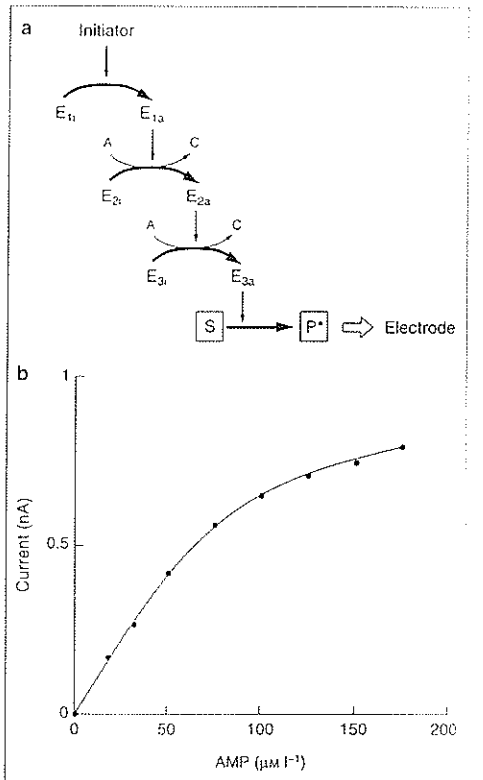

Figure 16. Schematic illustration of sequential activation of enzymes in biosensors. A and C represent co-reactants. (b) AMP-concentration dependence obtained with a glycogen phosphorylase *b*/alkaline phosphatase/mutarotase/glucose oxidase electrode in presence of glycogen, oxygen, and phosphate. (Reproduced from Wollenberger and Scheller, 1993, with permission of Elsevier Sc. Publisher).

Muscle glycogen phosphorylase *b* is active only in the presence of AMP, which acts as an allosteric activator. The proportion of the active enzyme can be determined primarily by the rates of phosphorylation in presence of dispersed glycogen and ortho phosphate with and without AMP, with the following reaction sequence immobilized in the membrane of a hydrogen peroxide indicating electrode (Wollenberger and Scheller, 1993):

$$\alpha\text{-Glucose-1-phosphate} \xrightarrow{\textit{alkaline phosphatase}} \alpha\text{-glucose} + P_i \qquad \text{Eq. (18)}$$

$$\alpha\text{-Glucose} \xleftrightarrow{\textit{mutarotase}} \beta\text{-glucose} \qquad \text{Eq. (19)}$$

$$\beta\text{-Glucose} + O_2 \xrightarrow{\textit{glucose oxidase}} \text{gluconolactone} + H_2O_2 \qquad \text{Eq. (20)}$$

At the compromise pH of 7.5 the sensitivity is sufficient to measure 1 μmol/l glucose–1–phosphate. The rate of glucose–1–phosphate formation represents the

activity of glycogen phosphorylase *b*. The three-enzyme sensor can be used for the determination of 0.005–0.2 U/ml glycogen phosphorylase *a* and *b*.

As implied above, glycogen phosphorylase *b* activity is controlled by the concentration of AMP. For the determination of AMP glycogen phosphorylase *b* was coentrapped with its substrate glycogen in a separate compartment in front of the above three enzyme layer.

The sensor responded repeatedly to micromolar concentrations of AMP with a response time of 20 s. Removal of AMP resulted in the establishment of the baseline, i.e. an inactivation of phosphorylase *b*. The calibration plot of current versus AMP concentration gives a linear range from 5 to 75 μmol/l (*Figure 16b*). Increasing the AMP concentration above 150 μmol/l did not further magnify the response. The increase in the apparent K_M value from 2 μmol/l in solution to 50 μmol/l in the membrane strongly supports the assumption of diffusion control.

Since the effect of AMP is an enzyme activation, the AMP response reflects an amplification of the probe sensitivity, which is considerably higher than that for glucose-1-phosphate. The reagent-free arrangement could be used for about 20 repetitive AMP measurements. Then the sensitivity declines owing to exhaustion of the substrate, glycogen.

Conclusions

The performance of biosensors can be tailored by coupling enzymatic reactions to the base transducer. Using cycling enzymatic reactions, sequentially acting enzymes and competing reactions a wider range of substances becomes measurable and also the selectivity and sensitivity of the probe is improved.

The investigation of the allosteric activation of enzymes in sensor membranes opens up a new way of sensor performance. The covalent chemical modification of physiologically important proteins make their effectors accessible to bioelectroanalysis. Thus, biosensors are not only suitable for the measurement of substrates, enzyme activities, inhibitors, and prosthetic groups of enzymes, but also allow the determination of activators of allosteric enzymes. Future prospects in interdisciplinary biosensor research are expected from developments in fields such as microelectronics, bioengineering, and polymer chemistry. This might include chemical and genetic modification of proteins, procedures for oriented immobilization of the biomolecules, and microstructured transducers. Thus, sensors with novel characteristics are envisaged that will further accelerate their practical application.

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