An enzyme biosensor based on gold interdigitated thin film electrodes for water quality control

M. Marrakchi1*, S.V. Dzyadevych1,2, Ph. Namour3, C. Martelet1, N. Jaffrezic-Renault4

1 CEGELY, UMR-CNRS 5005, ECL-Lyon, 69134 Ecully Cedex, France, Email: mounamarrakchi@yahoo.fr
2 Laboratory of Biomolecular Electronics, Institute of Molecular Biology and Genetics, National Academy of Science of Ukraine, 150 Zabolotnogo Street, Kiev 03143, Ukraine
3 Cemagref of Lyon, 3 bis quai Chauveau, 69336 Lyon cedex 09, France
4 Laboratory of Analytical Chemistry, UMR-CNRS 5180, Claude Bernard University Lyon 1, 69622 Villeurbanne Cedex, France

Abstract

A conductometric proteinase K biosensor for organic matter monitoring in rivers has been developed. In fact, with approximately 30% of the total Chemical Oxygen Demand (COD), proteins were chosen to be used as indicators of urban pollution. Proteinase K hydrolyzes proteins into different ionic amino-acids which results in local conductivity changes. In this work, we began with the optimization of biosensor response using bovine serum albumin (BSA) as standard protein. A stable biosensor with a constant repeatability and a detection limit about 0.5 µg/mL BSA were obtained. Then, response biosensor was tested with samples of rivers water. Good correlations between conductance changes and values given by standard methods (chemical oxygen demand and protein concentration evaluated by microBCA protein assay) have been shown.

Keywords: Gold electrode, biosensor, proteinase K, water quality

Introduction

The increasing demand by citizens and environmental organizations for cleaner rivers and lakes, groundwater and coastal beaches has been evident for a long time. It has been once

*Corresponding author. Tel.: +33 4 74 47 21 44; Fax : + 33 4 74 45 52 53. E-mail address: mounamarrakchi@yahoo.fr
more confirmed by the last European Water Framework Directive (Directive 2000/60/EC, 2000). The new European Water Policy will get polluted waters clean again, and ensure that clean waters are kept clean. There is a number of objectives in respect of which the quality of water is protected. The key ones at the European level are the general protection of the aquatic ecology, a specific protection of unique and valuable habitats, the protection of drinking water resources, and the protection of bathing water. In addition, the directive which introduced new concepts of “good chemical state” and “good ecological state” requires the evaluation of polluting fluxes. It is clear that the requirements of most traditional analytical methods, both in terms of time and costs, make them unsuitable for the application of this directive. Water quality control needs an early warning system for on line and in situ pollution monitoring.

As proteins constitute more than 30 % of total chemical oxygen demand (COD) in the effluents, they seem to represent a good indicator of the organic matter content (Namour, 1999). However, the most widely used methods for protein analyses are based on classical laboratory methods grounded on colorimetric procedures which are not convenient for in situ monitoring of organic matter content. In this context, biosensors appear as a suitable alternative or as complementary analytical tools (Rodríguez-Mozaz, 2005).

A biosensor associates a bioactive sensing layer with any suitable transducer giving a usable output signal. The characteristics and performance of such devices appear attractive: sensitivity, reliability, handiness, simplicity, rapidity and cheapness. Many biosensor-based analyzers are now available or are under development by many companies. The use of an enzyme in association with a specific electrode was the first proposed type of biosensor. The enzyme is immobilized on the surface of the electrochemical transducer, and the electrical signal is obtained (Clark, 1962; Updike, 1967). Many enzyme-based systems were used for
environmental monitoring (Xavier, 2000; Abad, 1998; Supruna, 2004; Anh, 2005) and many of them have been reviewed by Karube et al. (Karube, 2000) and Rogers (Rogers, 2006).

In this work, a protease “proteinase K” was immobilized on interdigitated conductometric gold electrodes. The conductometric transducers have important advantages. In fact, they do not need the use of reference electrode; they operate at low-amplitude alternative voltage preventing Faraday processes on electrodes; they are light insensitive, they offer a good ability to miniaturization and a high-level integration by using a cheap thin film standard technology. The Proteinase K is an endolytic serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids (Ebeling, 1974). The enzyme was immobilized by cross-linking with glutaraldehyde. The resulting conductivity changes are produced by enzymatically catalyzed hydrolysis of the substrate. First, conductometric biosensor has been optimized by using bovine serum albumin (BSA) as substrate. Then, biosensor response was tested with different samples of natural rivers water and compared with values given by standard methods.

Experimental

Materials

Proteinase K (EC 3.4.21.64, freeze-dried from Tritirachium album) and bovine serum albumin (BSA) were purchased from Sigma. Glutaraldehyde (grade II, 25 % aqueous solution) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other reagents were of analytical grade and were used without any further treatment. Millipore Milli-Q nanopure water (resistivity 18.2 Ωcm) was used throughout for the preparation of solutions.
Sensor design

Two identical pairs of gold interdigitated electrodes (thickness 0.5 mm, dimensions 5×30 mm) were fabricated by vacuum deposition on a ceramic substrate (sintered aluminum oxide) at the Institute of Semiconductors Physics, Kiev, Ukraine (Fig.1). An intermediate layer of chromium (0.1 µm thick) was used for better gold adhesion. Each finger of the electrode was 20 µm wide and 1mm long, with 20 µm spacing between fingers of the electrodes in the pair. The sensitive area of each electrodes pair was about 1×1.5 mm. To define the sensitive area of the transducer, the central part of the chip was covered with epoxy resin.

Enzyme immobilization

The enzyme membrane (Fig. 2) was prepared on the transducer surface by cross-linking of proteinase K with bovine serum albumin (BSA) in a saturated glutaraldehyde vapor (Dzyadevich, 1994; Marrakchi, 2006; Anh, 2004). Two mixtures were prepared. First, a mixture of 4% (w/w) of proteinase K and 6% of BSA was prepared in 20 mM phosphate buffer pH 7.5 with 10% of glycerol. As a differential experimental set-up was used, we prepared two electrodes: the working one coated with the enzyme membrane and the reference one with only a mixture of 10% BSA and 10% glycerol in phosphate buffer. Then, the interdigitated electrodes were placed in saturated GA vapor for 20 minutes (optimum time of immobilization showed in (Marrakchi, 2005)). After exposure, membranes were dried at room temperature from 15 to 30 min.

Measurements

Measurements were conducted in open cell filled with 5 mM phosphate buffer pH 7.4 magnetic-stirred at room temperature. Generator was employed to generate sinusoidal wave of 100 KHz frequency with small-amplitude alternating voltage (10 mV peak-to-peak about 0
These conditions were used to reduce faradic processes, double-layer charging and concentration polarization at the microelectrode surface. After stabilization of output signal, substrate concentrations were increased stepwise by adding defined volumes of concentrated solution. Output differential signal between the electrodes covered with immobilized enzyme and those with the blank membrane after low noise differential amplifier fed into a Stanford Research Systems SR510 Lock-in amplifier supplied with a reference signal from its internal oscillator. The use of this differential mode makes possible the elimination of all non specific charge variation such as variation of the ionic strength. It has been shown in a previous work that the salinity of the measuring media nearly did not influence the sensor response (Marrakchi, 2005). Thus, the output of the lock-in amplifier is directly proportional to the cell conductance and the responses of the biosensor were recorded as a function of substrate concentration.

Storage

Biosensors are prepared and then stored, between the experiments, at 4°C in the 5 mM phosphate buffer solution, pH 7.4.

Results and discussion

Biosensor development

For protein detection, an enzyme: proteinase K was immobilized on direct contact with the sensor chip. Proteinase K belongs to the class of serine endopeptidase. This protease was chosen because of its strong proteolytic activity on both denatured and native proteins and its large range of pH optimum (Ebeling, 1974). The hydrolyze of proteins in contact of the enzyme generates charged peptides and/or free amino acids (figure 3) which lead to local conductivity changes in the layer containing the immobilized enzyme.
The dependence of the steady-state responses of the proteinase K based conductometric biosensor on the concentration of a standard protein BSA (Bovine Serum Albumin) is shown in figure 4. The steady-state response is the point at which the output signal is getting saturated. The linear range for BSA determination was from 0.8 to 6 µg/mL with good sensitivity. After 6 µg/mL, we have a saturation phenomenon. This range of response obtained with the biosensor is in adequacy with the values of the real concentrations of proteins in rivers water (Namour, 1999).

Statistical analysis of biosensor response after many times repetitions as AFNOR XP T90-210 method (Afnor, 1999) showed that we have a good reproducibility, a constant repeatability and a detection limit about 0.5 µg/mL BSA. The study of the biosensor stability showed that we have a long period for using the conductometric enzyme sensor. In fact, the sensor is working for more than one month.

Because of the perspectives of using our conductometric biosensor directly on a river study site, it was interesting to know the effect of the temperature on its response given the change of water temperature between the different seasons. As we can see in figure 5, our biosensor response depends on the temperature. Up to 30°C for higher temperature, higher response, that’s why for rigorous analysis, temperature must be taken into account.

*Analysis of rivers water*

After the development of the proteinase K conductometric sensor, we tested biosensor response after the addition of some samples of water from different rivers point near Lyon (Rize downstream D112, Rize downstream bridge of Cusset, Jonage channel, Rhône Fessine, Chaudanne drain, Chaudanne downstream Leclerc, Yzeron bridge of Chabrol), France. The water samples of the rivers were filtered beforehand through a filter of 0.45 µm to eliminate insoluble compound and microorganisms. The elimination of the microorganisms is important to improve the stabilization of physicochemical characteristic of the samples. In the beginning
the idea was to report biosensor response, after river water injection (diluted if necessary) on the BSA calibration curve to obtain protein concentration in the sample. However, the experiences showed that BSA cannot be used as a standard for the determination of water concentration of protein. In fact, proteins found in natural water are different in size and kind which changes the way of their hydrolysis and analysis by the biosensor, in comparison with the BSA which is a big and complex protein.

In this way, the river water was also analyzed by standards methods to compare it with values given by the biosensor. As proteins were chosen as an indicator of the organic matter in the effluents, we started with the comparison of sensor response and dissolved organic carbon (DOC) values of the different river’s water. The results are shown in figure 6. A quite good linear correlation between the chemical oxygen demand and the response of the biosensor was obtained. This shows that the biosensor can well be used to estimate the organic matter content in the river’s water.

Finally, we used the “Pierce BCA Protein Assay” for the colorimetric detection and quantification of total protein in the different samples (fig. 7). We obtain a good correlation with some differences due to the less good sensibility of the classical method to low protein concentrations.

**Conclusion**

The proteinase K biosensor developed seems to have big potentialities for protein determination in natural water. In fact, the biosensor shows a good reproducibility (3.3 %) and sensibility (detection limit about 0.5 mg/L BSA) and a constant repeatability. In addition, the fast response (7 or 8 minutes) and the capacity of the biosensor to work at low temperatures (10°C) makes it very attractive for in-situ control of water quality.
However, this biosensor is not performed to replace traditional methods but to be used as an early-warning system for on line and in situ pollution monitoring in rivers as the new European Water Policy requires. It can be an analytical tool to save time and costs, with a possibility of making real-time decision on local environmental problems.

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Figure Captions

**Figure 1.** (a) General view of the experimental setup for conductimetric measurements in a differential mode  
(b) Microscopic view of the gold interdigitated microelectrodes

**Figure 2.** Schematic representation of the enzymatic membrane deposited on the gold interdigitated microelectrode

**Figure 3.** Schematic representation of the cleaving reaction with Proteinase K

**Figure 4.** Dependence of biosensor response (µS) on BSA concentration (in 5mM PBS buffer, pH 7.4)  
a: curve of response with saturation effect beginning for 8 µg/mL BSA  
b: linear calibration curve for BSA concentration between 1 and 6 µg/L

**Figure 5.** Effect of temperature on the biosensor response  
(in 5mM PBS buffer, pH 7.4, for a 10µg/ml of BSA)

**Figure 6.** Comparison of biosensor response with DOC (dissolved organic carbon) values in samples of water from different rivers point near Lyon (France)

**Figure 7.** Comparison of biosensor response (µS) with protein concentration values (µg/mL) given by BCA protein Assay values (samples of water from different rivers point near Lyon, France)
Figures

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