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Short communication

Enzymatically modified ion-selective electrodes for flow injection analysis

Robert Koncki *, Izabela Wałcerz, Ewa Leszczyńska

Department of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland

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Abstract

The application of potentiometric biosensors based on membrane ion-selective electrodes for flow injection analysis (FIA) is presented. The biosensors have been obtained by covalent binding of enzyme molecules directly to surface of ion-selective membranes. The biosensor/FIA systems enable the determination of urea and penicillin in the millimolar ranges of concentration. About 30 samples per hour can be analysed in the performed FIA systems. The operational stability of the bioanalytical systems exceeds 1 month. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Flow injection analysis; Ion-selective electrodes; Enzyme immobilization; Urea; Penicillins

1. Introduction

Ion-selective electrodes are well known analytical tools suitable for the simple and fast determination of the activity of many cations and anions [1]. Although ion-sensitive potentiometric sensors do not respond directly to non-ionic species, but certain modifications can be done to broad the range of the determinable analytes. For example, determination of gaseous species is possible due to application of the additional gas-permeable membranes [2]. Another solution is the use of additional membranes which selectively convert non-ionic analytes into ionic species determinable by ion-selective electrodes. Potentiometric enzymatic electrodes are a large group of such sensors [3,4]. These biosensors enable highly selective determination of many important organic and bioorganic compounds: amides, esters, amino acids, acylcholines, β-lactam antibiotics, sugars, purine compounds, etc. and might be useful in clinical, pharmaceutical, food and biochemical analysis. All above mentioned biosensors can be based on ion-selective electrodes sensitive to hydrogen and ammonium ions. Most of them are based on polymeric membranes containing appropriate ionophores

* Corresponding author. Fax: +48-22-8225-996; e-mail: rkoncki@chem.uw.edu.pl.

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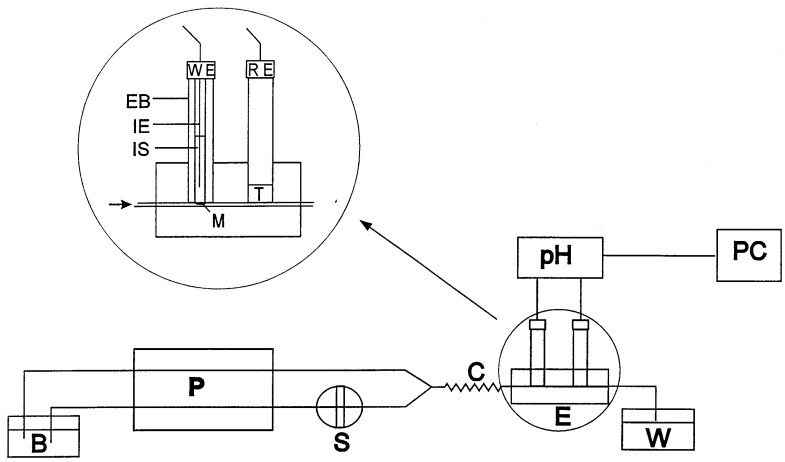

Fig. 1. Scheme of the FIA set-up: carrier buffer (B); peristaltic pump (P); injection sample unit (S); mixing coil (C); flow-through detector cell (E); pH-meter (pH); data collecting computer (PC); waste (W). Flow cell with mounted enzyme electrode is shown in the insert: biosensor (WE); electrode body (EB); internal electrode (IE); internal solution (IS); ion-selective membrane with enzyme layer (M); reference electrode (RE); teflon mounting element (T).

which determine the sensitivity and selectivity of the internal potentiometric electrode.

The application of biosensors in flow injection analysis (FIA) conditions strongly improves their analytical features [5,6]. Measurements are faster, more reproducible and less work-consuming. Moreover, FIA enables the automation of the analytical procedure, including pretreatment of sample before measurement. FIA techniques are based on non-equilibrium measurements and therefore require high sensitivity and short response time of applied biosensors. Moreover, operational stability of the biosensors is essential as FIA systems are designed for frequent use over a long period of time.

In this paper we present the potentiometric biosensors based on ion-selective membrane electrodes for FIA systems, which meet all the above mentioned requirements. An urea biosensor based on an ammonium ion-selective electrode and a penicillins biosensor based on a pH-electrode, were studied. Both biosensors were obtained by covalent linking of enzyme layers (urease and penicillinase, respectively) directly to the surface of respective ion-sensitive membrane. The FIA systems with the enzyme electrodes can find wider applications in pharmaceutical and biomedical analysis.

2. Experimental

2.1. Materials and reagents

Materials used for ion-selective membranes preparations: nonactin, tridodecylamine (ionophores), and bis-(2-ethylhexyl) sebacate (DOS, plasticizer) were from Fluka (Switzerland). Carboxylated poly(vinyl chloride) (PVC-COOH, membrane matrix and immobilization support) was purchased from Aldrich (Germany). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for enzyme immobilization and enzymes: urease (EC 3.5.1.5.) and penicillinase (EC 3.5.2.6.) were obtained from Sigma (St. Louis, MO). Analytical grade reagents and double distilled water were used for all experiments.

2.2. Apparatus and flow injection analysis set-up

The scheme of the FIA set-up is presented in the Fig. 1. Ion-selective electrode bodies (Philips model IS 561, Moller Glasblaserei, Switzerland) were used for the construction of enzymatically modified membrane electrodes. The potentiometric biosensor and the reference calomel electrode (Philips model R11) were coupled to digital pH-meter (Radiometer model PHM 85, Denmark)

connected to a data collecting PC via RS232 interface. Both electrodes were mounted into small flow cell (≈ 10 μl) through which carrier solution was pumped (flow rate: 1.3 ml min^{-1}) using multichannel peristaltic pump (Minipuls 3, Gilson, France). The length of mixing coil was 20 cm. The sample dilution in the FIA system was 4–6. For sample injection home-made rotary injection valve was used. A sample injection volume was 150 μl. Both values: flow rate and sample injection volume were compromise between the increase of system sensitivity and the decrease of the time of single determination. The increase of flow rate resulted in shortening of the response time, but it also decreased the sensitivity of the system, whereas the increase of sample injection volume caused the opposite effects.

2.3. Biosensors preparation

Ion-selective membranes were prepared according to the standard procedure with compositions as follow: nonactin (3%), DOS (64.5%), and PVC-COOH (32.5%), and tridodecylamine (1%), DOS (66%) and PVC-COOH (33%) for ammonium ion and pH sensitive membranes, respectively. For the enzyme immobilization on the surface of ion-selective membrane freshly prepared solutions containing respective enzyme (500 U ml^{-1} of urease or 1500 U ml^{-1} of penicillinase) and EDAC (5mg ml^{-1}) were deposited and left for 24 h. Then electrode was washed in vigorously stirred 0.1 M phosphate buffer of pH 7.0, to remove an excess of the unbound protein. Detailed procedures of fabrication and properties of urea and penicillins biosensors were described in our previous papers [7,8].

3. Results and discussion

3.1. Biosensors

The enzymatic layer of the urea biosensor contains urease which catalyses hydrolysis of urea:

$$(H_2N)_2CO + 3\ H_2O \rightarrow 2\ NH_4^+ + HCO_3^- + OH^-$$

Ammonium ions formed at the membrane surface change the potential of the ammonium ion-selective electrode. The enzymatic layer of the penicillin biosensor contains penicillinase (β-lactamase). The enzyme catalyses the hydrolysis of penicillin:

$$\text{penicillin} + H_2O \rightarrow \text{penicillonic acid}$$

Hydrogen ions produced in the course of the biocatalytic process change the potential of the applied pH-electrode. In both cases the analytical signal of the biosensor (changes of the bioelectrode potential) is proportional to the concentration of the analyte (enzyme substrate).

The choice of the proper method of enzyme immobilization is a crucial step in biosensor construction, determining its quality. An ideal enzyme membrane is expected to be active, stable, thin and easy to prepare. High activity of biosensing layer implies high sensitivity of the biosensor. In most cases the stability of the enzyme membrane is responsible for the lifetime of the biosensor. The response time of the biosensors is generally determined by the thickness of enzyme layer, as the species transport within the membrane determines the dynamics of the whole process. The method of the enzyme immobilization, used in this work, is based on the covalent binding of enzyme molecules directly to the surface of an ion-selective membrane so it allows to obtain sensitive, stable, and fast responding biosensors [7,8]. Direct contact of enzyme with an ion-sensitive membrane enables the effective monitoring of the course of a biochemical processes, which is a source of the analytical signal. As the monomolecular enzyme layer is extremely thin and there is no need to apply any additional protective membranes, the response time of the biosensor is short and similar to that of the internal potentiometric electrode. The high durability of the immobilized enzymes results in long lifetime of the biosensors.

3.2. Biosensor/flow injection analysis system

The above mentioned advantages of the biosensors (their high sensitivity, stability, and rapid response) predestine them for the application in

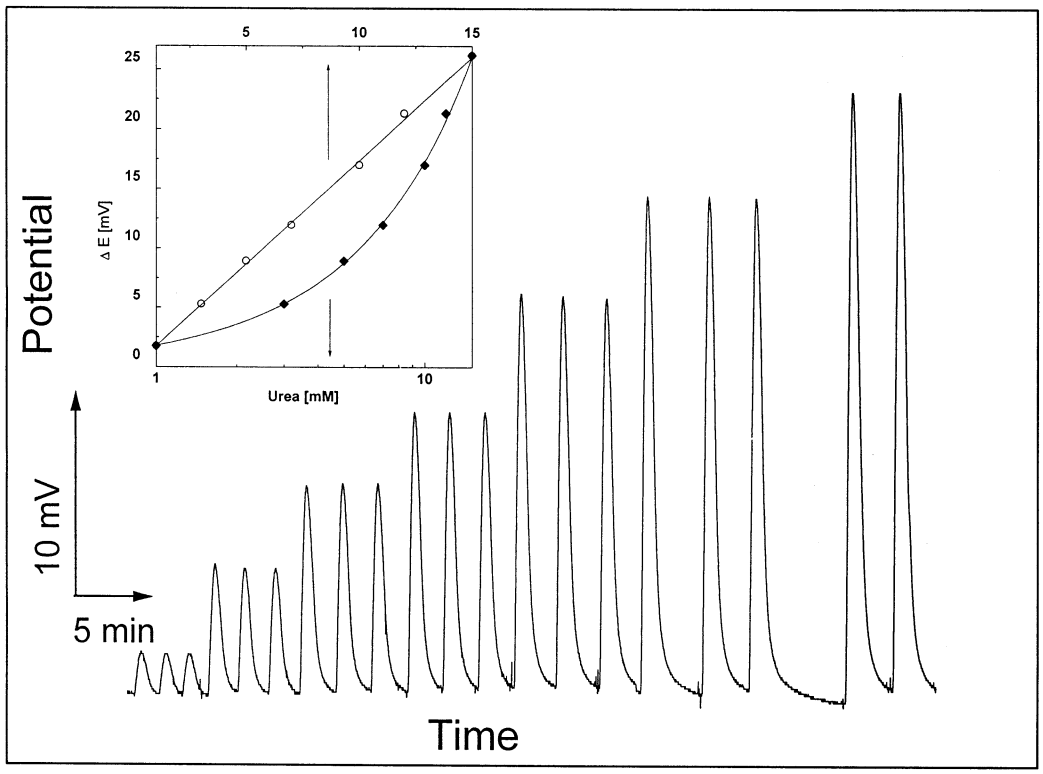

Fig. 2. Analytical response of the urea biosensor in the FIA system. Calibration graphs (insert) are presented in linear and logarithmic scales. The measurements were performed in 0.02 M phosphate buffer of pH 6.0, containing 0.15 M NaCl.

FIA. Moreover the biosensors can be easily adopted for FIA systems. Simple through-flow cell with incorporated enzyme electrode is shown in the Fig. 1 (insert). As PVC-membranes with immobilized enzyme can be casted in various forms, it is possible to perform lot of detector cells in different measurement configurations [6].

The analytical response of the urea biosensor in the FIA system was rapid and reproducible (Fig. 2). The system enabled around 30 injections of urea samples per h. The optimized measurement conditions allowed the determination of urea within the linear range 1–15 mM with good regression coefficient ($r = 0.999$) (Fig. 2, insert). For higher urea concentration the analytical response was linear in the logarithmic scale. The base-line potential was stable during measurements (6–8 h daily). After 1 month of everyday use no significant decay of sensitivity was observed.

Fig. 3 presents the analytical response of the penicillins biosensor in the similar FIA system for penicillin V and penicillin G. This FIA system was also sensitive, fast, and reproducible. The time of a single determination was ca 2 min. The operational stability of the system exceeded 1 month. The system enabled β-lactam antibiotics determination in the millimolar range of concentration (Fig. 4). Under optimized conditions (5.0 mM phosphate buffer of pH 7.0), the linear analytical responses in the range 1000–10000 U ml^{-1} (biological activity units) with satisfactory regression coefficients ($r > 0.998$) were obtained.

3.3. Analytical applications

Both FIA systems with enzyme electrodes can be applied for determination of the analytes in real samples. The FIA parameters (flow rate, injection volume of sample) were as described in

Section 2.2. The FIA system for urea determination was successfully used for determination of urea in control serum samples within normal and pathological ranges of concentration (Table 1). The elimination of effects from alkaline cations was achieved by adjusting the sodium and potassium ions concentrations in the working buffer (carrier) to their levels in the analyzed samples. It is common procedure of elimination of such interferences [9].

The FIA system with penicillins biosensor was used for the determination of penicillin V and G in β-lactam pharmaceutical products (Table 1). Because of high activity of penicillins in these drugs diluted samples were analyzed. This dilution eliminated interferences from additives and

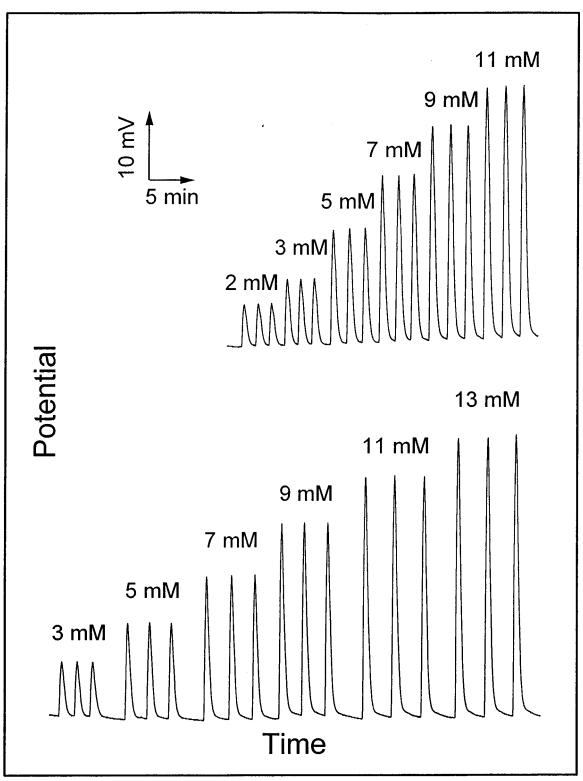

Fig. 3. Analytical response of the penicillins biosensor in the FIA system for penicillin V (top) and penicillin G (bottom). Concentrations of penicillin are given in the figure. The measurements were performed in 5.0 mM phosphate buffer of pH 7.0.

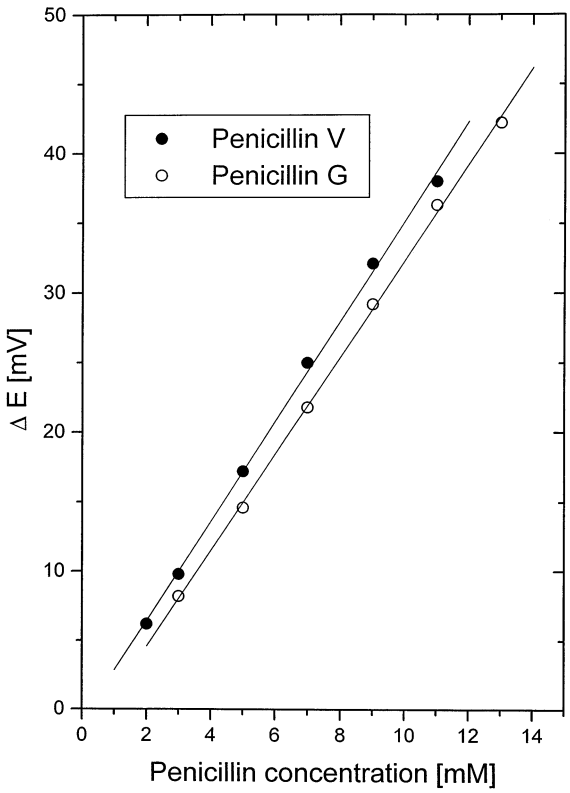

Fig. 4. Calibration graphs for penicillin V and G. The same conditions of measurements as for Fig. 3.

in consequence no sample pretreatment was needed. The precision and accuracy of such analysis is better then 2%. In both cases the results were comparable to those obtained using standard reference methods (Table 1).

These primary results show that application of the described biosensors in FIA systems for the analyses of more complex real samples, e.g. fermentation broth (penicillins and urea), ceratolytic pharmaceuticals (urea), and physiological fluids (urea) is possible.

4. Conclusions

The potentiometric biosensors based on the membrane ion-selective electrodes with covalently bound enzyme are suitable for fast, selective, and accurate analysis. High sensitivity and

Table 1
Comparison of the results of urea and penicillins determination in real samples using described FIA systems and respective reference methods

Analyte/sample	Presented method	Reference value
Urea/serum (mM)	6.4 ± 0.1	7.57 ± 1.82[a] 6.31 ± 1.51[b]
Urea/serum (mM)	22.0 ± 0.5	21.9 ± 2.0[a] 22.9 ± 1.0[b]
Penicillin V/drug (U/mg)	1480 ± 30	1500[c]
Penicillin G/drug (U/mg)	1580 ± 30	1590[c]

[a] Enzyme-kinetic-UV method (Cormay serum standard).
[b] Kodak–Ektachem method (Cormay serum standard).
[c] HPLC method (β-lactam antibiotic in pharmaceutical form).

short response time of the biosensors allow their application in flow injection analysis. Up to 30 samples per hour can be analyzed in the performed FIA systems. Long lifetime of the applied biosensors results in long term stability of the systems exceeding 1 month of everyday use. The presented FIA systems seems to be useful for pharmaceutical and biomedical analysis and can make an attractive alternative for routine methods of determination especially in respect of simplicity, low costs, and short time of analysis.

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