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Creatinine biosensor based on ammonium ion selective electrode and its application in flow-injection analysis

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Abstract

A new, highly sensitive, fast responding and stable potentiometric biosensor for creatinine determination is developed. The biosensor is based on an ammonium ion-selective electrode. Creatinine deiminase (EC 3.5.4.21) is chemically immobilized on the surface of the polymeric ion-sensitive membrane in the form of monomolecular layer using a simple, one-step carbodiimide covalent attachment method. The resulting enzyme electrodes are useful for measurement under flow injection analysis (FIA) conditions. The biosensors exhibit excellent operational and storage stability. The enzyme electrodes retain over 70% of initial sensitivity after ten weeks of work under FIA conditions. The storage stability at 4 °C is longer than half a year without loss of sensitivity. Under optimized conditions near 30 samples per hour can be analyzed and the determination range (0.02–20.0 mmol l^{-1}) fully covers creatinine concentrations important from clinical and biomedical point of view. The simple biosensor/FIA system has been successfully used for determination of creatinine in urine, serum and posthemodialysate samples.

Keywords: Creatinine; Biosensor; Potentiometry; Flow-injection analysis

1. Introduction

Creatinine is one of the most requested analytes in modern clinical analysis. Determination of this metabolite in various biological fluids is useful for evaluation of renal, muscular and thyroid dysfunctions. Such analyses are helpful for biomedical diagnosis of acute myocardial infarction as well as for quantitative description of hemodialysis therapy.

Many chemical methods employed for creatinine determination are based on well-known reaction between the analyte and alkaline picrate, developed over one hundred years ago by Jaffe. However, these spectrophotometric methods are time-consuming, laborious and not very specific as many other metabolites and drugs can interfere [1–3]. Highly selective and relatively fast creatinine determinations in biofluids are possible with several chromatographic methods [3,4]. Unfortunately, equipment for these separation techniques is expensive and therefore not suitable for routine analyses. Therefore development of inexpensive and rather selective creatinine biosensors is promising. Various enzyme electrodes for creatinine have been reported in the literature and their principles and designs have been recently reviewed [5]. Many amperometric biosensors are based on trienzyme system that sequentially converts creatinine to creatine, creatine to sarcosine and finally sarcosine to glycine [3,5–8]. The source of the analytical signal is the electrochemical detection of oxygen consumed or hydrogen peroxide generated in the course of the final enzyme reaction. Monoenzymatic biosensors are based on the detection of ammonia generated in the course of enzymatic hydrolysis of creatinine by creatinine deiminase [5]. Ammonia can be detected amperometrically with polypyrrole [9] or polyaniline [10] modified electrodes. In potentiometric monoenzymatic biosensors the role of internal sensor is played by gas sensitive electrodes [11–13], or by pH-sensitive devices [14,15].

Biosensors can be applied as disposable analytical devices as well as sensors for repeated use. In the latter case enzyme electrodes are especially useful under flow injection analysis (FIA) conditions because of shorter time and lower cost of single determination and significant improvement of parameters of analysis such as selectivity, reproducibility and accuracy. FIA techniques are based on non-equilibrium measurements and therefore high sensitivity and short

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response time of applied biosensors is necessary. Moreover, high operational stability of the biosensors is required, as FIA systems are designed for frequent use over a long period of time. Biosensors dedicated for FIA should be stable, sensitive, reversible and fast-responding. The choice of the 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 the membrane implies high sensitivity of the enzyme electrode. The stability of the enzyme layer determines the lifetime of the biosensor. The response time of the bioelectrode is generally determined by the thickness of enzyme layer, as the species transport within the membrane limits the dynamics of the whole process of analytical signal generation.

Recently, a general method for preparation of biocatalytically modified membrane based sensors fulfilling such requirements has been developed [16,17]. The method is based on covalent binding of enzyme molecules directly to the surface of polymeric ion-sensitive membranes (potentiometric or optic). In this work, similarly prepared the monoenzymatic potentiometric creatinine electrode based on ammonium ion selective electrode modified with creatinine deiminase (EC 3.5.4.21) is presented. The source of the analytical, potentiometric signal of the biosensor are ammonium ions generated directly at the surface of the ion-sensitive membrane of the electrode in the course of the enzymatic degradation of creatinine. The biosensor is suitable for use under FIA conditions. The application of this simple bioanalytical system based on such biosensor for fast and accurate analysis of real biomedical samples is also demonstrated.

2. Experimental

2.1. Reagents and materials

Materials used for ammonium ion-selective membrane preparation: nonactin (ionophore) and bis-(2-ethylhexyl)-sebacate (DOS, plasticizer) were obtained from Fluka (Switzerland). Carboxylated poly (vinyl chloride) (PVC-COOH, membrane matrix and immobilization support) were from Aldrich (Germany). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) for immobilization procedure and creatinine deiminase (creatinine iminohydrolase, EC 3.5.4.21, 25–50 U mg^{-1} lyophilized powder from microorganism) were from Sigma (USA).

Cation exchanger (Dowex 50WX8, mesh 200–400) was from Aldrich (Germany). All other organic and inorganic reagents were of analytical grade and were used without further purification. Experiments were carried out using solutions prepared with doubly distilled water immediately prior to use.

For reference creatinine determinations in real samples the manual procedure for kinetic spectrophotometric Jaffe method was applied using reagent kit no. 555A from Sigma (USA).

Urine samples were from healthy volunteers. Certified serum standards were from Cormay (Poland). Postdialysate samples were prepared using concentrates for hemodialysis from Karima (Poland).

2.2. Biosensor construction

Ammonium ion selective membranes were prepared according to a common procedure [16] by dissolving about 300 mg of the membrane components in 3 ml of tetrahydrofuran. The solution was spilled out on a PTFE plate inside a glass ring (25 mm diameter) and left overnight to dry. The composition of membranes after solvent evaporation was: 3 wt.% of nonactin, 32 wt.% of PVC-COOH 65 wt.% of DOS. The thickness of the formed membrane was around 0.5 mm. The discs of 5 mm diameter were cut out and attached to electrode bodies filled with inner solution (0.1 M NH_4Cl). The silver/silver chloride electrode played a role of internal electrode.

To immobilize the enzyme layer on the membrane 45 μl of freshly prepared water solution containing EDAC (6 mg ml^{-1}) and creatinine deiminase (650 U ml^{-1}) was deposited on the membrane surface and left for 6 h. Residues of unbounded enzyme were desorbed from the biosensor surface by immersing the electrode for 1 h in vigorously stirred 0.1 M phosphate buffer of pH 6.9.

2.3. Instrumentation and measurements

The enzymatically modified membranes were attached to electrode bodies Philips IS 561 from Moller Glasblaserei (Switzerland). The biosensor and a saturated calomel reference electrode (also from Moller Glasblaserei, model R 11), were mounted into a small (0.01 ml) laboratory-made flow-through electrode cell made of PTFE. The cell was part of a simple double-channel FIA system consisted of Minipuls 3 peristaltic pump (Gilson, France), laboratory-made injection valve and mixing coil (20 cm). The volume of the injection loop was 0.50 ml. Commonly used flow rate of the carrier solution was 2.30 ml min^{-1}. The electrodes were coupled to digital pH-meter (model OP208/1, Radelkis, Hungary) connected with a data collecting personal computer. Similar FIA setups were shown previously [17–19].

For optical reference determinations of creatinine at 500 nm wavelength Shimadzu 2401/PC spectrophotometer (Japan) and disposable polystyrene 1 ml cuvettes from Sarstedt (Germany) were used. The analytical procedure was performed according to the Sigma protocol.

3. Results and discussion

The method applied for the biosensor preparation is based on covalent binding of enzyme molecules directly to the surface of polymeric ammonium ion-sensitive membrane made of carboxylated poly(vinyl chloride) matrix. On the

surface of the membrane reactive groups are present and the material is a ready-to-use support for enzyme immobilization [16]. The carboxylic groups allow chemical binding of biomolecules to the surface using a simple one-step carbodiimide method. Carbodiimide activites the carboxylic groups to allow formation of imide bonds between these groups and amine groups of the enzyme molecules under mild conditions acceptable for many often unstable biologically active proteins. These covalently linked enzyme molecules form a stable monomolecular layer exhibiting high biocatalytic activity. Direct contact of enzyme with sensing membrane enables effective electrochemical monitoring of biocatalytic processes, which are a source of the analytical signal. As the monomolecular enzyme layer is extremely thin and strongly bound to the polymer there is no need of additional protecting membranes and therefore response time of such biosensor is short, similar to that for internal electrode. In other words, there are no limitations in substance transport into the biolayer as the "naked" enzyme molecules are exposed to analyzed solution. The high durability of the chemically immobilized enzyme layer resulted in the long lifetime of the enzyme electrode. Other enzyme electrodes obtained using this methodology meet all mentioned requirements for biosensors designed for FIA, as it was recently demonstrated for β-lactam antibiotics [17,18] and urea [17,19] detection.

The developed creatinine biosensors exhibit very long operational and storage lifetimes. In the course of the operational stability test 3–5 h-long control calibrations were performed everyday and the investigated enzyme electrodes retained over 70% of initial sensitivity after 72 days of such semi-continuous work (Fig. 1). The storage stability investigated at 4 °C was found to be longer than half a year without loss of sensitivity. Longer stability tests were not performed.

The behavior of the creatinine enzyme electrodes under FIA conditions is typical. Both, an increase in flow rate and a decrease in sample injection volume caused a decrease of peak height and shortening of time necessary for peak formation. The selection of these physical conditions for measurements is a result of compromise between required sensitivity and frequency of determinations.

The effects from buffer pH and concentration were investigated in range 6.5–9.5 of pH and 0.010–1.0 M (TRIS/HCl buffers), respectively. No significant effect from buffer concentration was found. In range of pH from 7.0 to 9.0 the recorded analytical signals were similar. Out of this range significant decrease of the biosensor sensitivity was observed. In more acidic solutions this decrease was dramatic and irreversible. For further experiments as optimal pH of 8.1 was chosen, due to maximal buffer capacity of the carrier buffer.

Further investigations were devoted to the selectivity of the biosensor/FIA system. Due to high specificity of the applied enzyme, other compounds such as creatine, sarcosine, urates, ascorbates, natural amino acids, glucose and urea did not effect the response of the enzyme electrode. We found only significant interferences from ammonium, sodium and potassium cations. Obviously, the source of this non-selectivity is caused by sensitivity of internal ion-selective electrode to primary ion and its poor selectivity to alkaline cations. The results obtained confirmed that a removal of the cations before real sample analysis with the biosensor is necessary.

The wide range of determination of the system (0.02–20.0 mM) fully covers creatinine levels present in clinical samples. Under optimum conditions the detection limit is lower then 0.015 mM. Physiological level of serum creatinine varies from around 0.05 to 0.11 mM. Levels higher than 0.14 mM required further clinical investigations and those over 0.5 mM indicate severe renal dysfunction. In extremely pathological conditions concentration of creatinine in blood can exceed 1 mM. Creatinine levels in postdialysate liquid

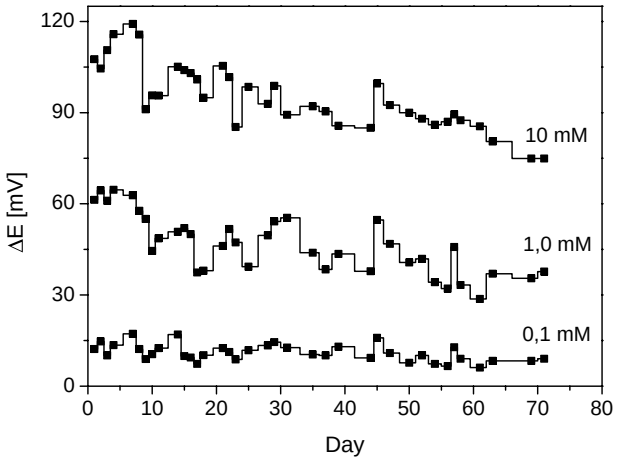

Fig. 1. Operational stability of the creatinine enzyme electrode. Measurements performed in 0.1 M TRIS/HCl Buffer, pH 8.1. Creatinine concentrations are given in the figure.

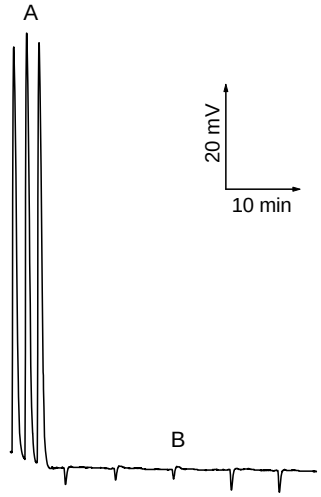

Fig. 2. Signal of the biosensor for samples of dialysate fluid injected into the FIA system without (A) and with (B) use of the cationite column. The samples contain sodium, potassium, magnesium and calcium cations at levels 140, 3.0, 1.0 and 2.0 mM, respectively.

vary from around 0.06 up to 0.35 mM. The highest levels of the analyte, up to several milimoles per liter, are found in urine samples from healthy people.

Although the response of the enzyme electrode is not influenced by other metabolites usually present in physiological samples, serious source of interferences are, as early mentioned, NH_4^+, K^+ and Na^+ cations (Fig. 2). Concentration of endogenous ammonia in human urine is normally as high as 10 mM. Physiological level of ammonia in serum is lower than 0.05 mM, but for patients with hepatic coma it can increase up to 0.2 mM. Moreover, ammonia content of freshly drawn blood rises rapidly on standing (due to enzymatic degradation of glutamine and other labile compounds) to two to three times its normal level. Typical levels of potassium and sodium in physiological fluids are at few and over 100 mM, respectively. Fortunately, all these cations can be easily removed from samples using common cation exchangers. We found that filling of the injection loop with all kinds of tested samples through 4 cm long exchanger column (containing 0.3 g of Dowex) is a sufficient way for achieving separation. High efficiency of the separation was confirmed in the experiments with non-modified ion-selective electrode mounted into the FIA system (Fig. 2). In all cases the signal of the electrode was not influenced by the injected samples. On the other hand, the analyte did not interact with the exchanger, because at pH 8 creatinine predominantly exists in the zwitterionic form. Experiments with detection of creatinine in model buffer solutions with

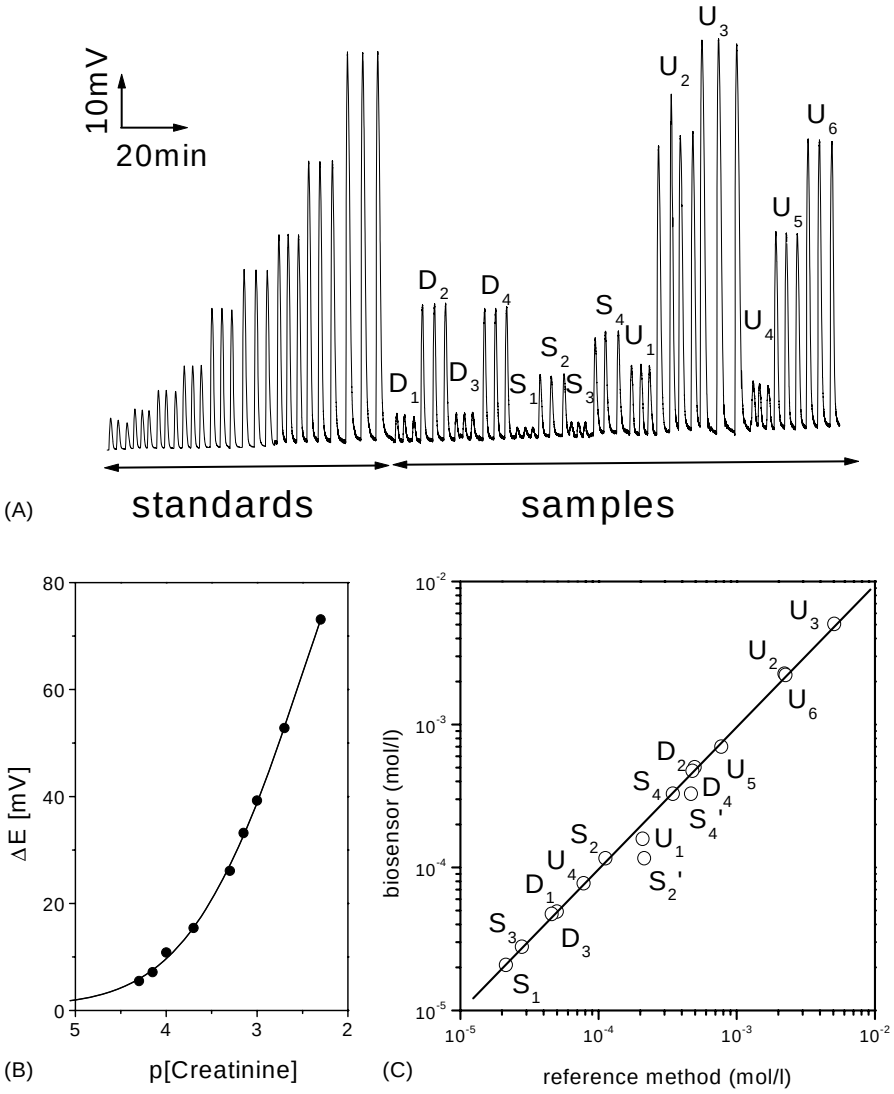

Fig. 3. (A) Run-time data trace of measurements with developed bioanalytical system consisted of calibration and analysis of real samples. (B) Corresponding calibration graph. (C) Correlation of creatinine concentrations in real samples determined by the enzyme electrode and by the reference method. Analyzed samples: D1—dialysate sample with lower creatinine level, D2—dialysate sample with upper creatinine level, D3—Low-potassium dialysate sample with lower creatinine level, D4—Low-potassium dialysate sample with upper creatinine level, S1—10-fold diluted standard of physiological human serum, S2—undiluted standard of physiological human serum S3—10-fold diluted standard of pathological human serum, S4—undiluted standard of pathological human serum, U3, U6—undiluted human urine, U2, U5—5-fold diluted human urine, U1, U4—50-fold human urine. For sample dilution carrier buffer has been used. S′2 and S′4—results of analysis by Jaffe method for undeproteinized samples S2 and S4, respectively.

and without use of the column lead to the same results. Regeneration of the cation-exchange column has been performed off-line by washing the resin with hydrochloric acid and subsequently with the carrier buffer.

Typical response of the enzyme electrode under optimized FIA conditions (given in Section 2) is shown in Fig. 3 (insets A and B). As can be seen from inset C (Fig. 3), the results of analysis of real samples obtained using the reported biosensor/FIA system are fully comparable with those obtained using standard reference method. A good correlation between both methods is evident in wide concentration range for nearly all kinds of samples ($Y = (1.00(\pm 0.009) \times X) + 1.8 \times 10^{-5}(\pm 1.5 \times 10^{-5})$, $R = 0.9994$, S.D. $= 4.7 \times 10^{-5}$, $n = 14$). Only in case of undeproteinized serum samples (S′2 and S′4), higher levels of creatinine were determined using the reference method. Evidently, this is an effect of non-specificity of the Jaffe method caused by high content of proteins in the samples. For deproteinized (S2 and S4) and diluted (S1 and S3) serum samples the results of analysis are just well correlated, although in the last case the creatinine levels are near of the detection limit of the biosensor/FIA system. The results shown in Fig. 3C clearly confirm that simple and inexpensive biosensor reported in this paper enables accurate, sensitive and selective analysis of real clinical samples containing low levels of the analyte in a complex matrices.

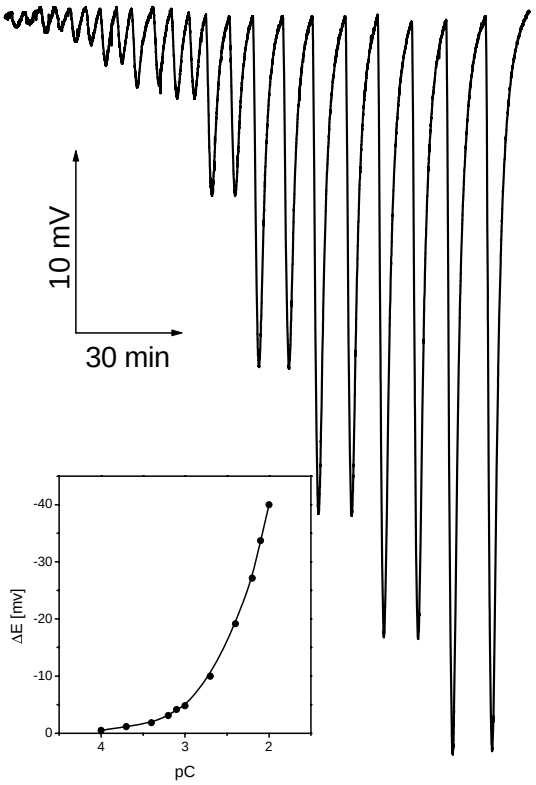

Fig. 4. Calibration of the pH-enzyme creatinine electrode in the FIA system. Measurements performed in 5.0 mM phosphate buffer, pH 6.0. Corresponding calibration graph is given in the inset.

As reported earlier [16], the preparation method used in this work is general, and could be used for construction of various kinds of biosensors. By changing the ionophore (*N*-tridodecylamine instead of nonactin) in the PVC-COOH matrix of the biosensor we have obtained pH-enzyme creatinine electrode. Protocols for ion-selective membrane preparation and enzyme immobilization were the same. The resulted biosensor exhibited similar operational and storage stabilities and the dynamics of the response, and therefore it is suitable for measurements under FIA conditions (Fig. 4). Contrary to the biosensor based on ammonium ion-selective electrode, the analytical response of this pH-enzyme electrode was not affected by ammonia and alkaline cations. Unfortunately, the main drawback of this creatinine biosensor, common to all pH-based enzyme biosensors [20], is strong dumping effect of the buffer capacity of samples on its analytical signal. This inconvenience limits the practical utility of the biosensor. Despite this disadvantage, this creatinine pH-enzyme electrode is useful for analysis of samples having a low buffer capacity. We found that the simple FIA system based on this biosensor is useful for analysis of real samples of human urine. Unfortunately, for samples with higher buffer capacity or lower creatinine levels (serum and postdialysate), the detection limit of the FIA system based on the pH-enzyme electrode was not sufficient.

4. Conclusion

A sensitive, fast-responding and stable potentiometric enzyme electrode for determination of creatinine has been developed. The biosensor is especially useful under FIA conditions. Simple FIA system based on the biosensor has been successfully used for accurate, fast and selective analysis of real samples important from clinical point of view. The biosensor could be also applied as sensing element of bioanalytical devices dedicated for biomedical diagnostic and control of hemodialysis treatment. A prototype of hemodialysis monitor based on the enzyme electrode is under final examination [21].

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