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Short communication

Application of creatinine-sensitive biosensor for hemodialysis control

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ABSTRACT

The highly sensitive and selective potentiometric biosensor for creatinine determination has been developed by us earlier. In it, pH-sensitive field effect transistors were used as transducer and immobilized creatinine deiminase (EC 3.5.4.21)—as a biosensitive element. In the work presented, we optimized this biosensor for creatinine analysis in real samples of dialysate in patients with renal failure. The optimized version of biosensor was applied for on-line monitoring of the level of creatinine in the patient's dialysate fluid in the course of dialysis session. High correlation between the biosensor analysis and traditional Jaffe method was demonstrated.

1. Introduction

Creatinine is one of the most important, analytes used for determination of kidney and muscular dysfunction. Abatement of kidney function and consequent accumulation of toxic products of protein metabolism is hazardous for human health. It is a case when blood purification with an artificial kidney is an effective therapy to save the patient's life (Koncki, 2008). Blood dialysis results in washing-out toxic substances, in particular, creatinine, which is the main marker of the kidney disease. Creatinine is a non-threshold substance excreted together with urine, its amount in urine and blood serum is basically determined by the muscular mass and renal excretion ability. Therefore an increase in creatinine level is usually a sign of a decrease in the kidney functional activity. This metabolite belongs to a fraction of residual blood nitrogen. Its concentration in blood serum is rather stable and used in routine clinical practice for both diagnostics and the monitoring of kidney diseases (Wyss and Kaddurah-Daouk, 2000). Blood purification by artificial kidney is a protracted and unpleasant procedure; therefore an express-method of control of hemodialysis efficiency is required to reduce its duration and thus the time of patient's discomfort.

There are a number of methods for creatinine determination. The first of them, a colorimetric method using Jaffe reaction, was first proposed in 1886. It is based on the interaction of creatinine with picric acid in alkaline medium resulting in formation of orange colored tautomer creatinine picrate (Jaffe, 1886). This method is still in use, despite its insufficient specificity determined by low specificity of Jaffe reaction. The further scientific efforts were thus trended at the creation of more specific methods. Numerous instrumental methods were developed, they are: high-pressure liquid chromatography (Dobberpuhl and Dash, 2003; Merás et al., 2005; George et al., 2006; Samanidou et al., 2002.), ion chromatography (Yokoyama et al., 2000, 2005), micellar electrokinetic chromatography (Burke et al., 1999; Tran et al., 1997; Poboży et al., 2003), chromatography using fluorescent indicators (Yao and Kotegawa, 2002), capillary electrophoresis (Costa et al., 2007), capillary zone electrophoresis (Zinellu et al., 2006; Tuma et al., 2005; Rodrigues et al., 2004; Zinellu et al., 2005), capillary isotachophoresis (Kvasnička and Voldřich, 2000) and tandem mass spectrometry (Huškova et al., 2004.). However, all these methods are very complicated and unsuitable for creatinine measurement in real-time mode.

At present, only biosensor methods make on-line determination of creatinine possible (Radomska et al., 2004a,b; Poboży et al., 2003; Premanode and Toumazou, 2007; Tombach et al., 2001). Their basic difference from conventional instrumental analytical approaches is the benefit of qualitative and/or quantitative analysis in on-line mode without or with minimal sample pretreatment. Though some biosensor methods have been developed and different kinds of sample pretreatment have been proposed, precise determination of creatinine in biological liquids is still an actual challenge. The main disadvantage of amperometric biosensors is usage of

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complicated bioselective elements. A three-enzyme system initiates the cascade of three enzymatic reactions, which can be a reason of low selectivity of analysis and an essential error in creatinine measurement. The main weakness of potentiometric biosensors based on ammonium ion-selective electrode is an influence of endogenous NH_4^+ on the results of analysis.

The creatinine sensitive biosensor based on pH-sensitive field effect transistor and immobilized creatinine deiminase (EC 3.5.4.21) has been developed and tested in model solutions (see our previous paper Soldatkin et al., 2002).

Therefore, the main goal of this work was optimization of the biosensor developed for application for on-line analysis of metabolite creatinine in dialysis fluid in the course of hemodialysis.

2. Experimental

2.1. Materials

The preparation of enzyme creatinine deiminase (EC 3.5.4.21) with activity of 36 U/mg was purchased from "Sigma" (Germany); bovine serum albumin (BSA) (fraction V) and 25% aqueous solution of glutaraldehyde (GA) and creatinine were purchased from "Sigma–Aldrich Chemie" (Germany). Phosphate buffer solution (KH_2PO_4–NaOH), pH 7.4, produced by "Merck" (Germany) was chosen as a working buffer.

Bicarbonate post-dialysate samples for the experiments were obtained from Kiev Municipal scientific and practical centre of nephrology and hemodialysis. Bicarbonate dialysis fluid consists of 32 mM HCO_3^{2-}, 109.5 mM Cl^-, 3 mM CH_3COO^-, 138 mM Na^+, 1.75 mM Ca^{2+}, 2 mM K^+ and 0.5 mM Mg^{2+}.

2.2. Sensor elements based on pH-sensitive field-effect transistors

To ensure high reliability and stability of sensor elements, the *p*-channel MOS technology was used on *n*-type silicon substrates with specific resistance of 4.5 Ohm cm and ⟨1 0 0⟩ lattice orientation. The gate dielectric layer was formed from thermally oxidized 50 nm thick SiO_2 film and 50 nm thick Si_3N_4 film deposited in the low-pressure reactor. The gate area had zigzag-shaped geometry with the length to width ratio of 100, which provided sufficient gain factor of *p*-channel transistors. The *p*-type conducting buses covered with a dielectric layer were used to form electric contact to the transistor drain and source areas.

Based on the sensor elements described, the silicon chips containing two integral pH-sensitive field-effect transistors (pH-FETs) were prepared and mounted on the specifically designed printed-circuit boards for connection convenience. The contact from the chip to the board copper layer was made by ultrasonic welding with an epoxy glue sealing. This system makes it possible to implement differential mode of measurement, i.e. a sensitive bioselective membrane is deposited onto the gate of one transistor while the other serves as the reference. Due to this, an influence of changes in temperature, pH and ion strength of the solution as well as that of ambient light and electromagnetic effects on the measurement results are significantly lower.

The pH-FETs responses were measured using the circuit maintaining a constant value of the current in the transistor channel, enabling the output signal to follow the changes in potential near the transistor gate automatically. The threshold voltage for all pH-FETs was about −2.5 V. The measurement was performed under the following conditions: channel current of approx. 20 μA (the drift was about 0.2–0.3 μA per hour), drain-source voltage close to 1 V and the sensor bulk is connected to the circuit ground.

The topology, design and schematics of the pH-FETs elements were reported in details in Kukla et al. (2007).

2.3. Preparation of bioselective membranes

To prepare the working bioselective element based on creatinine deiminase, the solution containing 8% creatinine deiminase and 8% BSA was used. The enzyme portions were dissolved in 20 mM phosphate buffer, pH 7.4, with 10% glycerol added for enzyme stabilization and prevention of untimely drying up of the solution on the transducer surface. The mixture for preparation of a reference membrane was made in the same way but BSA in a final concentration of 16% was taken instead of the enzyme–BSA mixture. The solutions were deposited without delay onto entire sensitive surfaces of pH-FETs; 0.1 μl of each solution was used for this purpose. All the membranes had the same final content of total protein. The molecules of enzyme and BSA in bioselective and reference membranes were cross-linked by GA from saturated GA vapor (Nazarenko et al., 2005). Then the membranes were dried in the air for 15–20 min at room temperature. Prior to starting the measurements, the membranes were washed to remove an excess of in buffer solution.

2.4. Procedure of creatinine measurement in a model working buffer

The measurements were generally carried out at room temperature in 5 mM phosphate buffer, pH 7.4, intensively stirred in an open cell. Before usage, the biosensors were washed for a while in the buffer solution to obtain a stable basic output signal. The substrate concentrations were varied by adding aliquots of stock creatinine solution. A signal from the reference field-effect transistor with an inactive membrane placed on the same chip was subtracted from the signal on the transistor with an active bioselective membrane.

2.5. Creatinine measurement in dialysate samples using the method of standard additions (Aleskovskii et al., 1988)

The samples of dialysate of the patients suffering from renal insufficiency were tested. Creatinine concentration was measured at room temperature in an open vessel with intense stirring. Before measurements, biosensors were soaked in the working buffer solution until a stable baseline output signal was obtained.

First, the biosensor response was measured in the tested assay with unknown creatinine concentration, then definite aliquots of creatinine stock solution were added to the electrochemical cell and corresponding biosensor responses were determined. The curve was obtained by plotting the data; its linear approximation gave the point of intersection with the concentration axis (X), which corresponds to the creatinine concentration in the assay.

1 ml sample of the patient's dialysate was inserted into a cell filled with 5 ml of dialysis fluid (which did not contact with the patient's blood), i.e. the sample was 6-fold diluted. After obtaining the response to dialysate (the response time—about 2 min), the aliquots of creatinine stock solution were added, and the curve was plotted. The creatinine concentration was determined using the method of standard additions (Aleskovskii et al., 1988).

After each measurement the sensor was exposed to triple 5-min wash in fresh portions of dialysis fluid to obtain the baseline output signal.

3. Results and discussion

The operation of creatinine-sensitive biosensor is based on the reaction of creatinine hydrolysis catalyzed by creatinine deiminase:

$$\text{Creatinine} + H_2O \rightarrow \text{N-methylhydantoine} + NH_4^+$$

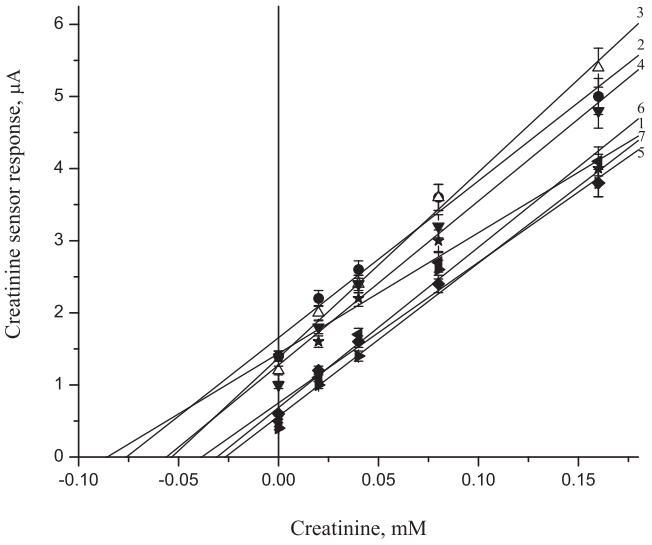

Fig. 1. Biosensor determination of the dynamics of creatinine concentration change in post-dialysate fluids in the course of dialysis. The dialysate samples after different time of dialysis: 1, 15 min; 2, 30 min; 3, 60 min; 4, 120 min; 5, 180 min; 6, 210 min; 7, 240 min. Measurements were done using the method of standard additions of creatinine: 0.02 mM, 0.04 mM, 0.08 mM and 0.16 mM.

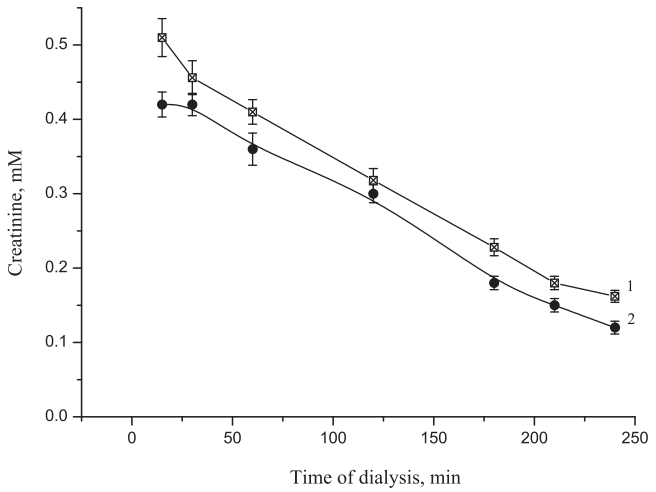

Fig. 2. Dynamics of creatinine leveling off in dialysate assays: 1, biosensor determination by method of standard additions; 2, biosensor method using calibration curve. Standard additions of creatinine: 0.02 mM, 0.04 mM, 0.08 mM and 0.16 mM.

The pH change induced by creatinine hydrolysis and proportional to the substrate concentration in tested solution is registered by the biosensor based on pH-FETs (Kukla et al., 2007).

The main working characteristics of the biosensor developed such as detection limit, dynamic range and selectivity in the model solution have been shown earlier (Soldatkin et al., 2002). The biosensor response to varying concentration of creatinine, buffer capacity and ionic strength has been tested too.

In the present work the biosensor response reproducibility and operational stability, which are the most important biosensor characteristics, were studied (the data are not presented). The biosensor was characterized by high reproducibility (the relative standard deviation of the signals did not exceed 1%) and high operational stability (the bioselective element activity did not change for 5–6 h). It means that the developed biosensor can be applied for determination of creatinine concentration in dialysis fluid during the hemodialysis sessions, which last for 3–4 h.

The enzyme biosensor was tested while analyzing creatinine in real samples of dialysate of the patients with renal failure. The male patient's dialysate produced by artificial kidney was sampled in the course of hemodialysis session with certain time intervals.

Creatinine concentration in assays of patient's dialysate was measured by the biosensor using two procedures—with standard additions and calibration curves. In case of the method of standard additions the samples tested (post-dialysate fluids) were introduced in the measurement cell with dialysis fluid considering the dilution (described in Section 2). The linear range of creatinine measurement in post-dialysate fluids (0.02–0.32 mM) does not differ from that for the model phosphate buffer (see Fig. S1 in a Supplementary file).

The linear approximation of the data obtained gives the point of intersection with the concentration axis (*X*), which corresponds to the creatinine concentration in the diluted assay (Fig. 1).

In case of the classical biosensor method we used the calibration curve (see Fig.S1 in a Supplementary file) for determination of creatinine concentration in the post-dialysate samples.

The dynamics of creatinine variation in the post-dialysate samples (that corresponds to its wash-out from patient's body) can be assessed from Fig. 2. As seen, after the first 15 min of dialysis the creatinine concentration remained high, essentially exceeding the norm (0.03–0.14 mM). After 4-h dialysis the creatinine level in the patient's post-dialysate was 0.16 mM (i.e. decreased by 70% compared to that during the first 15 min), which can be considered as normal for patients with renal insufficiency.

To evaluate the developed bioanalytical system, the data obtained were compared with the traditional Jaffe spectrophotometric method of creatinine determination in the patient's post-dialysate (Fig. 3).

We have estimated a correlation between the data on creatinine concentration in assays of patient's post-dialysate measured by the biosensor (using two procedures—with standard additions and calibration curves) and by the Jaffe method. The correlation coefficient in case of standard additions was 0.98 (Fig. 3, curve 1), for calibration curves—0.95 (Fig. 3, curve 2).

In Fig. 3 the slope of fitting line is larger than 1. Moreover, the fitting lines do not cross the *X*-axis in the "zero" point. Therefore, one of the creatinine detection methods brings a constant mistake in the determination. This can be associated with the fact that in our research we used a standard Jaffe protocol, developed for creatinine determination in blood serum and urine. We used this protocol for detection of creatinine in post-dialysis fluid. The composition of analyzed samples significantly differs (pH, salts content) from the

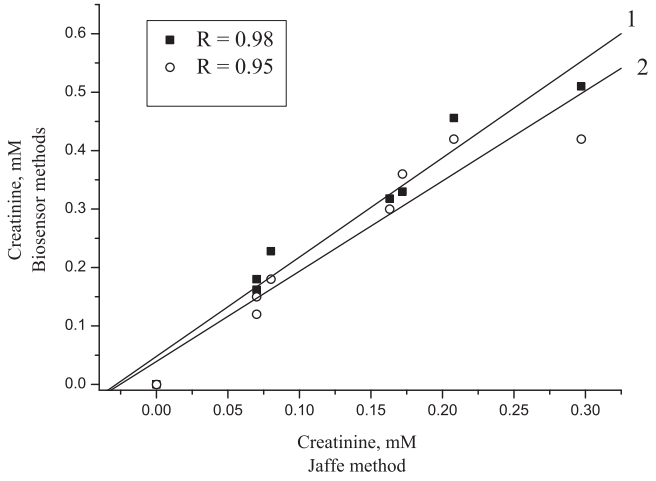

Fig. 3. Correlation between the data on creatinine concentration in dialysate samples obtained by two biosensor procedures (biosensor measurement using: 1, standard additions; 2, calibration curve) and Jaffe method.

composition of blood serum and urine, resulting in some decrease in effectiveness of formation of creatinine–picrate complexes.

Thus, both the methods of biosensor determination of creatinine can be used for on-line measurement of creatinine in patient's dialysate in clinical laboratories. The classical biosensor method using calibration curve can be considered as preferable because it is simpler and faster.

4. Conclusion

The biosensor developed earlier (Soldatkin et al., 2002) based on pH-sensitive field-effect transistors as a transducer and immobilized creatinine deiminase as a sensitive element was optimized and applied for real sample analysis. It provides a highly sensitive, selective and rapid method of creatinine analysis.

The dynamics of the creatinine concentration decrease in the patient's dialysate in the course of hemodialysis was on-line monitored by the biosensor. High correlation between the results obtained by the biosensor and with the traditional Jaffe method was revealed.

Thus, the biosensor developed can be applied for monitoring creatinine concentrations in post-dialysate fluids (control of the rate and quality of hemodialysis), as well as for screening creatinine level in blood serum and urine in norm and pathology while carrying out medical examination of population.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.02.062.

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