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Biosensor Measurement of Urea Concentration in Human Blood Serum

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***Abstract*—**An application of the highly sensitive and selective biosensor based on pH-sensitive field-effect transistor and immobilized urease for urea analysis was demonstrated in this work. The main analytical characteristics of the biosensor developed were determined; the conditions of urea measurement in real samples of blood were optimized. A conceptual possibility of application of the biosensor for detection of urea concentration in blood serum of patients suffering from renal insufficiency was shown.

***Keywords*—**Biosensor, blood serum, pH-sensitive field-effect transistor, urea, urease

I. Introduction

THE prospects for biosensors application in medicine have broaden with recent developments. Use of biosensors for monitoring urea content in the blood serum of patients with renal insufficiency looks especially promising. Namely urea is a biomolecule monitoring of which provides information on the kidneys condition [1]. Beside that, this metabolite plays the role of a marker for wide spectrum of low and medium molecular weight toxic substances that accumulate in blood of the patients with renal function impairment, and also is an indicator for the liver function diagnosis [2]-[4]. Determination of the urea concentration in blood serum is one of the most widespread tests in clinical laboratory diagnosis [5], [6]. Normal physiological concentration of urea in blood lies within the range of 2.5–8.6 mM. Significant increase in urea concentration is observed with chronic and acute renal insufficiency (50–70 and 120–150 mM respectively) [7]. When urea concentration exceeds 30 mM [8], [2] the patient has to be treated with hemodialysis therapy.

In contemporary laboratory diagnosis direct chemical methods are used for kidney disease monitoring, usually diacetyl monoxime method [9], and also enzymatic methods.

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However the mentioned methods are quite complicated since the analysis is based on the complex of reactions and specific sample preparation procedures are required, thus the methods are not suitable for on-line measurements. To work around these problems, novel highly sensitive and selective methods for express diagnosis of renal insufficiency are needed. High sensitivity and selectivity, low measurement duration, possibility of operation in the field, relative simplicity [10] and suitability for on-line measurements allow to propose the biosensoric method based on pH-sensitive field effect transistors with immobilized urease for urea detection.

II. Materials and Methods

A. Materials

The substances used in this work were the soy beans urease (EC 3.5.1.5) with activity of 66.3 U/mg from "Fluka" (Switzerland), bovine serum albumin (BSA) from "Sigma" (Germany), 25 % glutaraldehyde solution from "Sigma-Aldrich Chimie", and urea from "Sigma" (USA). The pH 7.4 phosphate buffer (KH_2PO_4-NaOH) from "Helicon" (Moscow, Russia) was used as a working buffer solution. Other used reagents were domestically produced with "chemically pure" or "pure for analysis" purity grades.

The human blood serum for urea detection experiments was provided by Kiev city center for nephrology and hemodialysis.

B. The pH-Sensitive FET-Based Transducer Design

In this work the sensor chips with differential pair of pH-sensitive field effect transistors produced at the JSC "Kwazar" facilities (Kiev) were used (Fig. 1). The developed sensor topology provides two identical *p*-channel field-effect transistors (FETs) on a single silicon crystal with the total area of 8x8 mm [11]. In order to eliminate possibility of parasitic conducting channel formation between the transistors a protective 50 um wide n^+-zone was formed on the crystal. Source and drain contacts for each of transistor elements were formed by extended $p+$ diffusion buses leading to the chip edge, where the contact to the *n*-bulk was also formed. Two-layer gate dielectric consisted of silicon oxide and nitride layers each 50 nm thick. Zigzag-like geometry of the transistor gate area with the channel width to length ratio of 100 provided sufficiently high transistor gain factor.

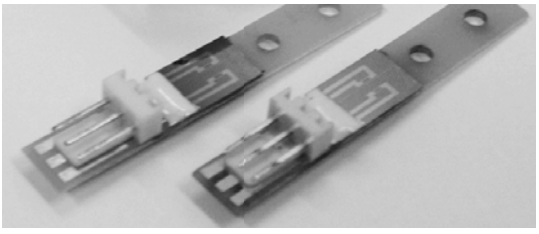

Fig. 1 General view of the pH-sensitive field effect transistor based sensor electrodes

Ion-selective properties of the transistors are conditioned by pH-sensitive dielectric layer of Si_3N_4 deposited on transistor gate area. Sensor elements used in this work demonstrated intrinsic pH-sensitivity of approximately 40 mV/pH and transconductance of 400-500 mkA/V, thus providing pH-sensitivity of the transistor channel current of 15-20 mkA/pH.

Response of the pH-FET sensors was measured by means of current-to-voltage converter circuit with the sensors working in the current source mode with active load. Threshold voltage of the used pH-FETs was about –2.5 V. Measurements were performed with the channel current magnitude of approximately 500 mkA, drain–source voltage of approximately 2 V, transistor bulk connected to the source [12] (Fig. 2). Described sensor electrodes allow effective implementation of the differential measurement mode when one of the transistors is used as a reference, and the other one is equipped with bioselective membrane deposited onto the gate area.

This mode of operation allows to significantly diminish the influence of such interfering factors as changes in temperature, pH and ionic strength of the solution, light and electromagnetic effects, upon the measurement results.

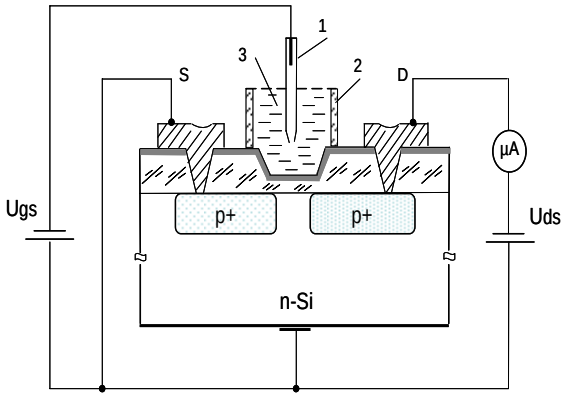

Fig. 2 Diagram of the ISFET connection during the measurements: 1) reference electrode, 2) sample cell, 3) analyzed solution; S and D designates respectively source and drain of FET

C. Preparation of Bioselective Membranes on the pH-FET Surface

In order to create a working urease-based bioselective membrane [13] the solution with 10 % urease and 10 % BSA content was prepared. Portions of enzyme were dissolved in 20 mM pH 7.4 phosphate buffer with 10 % glycerol, the latter used for stabilization of enzyme during immobilization and to prevent premature drying of the solution deposited on the transducer surface. The mixture for reference membrane was prepared in the same way but with the enzyme replaced with BSA at final concentration of 20 %.

Before deposition of membranes the working surface of transducers was degreased with ethanol and cleaned with distilled water. Obtained solutions were deposited with 0.1-2.5 mkl "Eppendorf" micropipette onto the working surfaces of pH-FETs until their full coverage. All membranes had equal final protein content. For polymerization of membranes the sensors were placed in saturated glutaraldehyde vapor. After that the membranes were dried during 15-20 minutes in air at room temperature.

Before the start of experiment the membranes were cleaned from remaining unbound glutaraldehyde by washing with the working buffer solution until the sensor baseline signal stabilized.

D. Determination of Urea Content in Model Solutions

Determination of urea concentration in model solutions was performed in the 5 mM potassium-phosphate pH 7.4 buffer at room temperature. The open type 1.5 ml sample cell with intensive stirring was used in experiments.

Before the experiment start the sensors with immobilized membranes were cleaned from unbound glutaraldehyde remains by washing them for 20-30 min. until the baseline stabilization. Substrate concentration was varied by addition of definite aliquots of the initial concentrated solution. After registration of each response the sensor was cleaned from reaction products by changing the working buffer at least three times every two minutes.

E. Determination of Urea Content in Blood Serum

Analysis of urea concentration was performed with 10 blood serum samples from the patients diagnosed with renal insufficiency. Measurement with biosensor based on pH-sensitive FETs with immobilized urease was conducted in 5 mM pH 7.4 phosphate buffer at room temperature in the open cell with intensive stirring. Urea concentration in the analyzed samples was determined from calibration curve.

Before the measurement blood serum sample was diluted by half with the working buffer, then 6 mkl of the mixture was placed into the 1.5 ml buffer-filled cell. Final dilution of the blood serum in the working cell was by factor of 500. After registration of each response the sensor was cleaned by washing with buffer solution until the baseline stabilization.

Verification of the determined urea concentration in blood serum was performed by classic spectrophotometric method based on diacetyl monoxime reaction [9] and with urease and peroxidase enzymatic system [14].

III. Results and Discussion

In the biosensoric urea analysis method, when urea contacts with biosensor's bioselective membrane the enzymatic hydrolysis takes place and the urea molecule dissociates to two ammonia ions and bicarbonate ion [7], [8]:

$$NH_2CONH_2 + 2H_2O + H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- \quad (1)$$

During this enzymatic reaction protons are absorbed from the solution, which results in increase of the working buffer pH within the bioselective membrane deposited onto the pH-FET surface and causes the change in the surface potential that is registered as the potentiometric biosensor response.

When determining urea concentration in the model solutions urease biosensor demonstrated typical enzymatic reaction kinetics. Obtained calibration curve for urea concentration determination was linear in the range from 0.02 to 0.16 mM (Fig. 3).

Minimum urea concentration detectable by the described biosensor matches the lower limit of the linear range of detection.

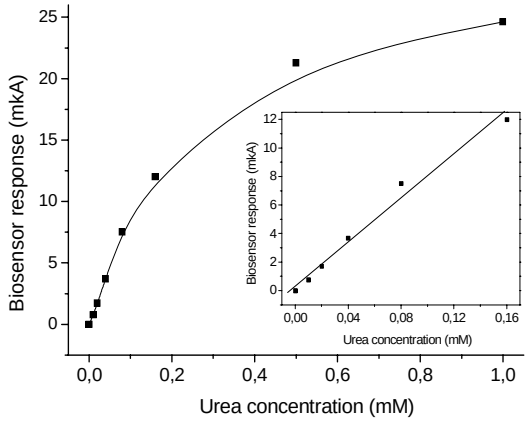

Fig. 3 Calibration curve for urea concentration determination in the model solution. Measurement was conducted in the 5 mM pH 7.4 phosphate buffer at room temperature

During the work with model solutions analytical characteristics of biosensor were investigated. The biosensor was characterized with high operational stability and reproducibility of responses, and also long-term storage stability. Linear range of the urea detection was sufficient for analysis of the blood serum samples from renal insufficiency diagnosed patients diluted by factor of 500.

When working with blood serum it is necessary to take into account presence of variety of substances that may cause non-specific change in the sensor signal. These substances may include proteins or low molecular mass blood components. Thus it was important to investigate their influence upon the biosensor response. A definite quantity of urease was added to the blood serum sample which then was incubated during approximately one hour, with occasional stirring, in order to remove the endogenous urea. Then this urea-free sample was placed into the biosensor cell (Fig. 4).

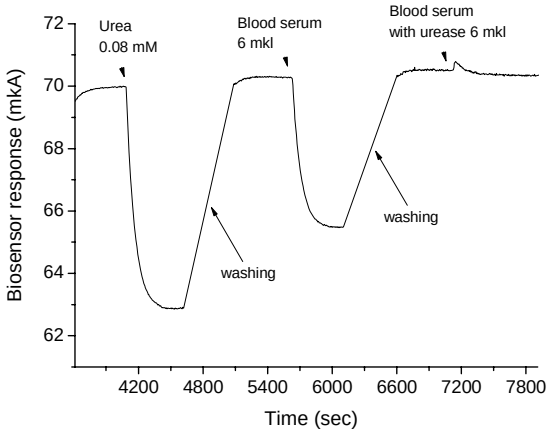

Fig. 4 Biosensor responses to introduction of substrate, blood serum with and without urease into the sample cell. Measurement was conducted in the 5 mM pH 7.4 phosphate buffer at room temperature

As can be seen from Fig. 4 introduction of the blood serum aliquot results in biosensor response similar to response toward pure urea. At the same time introduction of the blood serum previously incubated with enzyme does not lead to response signal appearance, apparently due to absence of urea in the sample.

Presented results demonstrate that the blood serum sampled from patients diagnosed with renal insufficiency and diluted by factor of 500 virtually does not induce non-specific response, and the developed urease biosensor is highly selective towards urea.

The influence of protein on the sensor response was also investigated (it is known that the content of protein in a healthy human blood is approximately 7.5 %). For this, bovine serum albumin was added to the working buffer in concentrations of 0.02 and 1 %. Fig. 5 shows calibration curves for urea concentration determination obtained in presence of protein in the said concentrations.

From the obtained data we can conclude that the presence of high-molecular protein fraction in buffer, taking into account dilution of the initial sample, does not lead to appearance of significant non-specific response. The emulated blood components thus practically did not influence the response of urease biosensor.

Next stage of the research was determining the urea content in the blood serum samples from the patients diagnosed with renal insufficiency. For this the classic biosensor method with calibration curve was used. Measurement was performed with 10 samples of blood serum diluted by factor of 500. The 5 mM pH 7.4 phosphate buffer was used as the working buffer.

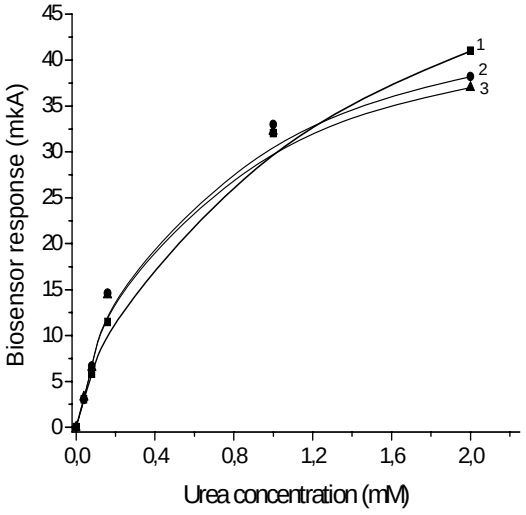

Fig. 5 Calibration curves for urea detection in presence of protein at various concentrations: 1) 5 mM pH 7.4 phosphate buffer; 2) 5 mM pH 7.4 phosphate buffer + 0.02 % BSA; 3) 5 mM pH 7.4 phosphate buffer + 1 % BSA

Before each measurement of the urea concentration in blood serum, sensor response to the 0.04 mM reference urea solution was measured thus providing the possibility of permanent control of biosensor calibration (Fig. 6).

Comparison of magnitudes of the obtained responses in solution with known urea concentration and in the blood serum sample allowed to estimate urea content in the latter.

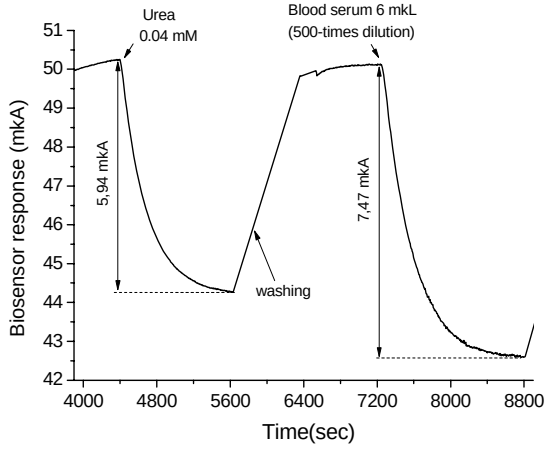

Fig. 6 Procedure for measurement of unknown urea concentration in the blood serum of a patient diagnosed with renal insufficiency. Measurement was conducted in the 5 mM pH 7.4 potassium-phosphate buffer at room temperature

One of the important characteristics of biosensor is the signal reproducibility, which was verified for urea concentration determination in both model solutions and actual blood serum samples. For this, sensor responses to the equal urea substrate concentration of 0.08 mM (for model solutions) and to 6 mkl of the blood serum admixed in buffer (for actual samples) were recorded during one workday at 30 min. intervals. As can be seen from Fig. 7, biosensor demonstrated high degree of signal reproducibility in both cases.

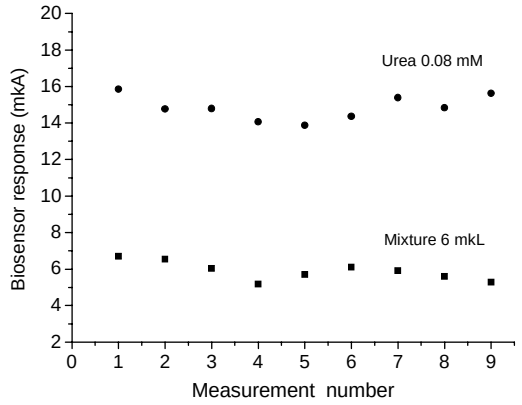

Fig. 7 Results of the biosensor signal reproducibility verification for determination of urea content in model solutions and blood serum samples. Measurement was conducted in the 5 mM pH 7.4 phosphate buffer at room temperature

Degradation of sensor functionality due to long-term storage in dry condition at +4 ºC also was investigated. The sensor was nearly daily used for urea analysis in blood serum (1-3 analysis procedures per day).

As can be seen from Fig. 8, the sensor performance remained stable during two weeks, then its activity rapidly drops. This can be explained by various reasons: membrane clogging by proteins contained in blood, enzyme being washed out of the membrane, or inhibitive influence of other blood components.

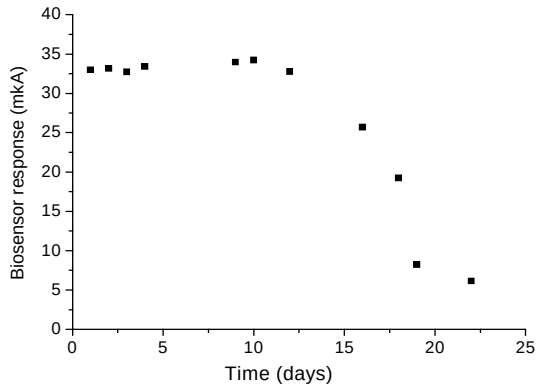

Fig. 8 Dependence of the urease biosensor response magnitude on storage time in dry condition at +4 ºC

IV. Conclusion

The method for determination of urea content in the blood serum of patients diagnosed with renal insufficiency was developed based on the designed potentiometric biosensor consisting of pH-sensitive field-effect transistors and urease immobilized by the glutaraldehyde vapor. It was shown that despite the multicomponent composition of the blood sample biosensor response remains selective towards urea. The latter can be explained by the use of biosamples diluted by factor of 500 and by differential mode of the sensor operation.

The high reproducibility of biosensor responses and stability of the sensor characteristics during storage and daily exploitation with the blood serum samples was shown too.

With the help of developed biosensor urea concentration analysis was performed for 10 samples of blood serum from different patients. High correlation of results (with correlation factor R = 0.97) obtained with urease biosensor and independent analytical method with the use of urease/peroxidase enzymatic system was demonstrated.

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References

[1] F. Kuralay, H. Özyörük, A. Yildiz, “Amperometric enzyme electrode for urea determination using immobilized urease in poly(vinylferrocenium) film,” *Sensor and Actuators B*, vol. 114, pp. 500–506, 2006.

[2] J. C. Chen, J. C. Chou, T. P. Sun, S. K. Hsiung, “Portable urea biosensor based on the extended-gate field effect transistor,” *Sensor and Actuators B*, vol. 91, pp. 180–186, 2003.

[3] K. Yoneyama, Y. Fujino, T. Osaka, I. Satoh, “Amperometric sensing system for the detection of urea by a combination of the pH-stat method and flow injection analysis,” *Sensor and Actuators B*, vol. 76, pp. 152–157, 2001.

[4] M. Gutiérrez, S. Alegret, M. del Valle, "Bioelectronic tongue for the simultaneous determination of urea, creatinine and alkaline ions in clinical samples," *Biosensor and Bioelectronics*, vol. 23, pp. 795–802, 2008.
[5] De J. V. Melo, A. P. Soldatkin, C. Martelet, N. Jaffrezic-Renault, S. Cosnier, "Use of competitive inhibition for driving sensitivity and dynamic range of urea ENFETs," *Biosensors and Bioelectronics*, vol. 18, pp. 345–351, 2003.
[6] R. Sahney, B. K. Puri, S. Anand, "Enzyme coated glass pH-electrode: Its fabrication and application in the determination of urea in blood samples," *Analytica Chimica Acta*, vol. 542, pp. 157–161, 2005.
[7] R. Koncki, "Recent development in potentiometric biosensors for biomedical analysis," *Analytica Chimica Acta*, vol. 599, pp. 7–15, 2007.
[8] G. Dhawan, G. Sumana, B. D. Malhotra, "Recent development in urea biosensors," *Biochemical Engineering Journal*, vol. 44, pp. 42–52, 2009.
[9] W. R. Fearon, "The carbamide diacetyl reaction: a test for citrulline," *Biochem. J.*, vol. 33, pp. 902–907, 1939.
[10] O. A. Boubriak, A. P. Soldatkin, N. F. Starodub, A. K. Sandrovsky, A. V. El'skaya, "Determination of urea in blood serum by a urease biosensor based on an ion-sensitive field-effect transistor," *Sensor and Actuators B*, vol. 26, no. 27, pp. 429–431, 1995.
[11] A. L. Kukla, A. S. Pavluchenko, Yu. V. Goltvianskyi, A. A. Soldatkin, V. M. Arkhypova, S. V. Dzyadevych, A. P. Soldatkin, "Sensor arrays based on the differential ISFET elements for monitoring of toxic substances of natural and artificial origin," *Sensor Electronics and Microsystem Technologies*, vol. 2, pp. 58–68, 2008.
[12] A. S. Pavluchenko, A. L. Kukla, Yu. V. Goltvianskyi, O. O. Soldatkin, V. M. Arkhypova, S. V. Dzyadevych, A. P. Soldatkin, "Investigation of stability of the pH-sensitive field-effect transistor characteristics," *Sensor Letters*, vol. 9, no. 6, pp. 2392-2396, 2011.
[13] S. V. Dzyadevych, V. N. Arkhypova, C. Martelet, N. Jaffrezic-Renault, J.-M. Chovelon, A. V. El'skaya, A. P. Soldatkin, "Potentiometric biosensors based on ISFETs and immobilized cholinesterases," *Electroanalysis*, vol. 16, pp. 1873–1882, 2004.
[14] http://www.terramedica.spb.ru/1d4_2007/slepushiva.htm