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Application of enzyme/zeolite sensor for urea analysis in serum

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ABSTRACT

Urea biosensor based on zeolite-adsorbed urease was applied for analysis of blood serum samples. It should be noted, that this biosensor has a number of advantages, such as simple and fast performance, the absence of toxic compounds during biosensor preparation, high reproducibility and repeatability (RSD = 9% and 4%, respectively). The linear range of urea determination by using the biosensor was 0.003–0.75 mM, and the limit of urea detection was 3 μM. The method of standard addition was used for analysis of serum samples with 500-fold dilution. Total time of analysis was 10 min. Good reproducibility of urea determination in real samples was demonstrated (RSD = 10%). Biosensor results were verified by using a common method of urea determination (diacetyl monoxime reaction). It was shown that by using this biosensor distinguishing healthy people from people with renal dysfunction becomes easier.

1. Introduction

Urea is the main end product of protein metabolism and it is synthesized in the liver from carbon dioxide and ammonia as a result of amino acid deamination. Once synthesized, urea is released from the liver to the blood and transported to the kidney, where it is filtered and excreted with the urine. A decreased urea level in the blood plasma is typical for patients with severe liver disease or insufficient protein intake and is an extremely rare occasion. A very high urea level in blood serum, called uremic syndrome, is associated with severe renal dysfunction. In this case it is necessary for either kidney transplantation or hemodialysis, which is much more preferable.

A physiological urea level depends on food consumed and is in a range from 2.5 to 7.5 mM [1]. A significant increase in urea concentration is observed during chronic and acute forms of renal disease (50–70 mM and 120–150 mM, respectively). Such abnormal urea levels can be reduced to 10 mM by hemodialysis or peritoneal dialysis. The urea level in dialysate may vary from 3 to 16 mM [2].

To establish an early-stage renal failure, the urea clearance should be determined, i.e. the ratio between the total amount of the substance excreted with the urine per day and the substance concentration in the blood. The normal urea clearance is 64–99 ml/min, depending on age and gender. Thus, complex and multiple tests for urea are required to diagnose different renal disorders in patients [3].

Considering the important biological role of urea as a diagnostic indicator of kidney failure and major uremic toxin, its determination is needed in medical diagnostics for clinical evaluation of renal function and monitoring the effectiveness of hemodialysis—the main treatment that saves the lives of millions of patients worldwide.

At present, a number of biosensors are developed for urea determination in biological samples. Among them are potentiometric [4–6], conductometric [7–9] and amperometric [10] biosensors. All biosensors for monitoring urea concentration use urease (EC 3.5.1.5) as a catalyst and bioselective element. Immobilization of urease is carried out by covalent binding [11], physical adsorption [12], inclusion in polymers [12–15] or binding to the transducer surface [16,17]. A number of problems are connected with immobilization methods, such as loss of enzyme activity in course of immobilization and reproducibility of immobilization. One of the approaches to overcome these problems is an application of various stabilizing agents, mediators, and nanomaterials.

Scientists display a deep interest in zeolites due to their specific properties. They have low toxicity, are tolerant to microorganisms and are resistant to mechanical, chemical and thermal damages [18]. For this reason zeolites can be used during work with multicomponent biological samples. Heat treatment can regulate the amount of surface −OH groups, which are important for zeolite immobilization in the bioselective element [19]. Some zeolites can be used as catalysts. The

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zeolite surface can be also modified with various organic and inorganic groups ($-NH_2$, $-SH$, $-Cl$, $-CN$, $-C_6H_5$, etc.) and has a high specific surface area, thus providing different interactions between zeolite, enzyme and transducer [20,21]. A wide range of modifications allows obtaining zeolites with diverse properties (for example, conductivity), which are potentially helpful for the biosensor development.

That is why we have developed a biosensor for urea determination based on a new method of immobilization—urease adsorption on zeolite. An application of this method will enable us to obtain highly reproducible biosensors without usage of toxic reagents.

2. Materials and methods

2.1. Materials

In this work we have used enzyme urease (EC 3.5.1.5), activity 66.3 U/mg, from Fluka (Switzerland); glycerol, bovine serum albumin (BSA, fraction V), and urea were purchased from Sigma-Aldrich Chemie (Germany). Potassium-phosphate buffer (KH_2PO_4-Na_2HPO_4) was of Ukrainian production. Other used inorganic compounds had an analytical grade.

Silicalite was synthesized in the Middle-East Technical University (Ankara, Turkey) from a gel whose composition was TPAOH: 5 and TEOS: 500 H_2O. Tetraethylorthosilicate (TEOS, 95% Acros) was used as a silica source, tetrapropylammoniumhydroxide (TPAOH, 25%) as a template. The mixture was continuously stirred for 6 h at room temperature, and then the resulting gel was placed for 18 h in an oven at 125 °C. The solid particles were centrifuged at 13,000 rpm, washed with deionized water and dried at 80 °C.

2.2. Conductometric transducers

We used the conductometric transducers manufactured in V. Lashkaryov Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (NASU) (Kyiv, Ukraine) in accordance with our recommendations. They are 5 × 30 mm in size and consist of two identical pairs of gold interdigitated electrodes deposited onto a ceramic support. The dimensions of the sensitive area of each electrode pair were about 1.0 × 1.5 mm. The width of each digit as well as the interdigital spaces was 20 μm. The general view of the conductometric transducer and the scheme of the measuring device have been presented in earlier publication [22].

2.3. Drop-coating of silicalite onto transducers

A silicalite layer was formed on the transducer surface by drop-coating. 10% silicalite solution in 5 mM PBS, pH 6.5, was used. 0.165 μl of the solution was deposited onto the active zone of each pair of electrodes, then the transducer was heated during 6 min up to 200 °C. This temperature had no effect on silicalite and did not influence the transducer working parameters. The procedure resulted in the formation of the silicalite layer in the electrode active zones (Fig. 1.).

2.4. Preparation of bioselective elements

In 2011 we developed a new method of enzyme immobilization by adsorption for bioselective element preparation. The procedure of urease adsorption on silicalite was as follows [23]. The transducers previously coated with silicalite (see 2.3) were used. Onto one pair of electrodes we deposited 0.15 μl of 5% urease solution in 20 mM phosphate buffer, pH 6.5, and 0.15 μl of 5% BSA in an analogous buffer—onto the other (reference) pair; then the transducer was exposed to complete air-drying (for 17 min). Neither glutaraldehyde nor any other auxiliary compounds were used. Next, the transducers were submerged into the working buffer for 20–30 min to wash off the unbounded enzyme. After experiments the transducer surface was cleaned from silicalite and adsorbed urease with ethanol-wetted cotton.

2.5. Experimental setup for conductometric measurements

A portable conductometric analyzer developed in the Institute of Electrodynamics (NASU) was used to determine changes in conductivity in the near-electrode buffer layer of two pairs of electrodes of each conductometric transducer (Fig. 2): 4 conductometric transducers (2) were fixed in the plastic holder (1) and connected to the module of secondary transducers (7) and measuring and control module (8), which processed signal and transmitted it to a personal computer via the RS-232C interface [24]. The module of secondary transducers includes 4 channels on the basis of an ac bridge and provides measurements in differential mode. Each of these bridge circuits includes both electrode pairs (working and reference) of conductometric transducer. Bridge circuits can be balanced on reactive and active components of impedance of conductometric transducers. This reduces the influence of non-informative parameters of transducers on the measurement results and allows obtaining stable metrological characteristics of the instrument. The analyzer is able to process the signals of all 4 channels simultaneously.

2.6. Measurement procedure

Measurements were carried out at room temperature in a 5 mM phosphate buffer solution, pH 6.5, continuously stirred in an open 2 ml cell. The substrate concentrations in the cell were varied by addition of different volumes of the stock solution. All experiments were repeated three times, and the mean or mean ± standard deviation (SD) results were plotted. Nonspecific changes in the output signal induced

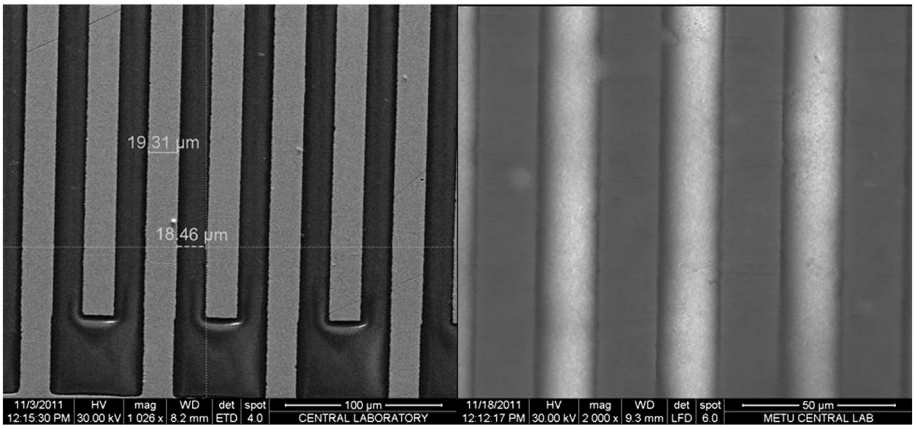

Fig. 1. SEM image of the active region of conductometric transducer with and without silicalite layer.

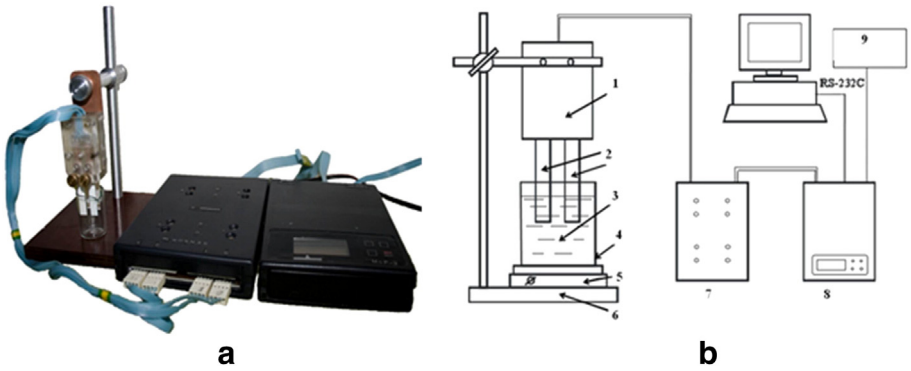

Fig. 2. Overall view (a) and scheme (b) of the portable conductometric analyzer: 1—fixing block, 2—biosensors, 3—buffer solution (4 ml), 4—working cell, 5—magnetic stirrer, 6—holder, 7—module of secondary transducers, 8—measuring and control module, and 9—power supply.

by fluctuations of temperature, medium pH, etc. were avoided due to usage of differential mode of measurement.

3. Results and discussion

3.1. Analytical characteristics of biosensors

Urease-based biosensors function due to the enzymatic reaction:

$$\text{Urea} + 2H_2O + H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^-$$

This reaction results in changes of the conductivity in the near-electrode layer of the solution that can be registered by the conductometric transducer [25]. These changes and, consequently, the biosensor response are proportional to the urea concentration. Usage of low toxic zeolites with high surface area and good adsorption properties led to formation of a uniform enzyme layer on the transducer surface with a high amount of immobilized urease. A large amount of pores inside the zeolites prevented them from changing the conductivity of the solution in the near-electrode layer; for this reason, zeolites did not influence significantly on the work of biosensor, but they were good urease carriers.

At the first stage of research we studied the sensitivity of the biosensor based on zeolite-adsorbed urease towards diverse urea concentrations. The dependence of response value on urea concentration in the analyzing sample was investigated and the calibration curve of the biosensor was plotted (Fig. 3). As seen, the linear detection range was up to 0.75 mM, which should be considered in further experiments for analyzing urea in real blood samples. The limit of urea determination was 3 μM.

Biosensor work strongly depends on the characteristics of the solution in which measurements take place. In our previous work we optimized the procedure of urease adsorption onto the silicalite surface during biosensor creation [23]. The influence of solution properties on biosensor work was also studied there. It was demonstrated that pH, buffer concentration and ionic strength influence on biosensor work. However in case of significant dilution of the serum sample (500-fold, see Section 3.3) these effects can be neglected because the concentration of added interfering substances will be too low to change biosensor characteristics.

3.2. Operational stability of biosensor and reproducibility of biosensor preparation

Repeatability and operational stability are important working characteristics of biosensors. To determine signal repeatability, the biosensor

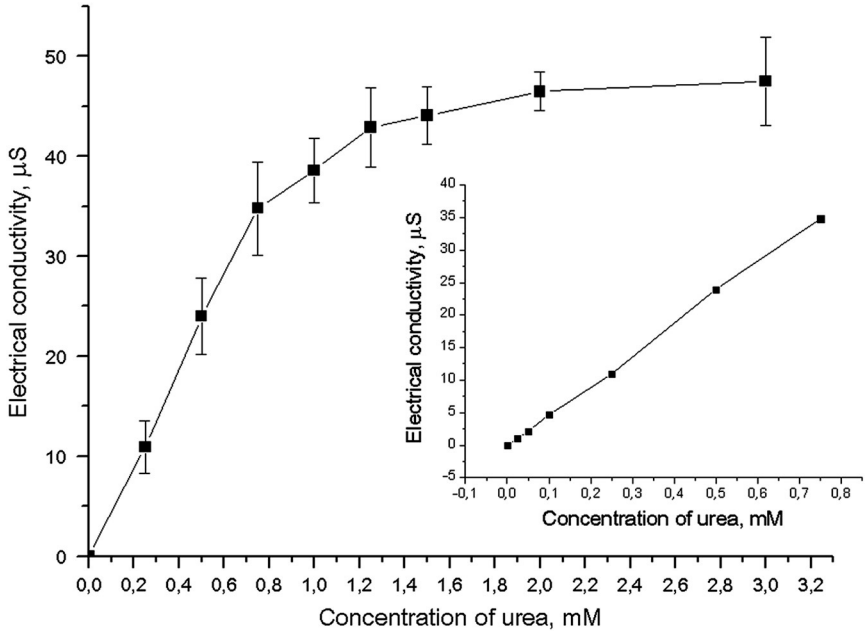

Fig. 3. Calibration curve of the urea biosensor. Measurements were done in 5 mM phosphate buffer, pH 6.5.

responses to the urea saturation concentration (2 mM) were measured during one working day with 30–35-min intervals. All the time between measurements the sensor was kept in the buffer solution upon continuous stirring. An error (relative standard deviation) of the urea measurement was 4%. It is considered to be quite acceptable, so the theoretically biosensor can be used for urea determination in assays of blood plasma and dialysate (Fig. 4, a).

A common disadvantage of biosensors with adsorbed enzymes is a gradual wash-out of the latter into the working solution due to weak enzyme-adsorbent binding. Therefore, an important subject of investigation was the sensor operational stability for the period of several days. During the experiment, the developed biosensors were stored dry at 4 °C. Over 10 days the response decreased to 90% of its initial value (Fig. 4, a).

At the next stage of our work, we have checked another important working characteristic—reproducibility of biosensor preparation. The experiments were carried out as follows. Several clean electrodes were drop-coated with two layers of silicalite, then urease was adsorbed, and two to three responses to the substrate (of saturation concentration) were measured. Next, the enzyme and silicalite layers were removed from the transducers with spirit-wetted cotton. The silicalite drop-coating and urease adsorption were repeated 5–8 times, and every time two to three responses were measured. The reproducibility error (relative standard deviation) was 9% (Fig. 4, b). The reason of low response dispersion can be a considerably weaker dependence of enzyme adsorption on environmental conditions as compared with other methods of immobilization. Therefore, the enzyme adsorption on silicalite is a more controllable method of immobilization than other traditional methods used in biosensorics.

3.3. Measurement of real samples

An important issue of our work was the measurement of blood serum in real samples. The experiment goal was to prove that conductometric biosensors based on a new method of urease immobilization are suitable for measurement in blood serum samples. We used the samples

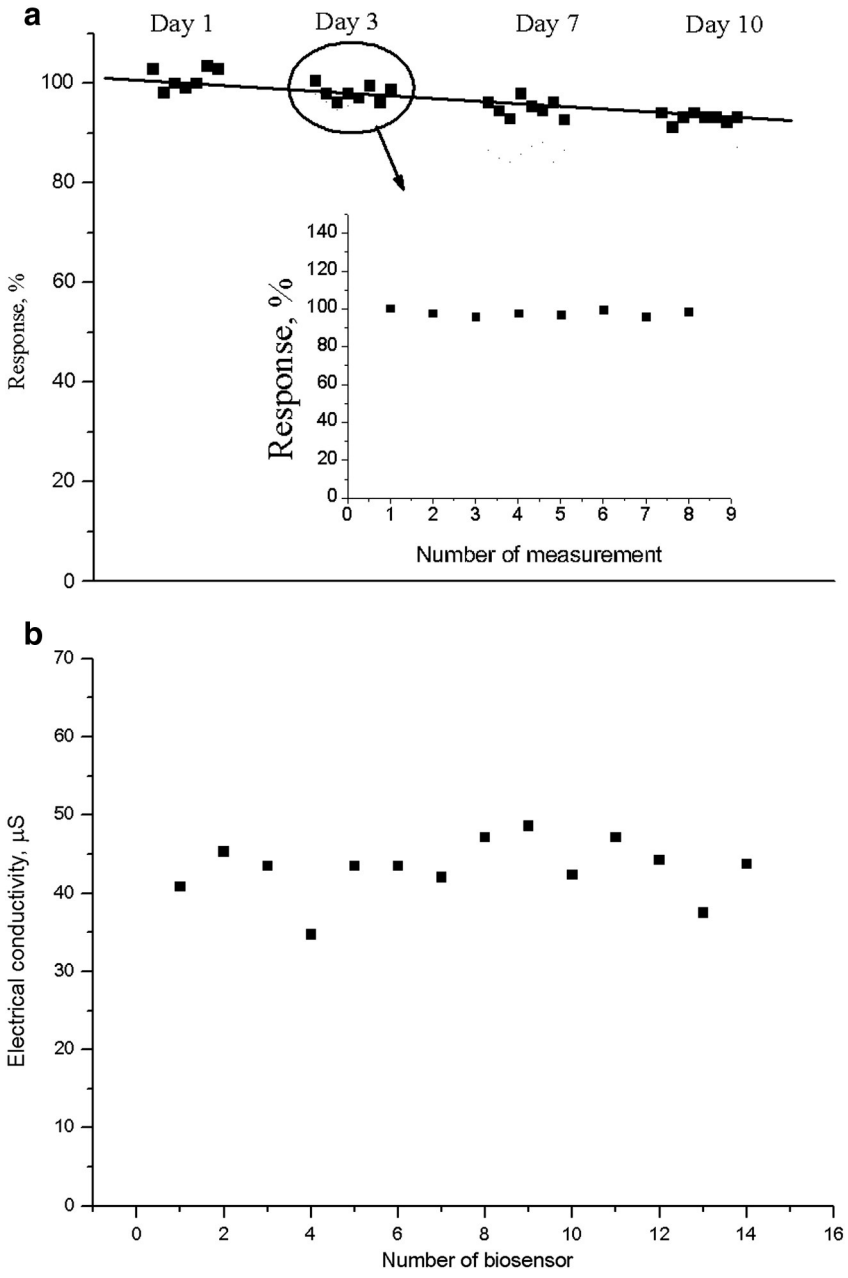

Fig. 4. Operational stability (a) and inter-reproducibility (b) of the developed biosensor based on silicalite-adsorbed urease. Measurement in 5 mM PBS, pH 6.5. Urea concentration—2 mM.

with excessive urea concentration taken from patients with renal failure, and two samples—from healthy people. Control determination of urea concentration using diacetyl monoxime reaction was carried out in "Kyiv municipal scientific and practical center of nephrology and hemodialysis" [26].

Urease is considered to be a good example of high-specific enzyme. Usually it is used for urea decomposition (Km = 1–4 mM). However, urease also splits hydroxyurea (Km = 1.25–1.6 mM), dihydroxyurea (Km = 12.5 mM), semicarbazide (Km = 60 mM), acetamide (Km = 750 mM), methylurea (Km = 220 mM), thioacetamide (Km = 83 mM), thiourea (Km = 70 mM), and formamide (Km = 1060 mM) [27,28]. Most of these compounds are not produced in human organism and thus cannot be found in blood. Even if some interfering compound will be present, its concentration will be insignificant after 500-fold dilution of a serum sample.

In our work we measured urea concentration in blood assays by the method of standard additions at 500-fold sample dilution. The examples of experiments are shown in Fig. 5. First, we obtained the biosensor response to the insertion of the blood serum aliquot into the measuring cell and plotted it on the ordinate axis. Next, three responses were obtained to three injections of 0.025 mM urea into the same cell. Plotting these results we obtained a straight line, the intersection of which with the abscissa axis corresponded to the urea concentration in the tested sample of blood serum (considering 500-fold dilution) (Fig. 5). Total time of analysis of one sample was about 10 min.

For each addition we carried out three to five repetitive measurements, and then calculated a mean value and standard deviation. As seen from Fig. 6, sensitivity and measurement accuracy of the biosensors were quite sufficient for precise differentiation of the serum samples taken from patients and healthy people.

The traditional method of diacetyl monoxime reaction was used as a control method of urea determination [29]. The principle of diacetyl monoxime reaction is that in the presence of thiosemicarbazide and iron (III) ions urea forms a complex with diacetyl monoxime, whose color intensity (measured at 540 nm) is directly proportional to the urea content in the biosamples tested. Different modifications of the reaction were proposed: addition of tryptophan, nitrites, phenylanthranilic acid, potassium persulfate, antipyrine, etc. Improved reaction is characterized by simplicity: only 15–20 min is required for it. The main disadvantages of the reaction are sensitivity of the urea-diacetyl monoxime complex to light and quick loss of color. Furthermore, reagents for the reaction are toxic, and modifications of urea also produce false results.

We revealed good correlation between the values of urea concentration in the samples of blood serum measured by the biosensor based on silicalite-adsorbed urease and those obtained by diacetyl monoxime reaction. The correlation coefficient was 0.995 (Fig. 6).

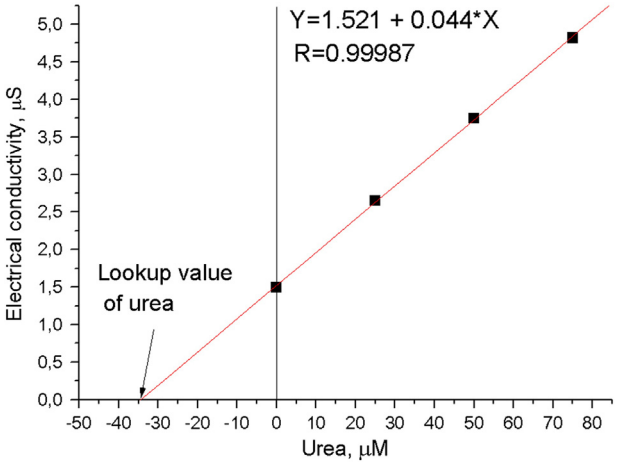

Fig. 5. Real experiment on urea determination by the developed biosensor using the method of standard additions. Measurement in 5 mM phosphate buffer, pH 6.5.

Number of sample	Methods of urea determination	
	Diacetyl monoxime reaction, mM	Biosensor, mM
1	27.2	28.9 ± 3.7
2	42.0	41 ± 3.9
3	47.4	49.2 ± 5.4
4	36.7	42.7 ± 2.6
5	33.1	37.9 ± 5.6
6	2.5 - 6.5	5.7 ± 0.8
7	2.5 - 6.5	4.5 ± 1.1

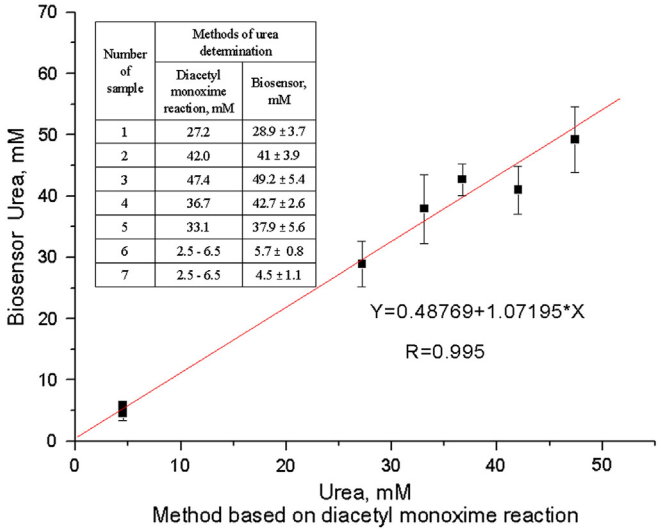

Fig. 6. Comparison of measurement by biosensors based on silicalite-adsorbed urease and by the method of diacetyl monoxime reaction. Measurement in 5 mM phosphate buffer, pH 6.5.

3.4. Repeatability of the biosensor signal

It was also important to show repeatability of the biosensor signal at measurement in real samples. For this purpose, a serum sample with a considerably higher than normal urea concentration was sequentially tested by the same biosensor ten times during one working day. The relative standard deviation between measurements was about 9%. Certainly, in this case repeatability is significantly worse than when testing model samples; however, this is a common effect for impure real assays, in particular, blood plasma (Fig. 7).

4. Conclusion

At this time, there are many different biosensors for urea determination [30]. Most of them are based on urease because it is a well-studied, cheap and stable enzyme; several enzyme systems are also used. Enzyme-based urea biosensor relies on different types of transducers: potentiometric [31,32], amperometric [33], conductometric [34], thermal [35], and optical [36]. Furthermore, there is a lot of communications about new modifications of immobilization procedures, and development of new urea-sensitive chemo- and biomembranes. Despite this,

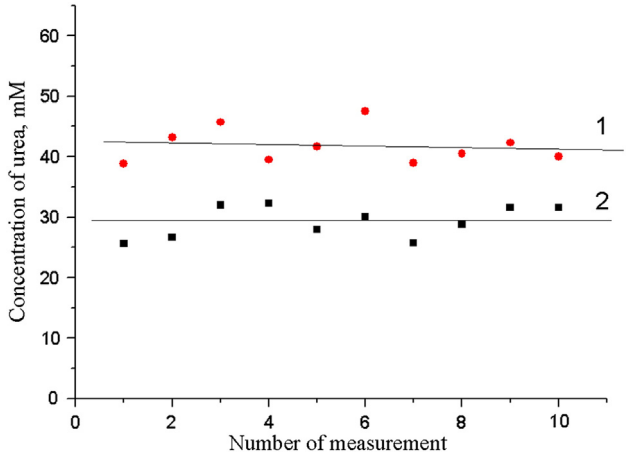

Fig. 7. Repeatability of the results of measurement of two serum samples (1—sample with 42 mM of urea, and 2—sample with 29 mM of urea) by the biosensor based on silicalite-adsorbed urease. Measurement in 5 mM phosphate buffer, pH 6.5, by using the method of standard additions (500-fold dilution).

each year many new biosensors for urea determination are being described. This happen because there is no ideal universal biosensor that can be used every day and development of new biosensor systems for precious, rapid, sensitive and selective determination of urea is an actual task. The proposed biosensor can fulfill some of the above-mentioned requirements.

We have created a highly sensitive and stable conductometric biosensor for urea determination using a new method of immobilization of the enzyme—urease adsorption on silicalite. The main advantages of our biosensor are good reproducibility of biosensor preparation, low cost of conductometric transducers, and absence of toxic compounds during biosensor preparation. This biosensor was used to analyze the urea concentration in a number of blood samples. The obtained results were in good correlation with the results of the traditional method of urea measurement based on the diacetyl monoxime reaction with a correlation coefficient 0.995. It was also shown that the proposed biosensor method is characterized by good reproducibility of results when working with blood samples; the measurement error did not exceed 10%.

The developed biosensor with improved analytical performance along with the upgraded procedure of biosensor analysis of blood samples may be used in the future in medical practice.

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