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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

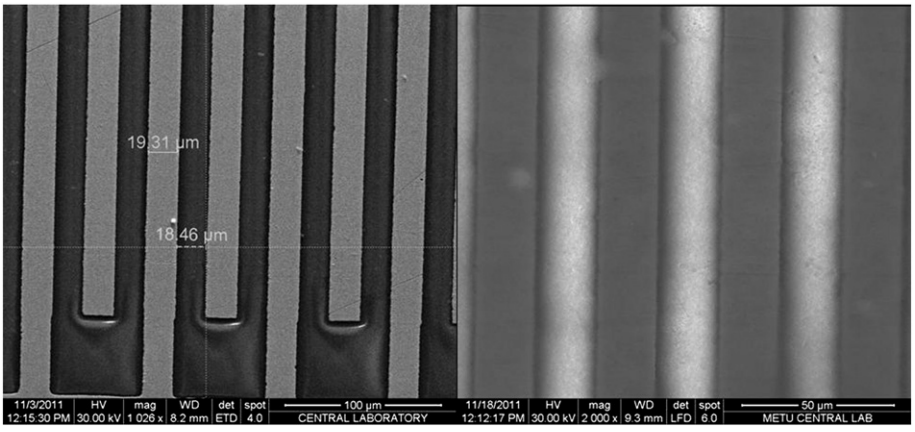

Fig. 1. SEM image of the active region of conductometric transducer with and without silicalite layer.