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Application of potentiometric biosensor based on recombinant urease for urea determination in blood serum and hemodialyzate

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ABSTRACT

Potentiometric biosensor based on two identical pH-sensitive field-effect transistors for urea determination was developed. Recombinant urease with low affinity to urea (K_m = 200 mM) was immobilized via entrapment in PVA/SbQ photopolymer on one transistor and served as a biorecognition element of the biosensor, while bovine serum albumin in PVA/SbQ photopolymer placed on the second transistor was used for reference. Biosensor was characterized by a wide range of urea determination: 0.5–15 mM linear range (operational detection range – 0.5–40 mM) and quick response time (1–2 min). Samples of blood serum and hemodialysate without urea caused no biosensor response. All this allowed analysis of blood serum samples that were diluted 10 times, what led to smaller measurement error in comparison with 100–500 times dilution in case of biosensors based on non-modified urease from soy beans. Urea concentrations in 10 samples of serum were determined; biosensor results correlated well with two control methods of urea measurement. Furthermore, kinetics of decreasing urea concentration in dialysate during hemodialysis was demonstrated. Biosensor measurements of dialysate samples were verified by traditional colorimetric determination of urea. Proposed biosensor can be effectively used for analysis of samples with different concentrations of urea and for hemodialysis control. What is more, characteristics of the biosensor make possible real-time measurements of urea concentration during hemodialysis by pumping dialysis fluid through the working cell. Biosensor showed no significant decrease of responses during 5 month of storage.

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1. Introduction

Urea is an organic substance which is widely distributed in living species. There is a balance between production (animals, microorganisms) and hydrolysis (microorganisms) of urea. Analysis of the urea concentration in various samples is essential for medical diagnostics, agro-food industry analyses, and in environmental monitoring [1]. In medical diagnostics, determination of the urea level is an important routine test since urea is the end product of protein metabolism and the main nitrogen component of urine, which is produced by the liver and excreted by the kidneys. Under certain pathological states, such as renal failure, hyperpyrexia, hyperthyrosis, leukemia, diarrhea, diabetes, urea concentration exceeds the normal range, which is 2.5–7.5 mM in the blood and 10–30 g (total amount) in urine collected during the day. Additionally, high level of urea in blood can be caused by chronic or acute renal failure (50–70 mM and 120–150 mM, respectively), urinary tract obstruction, dehydration, shock, gastrointestinal tract bleeding. These abnormal levels of urea can be reduced by hemodialysis or peritoneal dialysis (the urea level in dialysate may vary from 3 to 16 mM). Reduction in the urea level is very rare and may be caused by liver failure, nephrotic syndrome, and cachexia [2].

For the analysis of urea level, modern clinical laboratories mainly use spectroscopic methods, which rely on the pretreatment of samples, are time-consuming and unsuitable for real-time determination of urea.

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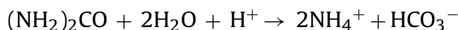
Biosensors based on enzyme urease are alternative for traditional methods of urea determination. Urease splits urea into ammonia and hydrogen carbonate by the reaction:



$$(NH_2)_2CO + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$$

which results in a decrease in concentration of hydrogen ions (pH increases) and formation of two new ions. Therefore this enzyme can be used for the development of different types of urea biosensors – potentiometric [1–9], amperometric [10–13], conductometric [14,15], thermal [16,17], optical [18–21].

Despite the large number of developed biosensors, almost all of them have one common disadvantage – a rather narrow linear range of urea determination. Therefore, when measuring high concentrations of urea it is necessary to dilute the samples by 200–500 times, which can lead to a significant increase in biosensor measuring error. These drawbacks can be avoided using low-affinity recombinant urease with large Michaelis constant (K_m) for biosensor creation instead of natural unmodified urease. This approach has to expand the biosensor linear detection range and shift it to higher urea concentrations.

This study was aimed at the development of a potentiometric biosensor based on low affinity recombinant urease (K_m = 200 mM) and its application for urea measurement in real samples of blood serum and hemodialysate.

2. Materials and methods

2.1. Materials

In this work, two urease types were used for comparison: recombinant urease (EC 3.5.1.5) expressed in *Escherichia coli*, activity of 150 U/mg, from Usbiological (USA) and unmodified urease from jack beans (*Canavalia ensiformis*) with activity of 66.3 U/mg from Fluka. Bovine serum albumin (BSA, fraction V), Nafion and urea were obtained from Sigma–Aldrich Chemie (Germany); PVA-SbQ – from Toyo Gosei Kogyo Co. Ltd. (Japan). The working phosphate buffer (KH_2PO_4-NaOH), pH 7.4, was prepared from reagents from Helicon (Moscow, Russia). Other compounds used were of analytical grade.

The samples of blood dialysate and serum for the measurements of urea content were obtained from Kiev municipal scientific and practical center of nephrology and hemodialysis (Ukraine).

2.2. Design of potentiometric transducers

Potentiometric transducers were based on pH-selective field-effect transistors and were produced at the JSC "Kwazar" facilities (Kiev, Ukraine) according to our recommendations. Each transducer contained a differential pair of pH-sensitive field effect transistors. Two identical p-channel field-effect transistors (FETs) were placed on a single crystal with the total area of 8 mm × 8 mm. Signals were recorded from both transistors and then signal from reference transistor (covered with BSA membrane) was subtracted from the signal of active transistor (covered with enzyme membrane). Transistors demonstrated 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.

More information about transistor structure and their photo can be found in [22], and description of a portative measuring device – in [23].

2.3. Preparation of biosensors

To prepare biosensors, urease was immobilized onto the surface of transducers via photopolymerization in PVA/SbQ. Aqueous solution containing 66% of PVA/SbQ and 10% of glycerol in 20 mM potassium-phosphate buffer, pH 7.4, was prepared. Right before the deposition onto the working surfaces of transducers, 10% solution of recombinant urease in 20 mM potassium-phosphate buffer, pH 7.4, was mixed with above-mentioned PVA/SbQ solution at 1:1 ratio and thoroughly mixed to obtain a homogeneous solution. The mixture for the reference membrane was prepared in the same way, but instead of the enzyme 10% BSA was used.

The resulting solutions were deposited onto the working regions of pH-FET transducers (enzyme-containing solution was placed onto one region of the transducer and BSA-containing solution – onto another region) until the regions were entirely covered (average volume of the mixtures deposited onto each sensitive area was 0.1 μl). The sensor chip with deposited membranes was placed under the UV lamp KF-4M (Ukrainian production) of 3.4 V/m^2 intensity at a distance of 10 cm from the biosensor to form bioselective membranes. The time of photopolymerization was 20 min.

2.4. Formation of additional Nafion-based membranes

Negatively charged polymer Nafion was used for the formation of additional membranes, which serve as an ion-selective barriers restricting the anions diffusion to the electrode surface [24]. An additional membrane was necessary to reduce the impact of components of hemodialysate and blood serum on the biosensor work.

Nafion solution (1%, w/w) in 10 mM phosphate buffer, pH 7.4, was prepared. After the urease immobilization, biosensors were kept in air during 10 min. Afterwards, 0.1 μl of Nafion solution was dropped over the enzymatic membrane and the same amount – over the reference membrane by using a micropipette. The transducers were kept in air for 15 min; during this time Nafion membrane was formed. Next, the biosensors were washed with the working buffer solution to wash out unbound components of membranes.

2.5. Measurement procedure

Measurements were carried out in the 5 mM potassium-phosphate buffer solution (KH_2PO_4-NaOH), pH 7.4, with intensive stirring at room temperature. The biosensor and Ag/AgCl reference electrode were placed into an open 1.5 ml measuring cell. The urea concentration in the working cell was obtained by the addition of aliquots of concentrated stock solution. The values of biosensor responses were calculated after reaching steady-state. After obtaining each response, biosensor was washed from products of reaction by changing working buffer (3 times with 2-min interval).

Non-specific changes in the output signal associated with fluctuations of temperature, medium pH, and applied voltage were compensated by using differential mode, i.e. measurement of the difference between the signals from two pH-FET electrodes (with enzyme and referent membranes), placed on the same transducer. All experiments were performed at least in three series.

2.6. Control methods of urea detection

Two control methods of urea determination in blood serum (a urease/glutamate dehydrogenase enzymatic system [25] and a diacetyl monoxime reaction [26]) were used for comparison. 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

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