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Conductometric urease microbiosensor based on thin-film interdigitated electrodes for urea determination

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he characteristics of conductometric urease microbiosensor based on thin-film interdigitated electrodes for urea determination have been studied. Urease has been immobilized by cross-linking with bovine serum albumin using glutaraldehyde. The resulting conductivity changes are produced by enzymatically catalyzed hydrolysis of urea. This process for both immobilized enzyme and soluble one is described by the classic laws of enzymatic kinetics. The influence of ionic strength, pH and buffer capacity of the samples on the biosensor response has been thoroughly tested. The results have been discussed concerning urea concentration analysis in human blood.

Introduction. Enzyme biosensors can play the major role in the fields of growing interest such as biomedicine and environmental science. These sensors seem to be very promising for such a purpose, since analytical systems based on them are simple, rapid and selective. The determination of urea in body fluids is one of the most frequent analysis in routine clinical laboratory. In mammalian liver, nitrogen derived from amino acid breakdown is converted to urea via a special catabolic pathway, the urea cycle, and is readily excreted by the kidneys [1]. An increased concentration of urea in blood and its reduced level in urine is therefore a strong indication for the renal disfunction.

In this work the alternative method for urease investigation based on a thin-film conductometric microsensor is proposed. The application of microfabricated conductometric transducers with thin-films interdigitated electrodes for the enzyme biosensors has been presented recently [2, 3]. The main advantage of conductometric detection is that almost all enzymatic reactions involve either consumption or production of charged species and therefore lead to the change in the ionic composition of the solution sample [4]. From the technological point of view it is important that thin-film metal electrodes are suitable for miniaturization and large scale production using inexpensive thin-film technology.

Although there is a great amount of information on the creation of biosensors for urea determination, the prospects for their practical use are not yet entirely clear. It is known that different factors of the analyzed liquid, blood in particular, affect the sensor characteristics in many different ways, and this effect should be taken into consideration when the analysis procedure is developed [5]. Therefore, we found it expedient to investigate thoroughly the properties of soluble and immobilized urease when they are influenced by

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factors typical for biological liquids, namely, ionic strength, buffer capacity and pH.

Materials and methods. *Materials.* Urease (EC 3.5.1.5, type B) with activity 12 I.U./mg was obtained from Olina (Lithuania), bovine serum albumin (BSA) was purchased from Sigma (USA) and 25 % aqueous solution of glutaraldehyde (GA) was va (Germany). All other reagents were of analytical grade.

Enzyme immobilization. Urease was immobilized using the procedure described earlier [5, 6]. 10 % (w/w) solutions of urease and BSA were prepared in 5 mM phosphate buffer (KH_2PO_4 — NaOH), pH 7.4. Prior to the deposition on the sensor chip these solutions were mixed in 5 % GOD, 5 % BSA and glycerol was added till 10 %. Glycerol prevents a loss of the enzyme activity during the immobilization and provides homogeneity of the membrane and its better adhesion to the sensor surface. Since a differential experimental set-up was used, a drop of the enzyme-containing mixture was deposited on the sensitive area of the measuring pair of electrodes while only a mixture containing 10 % BSA and 10 % glycerol was deposited on the reference pair of electrodes. Then the sensor was placed in a saturated GA vapour for 30 min. After exposure to GA, the membranes were dried at room temperature for 15 min. Before use the membranes were soaked in a 5 mM phosphate buffer, pH 7.4, for at least 30 min to equilibrate the membrane system.

Sensor design and measurements. The conductometric transducers consist of two identical pairs of gold interdigitated electrodes photolithographically patterned on a ceramic support with dimensions 5 mm × 30 mm (thickness 0.5 mm). The intermediate layer of titanium (0.1 μm thick) is first deposited for better gold adhesion. The sensitive area of each pair of electrodes forming a conductometric transducer is about 1 mm × 1.5 mm.

The original device consisting of an «Emocon-2» apparatus (production of Emocon Ltd, Kiev, Ukraine) coupled with the personal computer, a generator and an amplifier has been developed for the automatic monitoring of the biosensor system described. The generator provides the sinusoidal wave of 10.9 kHz frequency and 10 mV peak-to-peak amplitude about 0 V which is applied to two pairs of electrodes forming a miniaturized conductance cell. The low noise differential instrument — an amplifier measures the differential signal between the pair of electrodes covered with the immobilized enzyme and those covered with the «blank» membrane. The signal is monitored on IBM personal computer.

The measurements were carried out in daylight at room temperature (20—25°C) in a glass cell of about 2 ml volume. The interdigitated electrodes were immersed in vigorously stirred buffer solution and after stabilization of the

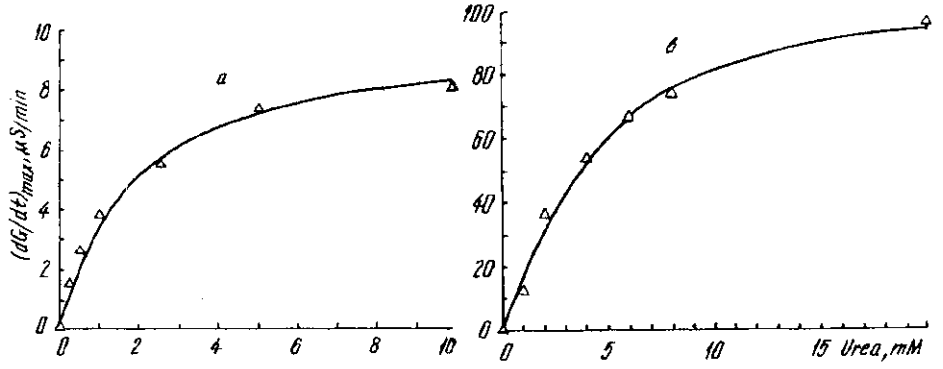

Fig. 1. The calibration curves for the urea conductometric biosensors with soluble (*a*) and immobilized (*b*) urease in the kinetic mode of measurement in 5 mM potassium phosphate buffer, pH 7.4

sensor output signal, the portion of substrate was added to the cell.

Results and discussion. In the case of urea assay, the resulting conductivity changes are caused by enzymatically catalyzed hydrolysis of the substrate:

$$\begin{matrix} H_2N \\ \diagdown \\ \quad C{=}O \\ \diagup \\ H_2N \end{matrix} + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-.$$

The typical calibration curves for the urea biosensors (with soluble and immobilized urease) in kinetic mode of measurement are shown in Fig. 1. These curves were replotted in the Eadie-Hoffstee coordinates and revealed the kinetic parameters enzymatic reaction. The hydrolysis kinetics for both immobilized and soluble enzymes is described by the Michaelis-Menten equation. K_m appeared to be identical to that measured by widely used methods [4, 7, 8] and its value was 1.33 mM for soluble urease and 3.73 mM for immobilized one correspondingly. This result suggests that reaction in the presence of the immobilized urease is controlled not only by the catalytic rate but also by the diffusion of substrates and products through the membrane.

The dependence of the enzymatic reaction maximum rate (kinetic response) on pH of the examined solution is presented in Fig. 2. In the case of the soluble urease, a maximum was observed at pH 6.5 in 5 mM potassium phosphate buffer. It does no with the results that we obtained earlier by the colorimetric determination of urea in 100 mM potassium phosphate buffer where the optimum pH for soluble urease was 7—7.5 [8]. This can be explained by noting that at low buffer capacity of the ammonia ions can not associate completely with the buffer species which are present in the sample. Thus, the enzyme actually works at higher pH than that of examined solution and it is just the reason of the apparent pH shift. In case of immobilized urease the optimum pH is wider which can be explained by the enzyme stabilization in the membrane.

The dependence of the reaction rate on the buffer concentration was also studied. In these experiments KCl was added to the solutions of different buffer concentration to ensure their identical conductivity. As seen from Fig. 3 the kinetic response for soluble urease greatly decreased with an increase of the buffer capacity of the solution. It means that the buffer species in the solution can associate with ammonia ions. For immobilized urease kinetic response

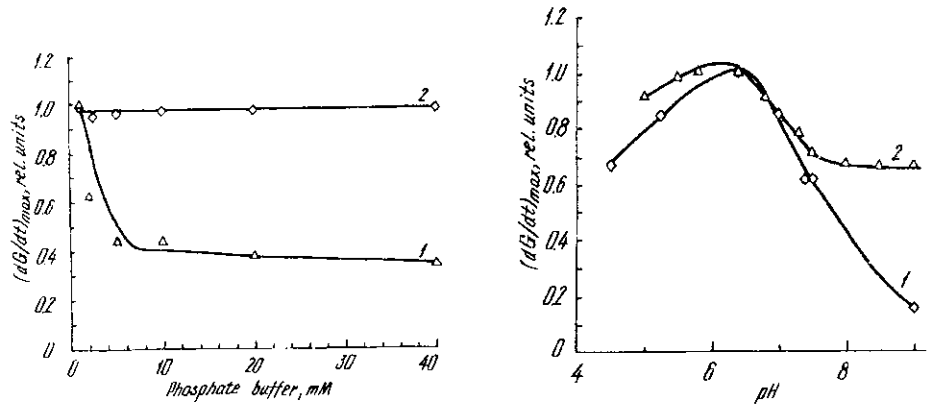

Fig. 2. pH dependence of the relative value of the enzymatic reaction rate for soluble (curve *1*) and immobilized (curve *2*) urease, 1 mM urea in 5 mM potassium phosphate buffer

Fig. 3. The dependence of the relative value of the enzymatic reaction rate for soluble (curve *1*) and immobilized (curve *2*) urease on the potassium phosphate buffer concentration, pH 7.4. In each case 0.5 mM urea was added

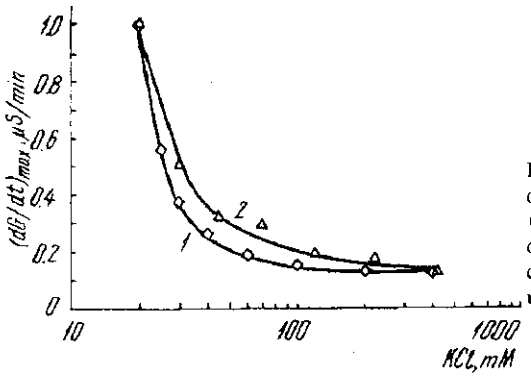

Fig. 4. The dependence of the relative value of the enzymatic reaction rate for soluble (curve *1*) and immobilized (curve *2*) urease on the KCl concentration. Measuring conditions: 5 mM phosphate buffer, pH 7.4, urea concentration — 1 mM

remains practically constant within the whole range of the buffer concentration tested.

Since biological liquids are featured by high concentrations of various salts it seemed important to ascertain whether the response of the conductometric urease biosensor depends on ionic strength. The dependence of the enzymatic reaction rate on the KCl concentration in the sample is shown in Fig. 4 for both soluble and immobilized ureases. As for the effect of KCl concentration on kinetic response of urease biosensor it was detected that an increase of the salt concentration within the 0 to 100 mM range resulted in about 80 % reduction of the signal value. Further increasing of the salt concentration up to 400 mM slightly changed the response value. From a practical point of view it is important to note that for the salt concentration above 100 mM the kinetic response of urease biosensor is practically independent on the ionic strength.

Conclusion. The enzymatically catalyzed hydrolysis of urea by the enzyme immobilized on biosensor chip is also described by the classic laws of enzymatic kinetics. The urease biosensor examined can be used for the determination of the urea concentration in biological liquids. Since urea concentration in human blood is much higher at pathologic states than in the normal (6—8 mM) [9]. For a blood sample diluted 20 times, the range of urea concentration falls within the operating region of the created biosensor. The content of low-molecular buffer components in human blood does not exceed 30 mM; therefore, after diluting a blood sample by the buffer, the capacity of the obtained solution is mainly determined by the diluting buffer. The ionic strength of the analyzed solution required for the biosensor response stabilization may be ensured by KCl introduction into the solution until the final concentration 100 mM. Under such conditions, the difference of the analyzed blood specimens in the ion composition will not affect the value of the biosensor response to urea, and thus, it will not distort the analytic results.

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Кондуктометричний уреазний мікробіосенсор для визначення сечовини на основі тонкоплівкових гребінчастих електродів

Резюме

Вивчено характеристики кондуктометричного уреазного біосенсора для визначення сечовини на основі тонкоплівкових гребінчастих електродів. Уреазу іммобілізовали ковалентною зшивкою з сироватковим альбуміном бика за допомогою глутарового альдегіду. Результуючі зміни провід-

ності викликано ферментативним гідролізом сечовини. Згаданий процес як для іммобілізованого, так і для вільного ферменту описується класичними законами ферментативної кінетики. Досліджено вплив на величину відгуку іонної сили, pH та буферної ємності розчину. Розглянуто питання стосовно визначення концентрації сечовини у крові людини.

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Кондуктометрический уреазный микробиосенсор для определения мочевины на основе тонкопленочных гребенчатых электродов

Резюме

Изучены характеристики кондуктометрического уреазного микробиосенсора для определения мочевины на основе тонкопленочных гребенчатых электродов. Уреазу иммобилизовали ковалентной сшивкой с бычьим сывороточным альбумином с помощью глутарового альдегида. Результирующее изменение проводимости вызвано ферментативным гидролизом мочевины. Этот процесс как для иммобилизованного, так и для свободного фермента описывается классическими законами ферментативной кинетики. Исследовано влияние на величину отклика ионной силы, pH и буферной емкости раствора. Рассмотрены вопросы, связанные с определением концентрации мочевины в крови человека.

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