Amperometric Urea Sensor
Enzyme Immobilization into Adjustable Membrane and Mathematical Characterization of the Biosensor

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Abstract: The prototype of amperometric biosensor for urea determination was designed. The enzyme electrode, made of a specially developed modified graphite (MG) paste, was produced by covering the electrode surface with adjustable membrane containing immobilized urease from *Canavalia ensiformis* (E.C. 3.5.1.5.). Simple methodology of urea determination in real time has been proposed. The experimental study and the mathematical model of the biosensor action have been performed.

1 INTRODUCTION

Rapid and simple determination of urea is very important in clinical analysis. Generally, abnormal urea concentration indicates kidney disease. The main procedure of urea level monitoring for patients is haemodialysis – blood filtering procedure. Filtered blood flows back to the patient and the leaked out dialysate is disposed as waste. Certainly, there are modern apparatus for blood dialysis using ratio Kt/V, where K is the dialyzer urea clearance, t is the treatment time, and V is the patient's urea distribution volume, to quantify the dialysis dose (Jensen et al., 2004). Unfortunately, the parameter Kt/V is not based on real time measurements. In fact, it is widely accepted that during 4 hours of haemodialysis Kt/V reaches value about 1.3 and this is an indication to finish dialysis procedure. Actually, urea level in blood is highly affected by stress, physical activity or nutrition and Kt/V would be relevant only if urea concentration was known just before dialysis started. Thus, it is very promising to determine the concentration in real time. Definitely, the non-invasive and cost-saving methods are preferable. Thus, in this study we propose amperometric detection system allowing detecting urea in waste product - dialysate at any time of the haemodialysis procedure.

Though the great number of urea determination methods are based on photometric or conductometric determination of NH^{4+} (Patton and Crouch, 1977; Soldatkin et al., 2014) or using a piezoelectric sensor (Miglior et al., 2007), their application for express analysis, and especially in turbid media, is rather complicated. In this case the amperometric biosensors are most promising. The electrochemical approach for rapid detection of urea have been proposed in (Sant et al., 2011) and also in our previous work (Razumiene et al., 2013).

The goal of this work was on a base of previous studies to design the urea analyser prototype. The core of this device is biosensor using especially developed modified graphite (MG) electrode and adjustible membrane containing immobilized urease. The mathematical model was proposed for characterisation of biosensor action.

2 EXPERIMENTAL

2.1 Preparation of Membrane

Poly(urethane-urea) (PUU) microparticles from poly(vinyl alcohol) (PVA) and hexamethylene diisocyanate (HMDI) were synthesized by one-step method in dimethyl sulfoxide/water (99/1 vol.%) solution according to previously described protocol (Budriene et al., 2007). Initial concentration of PVA was 0.1 M. Initial molar ratio of PVA and HMDI was 1.0:5.0.

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Immobilization of urease onto PUU microparticles was carried out in 0.1 M phosphate buffer solution, pH 7.2. The mixture of the enzyme, buffer and PUU carrier (in folowing ratio: 1540 U of urease for 0.5 g of polymeric carrier) was stirred at 25 °C for 30 min (immediately after synthesis) and then left at 4 °C overnight. Next day the immobilized enzyme was thoroughly washed with buffer and 2 µl of suspension were droped onto terylene film agglutinated to the rubber ring.

2.2 Preparation of MG and Biosensor

Modified graphite particles were synthesized from pristine graphite (Merck KGaA) by oxidizing it with potassium ferricyanide $K_3[Fe(CN)_6]$ in alkaline media. Titration analysis revealed the presence of small amount (0.14 – 0.17 mmol/g) of basic surface functional groups.

It was determined that the MG sample suitable for biosensor design contains a fine fraction of 63 % with an average diameter of the graphite particles of 20 nm.

MG powder was mixed with the pasting liquid consisting of 10 % polyvinyl dichloride in acetone and used for design of the electrodes.

Aiming to design working electrodes MG mixed with pasting liquid was extruded by forming tablet (Voitechovic et al., 2010). The tablet was sealed in a Teflon tube. Electrodes were washed with bidistilled water, and dried before use. Working urease-MG electrode (biosensor) was designed by mechanically attaching the membrane containing polymeric carriers with immobilized urease to the surface of MG. (Fig. 1).

2.3 Amperometric Measurements

Urea measurements were performed using a home-made analyser equipped with a three-electrode system comprised of a platinum plate electrode as auxiliary electrode, a saturated Ag/AgCl electrode as reference and urease-MG (2 mm diameter) as working electrode (biosensor) (Fig. 2).

The response of the biosensor to the addition of substrate was investigated under potentiostatic conditions at 0.2 V (vs. Ag/AgCl) in a stirred buffer solution. Phosphate buffer solution, pH 7.2, containing 1 M of urea or dialysate was used as a substrate. The specially created program and `Origin Pro 8.0` (free trial version from http://www.originlab.com, OriginLab Corporation, US) were used for data analysis.

2.3.1 Measurements in Dialysate

Dialysate was analysed using the developed analyser comprised with the biosensor. Prior the measurements analyser was tested with standard 1 M of urea solution. For each measurement 2, 3, 5, 7 and 10 µl of the solution were added into electrochemical cell containing of 1 ml of buffer solution. Taking into account that the concentration of urea in dialysate during the haemodialysis could be outside the working range of the biosensor, a dilution of the samples were necessary prior to analysis to adjust the sample concentration to the linear range of the biosensor. For this purpose, the samples of dialysate were 10 times diluted with buffer solution and analogous experiments were carried out by adding probes in the electrochemical cell.

2.4 Mathematical Model

Mathematical model was built aiming to have a tool for analysing behaviour of the biosensor, impact of its parameters and to lower number of required experiments (Amatore et al., 2006). The biosensor is considered as a reaction-diffusion system when defining its mathematical model (Baronas et al., 2010). Due to the biosensor symmetry, the model is formulated in the one-dimensional space – a line segment perpendicular to the active surface of the biosensor. The model is composed of three layers representing correspondingly the enzyme layer, terylene membrane and a Nernst diffusion layer that forms on the external surface of the terylene membrane. No diffusion of the urea and its products is assumed in the layer of the MG paste.

The urea detecting process is modelled as a two-step reaction. In the first step the urea (S) is detected by the urease (E) in an enzymatic hydrolysis reaction with production of intermediate compound carbamic acid (P) and ammonia (P'),

$$S \xrightarrow{E} P + P'. \quad (1)$$

The carbamic acid is finally electrooxidised (not in one-step mechanism) with production of ammonia and carbon dioxide (P'')

$$P \xrightarrow{e^-} P' + P''. \quad (2)$$

Electrons released in this reaction are collected by the MG electrode and form a biosensor response current.

Reaction (1) takes place in the thin layer, between the terylene membrane and the MG electrode. Kinetics of the urea and the carbamic acid

are described by the following reaction-diffusion equations:

$$\frac{\partial S}{\partial t} = D_1 \frac{\partial^2 S}{\partial x^2} - \frac{V_{\max} S}{K_M + S}, \quad (3)$$

$$\frac{\partial P}{\partial t} = D_1 \frac{\partial^2 P}{\partial x^2} + \frac{V_{\max} S}{K_M + S}, \quad (4)$$

where S and P are concentrations of the urea and the carbamic acid, D_1 is a diffusion coefficient, K_M stands for the Michaelis constant, V_{max} is the maximal reaction rate, t is a time from the start of the experiment and x stand for a distance from the electrode surface. Only mass transfer by diffusion is considered in the terylene membrane and the external Nernst layer,

$$\frac{\partial S}{\partial t} = D_i \frac{\partial^2 S}{\partial x^2}, \quad \frac{\partial P}{\partial t} = D_i \frac{\partial^2 P}{\partial x^2}, \quad i = 2,3, \quad (5)$$

where D_i, i=2, 3 are diffusion coefficients of the species in the corresponding medium.

The experiments start at a moment (t=0) when the urea is poured into the buffer solution, although it is still absent in the biosensor. We also assume zero concentration of the carbamic acid in the biosensor at this time.

Boundary conditions are defined for the external bound of the Nernst diffusion layer and the surface of the graphite electrode. On the upper boundary of the Nernst diffusion layer, constant concentration (S_0) is assumed for urea and the carbamic acid is absent,

$$S\big|_{x_3} = S_0, \quad P\big|_{x_3} = 0. \quad (6)$$

On the surface of the MG electrode, non-leakage condition applies to the urea. Electrode oxidation of the carbamic acid takes place on the surface of the electrode, thus, a gradient of the carbamic acid is considered to be equal to the rate of the oxidation process,

$$\left.\frac{\partial S}{\partial x}\right|_{x=0} = 0, \quad D_1 \left.\frac{\partial P}{\partial x}\right|_{x=0} = R_2, \quad (7)$$

where R2 stands for the rate of the oxidation process:

$$R_2 = \gamma k P_1\big|_{x=0}, \quad (8)$$

where k is a heterogeneous oxidation rate constant and γ is a rate of the active surface of the electrode to its area.

Response of the biosensor is derived from the current, produced the oxidation of the carbamic acid on the surface of the MG electrode. Response current at a time t is defined as:

$$i(t) = A n_e F R_2, \quad t > 0, \quad (9)$$

where A is an area of the active surface of the biosensor, n_e is a number of electrons exchanged in one reaction event and F stands for the Faraday constant.

The proposed model consists of a system of non-linear partial differential equations. Analytical solutions for such systems are known only for separate cases, therefore a numerical model was derived and results obtained by performing computer simulations.

MG paste is not represented in this model explicitly. It was assumed, that the MG paste increases active surface of the electrode and its impact can be modelled by increasing γ – ratio of the active electrode surface to its area.

3 RESULTS

3.1 Principle of Urea Biosensor

The urease-MG electrode (biosensor) is illustrated in Figure 1. The biosensor consists of a Teflon tube (6) with sealed tablet of MG (4), contact zone for MG (5), contact wire (7) and adjustable membrane comprising of immobilized enzyme (3), semipermeable film (2) and rubber ring (1). The amperometric urea detection principle is based on registration of oxidation current observed during the enzymatic reaction of the intermediate product in urease-catalyzed hydrolysis of urea (Laurinavicius et al., 2013).

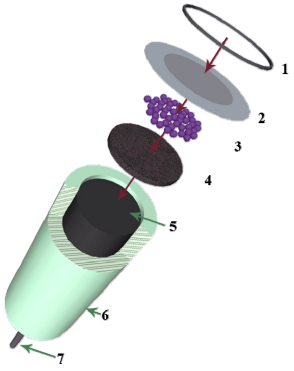

Figure 1: Principal scheme of urea biosensor.

The biosensor incorporated in to the three-electrode electrochemical cell is the core of proposed urea analyser.

3.2 Characterization of Urea Biosensor

Biosensor based on the urease-MG electrode after addition of urea in to electrochemical cell shows substrate-dependent anodic response. The biosensor response is fast (90 % of steady state current achieved in 10 s) and this feature is desirable for analytical instruments. The urea calibration curve and the linear range are presented in Figure 2.

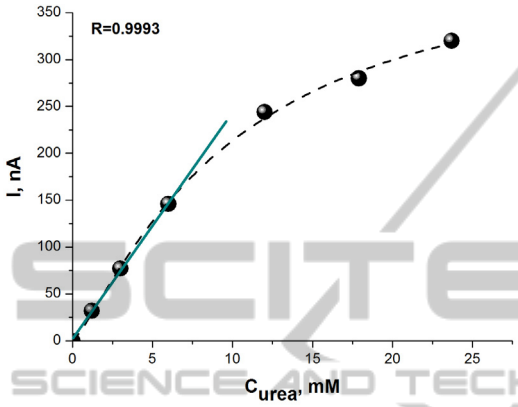

Figure 2: The urea calibration curve and the linear range (solid line). Applied urese-MG electrode potential 0.2 V, Phosphate buffer solution, pH 7.2.

While the linear range of urea biosensor is up to 6 mM the concentration of urea in dialysate during the haemodialysis will be outside the working range of the biosensor. Thus, for adjusting the sample concentration to the linear range of the biosensor a dilution of the samples were necessary prior to analysis and the sensitivity of the sensor allows it.

3.3 Urea Biosensor Stability

Stability of the biosensor designed using MG and membrane containing immobilized urease was investigated during 85 days (Fig. 3). The responses to the standard urea solution (3 mM) were periodically recorded at 20 °C and it was detected that the residual response of the biosensor was not less than about 50 % of initial magnitude over the period of 20 days. After 85 days the biosensors activity decreased up to 20 % of residual.

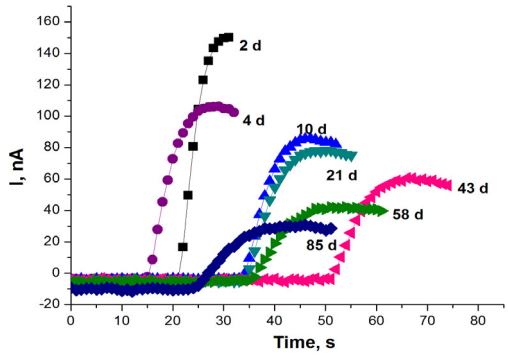

Figure 3: Intensity of responses to 3 mM of urea obtained by proposed analyser at 2 – 85 day.

As can be seen in Figure 3, not only intensity of responses but also the shape was changed. A physical explanation and a digital modelling of this ageing process are following.

3.4 Urea Analyser

The proposed urea analyser is shown in Figure 4.

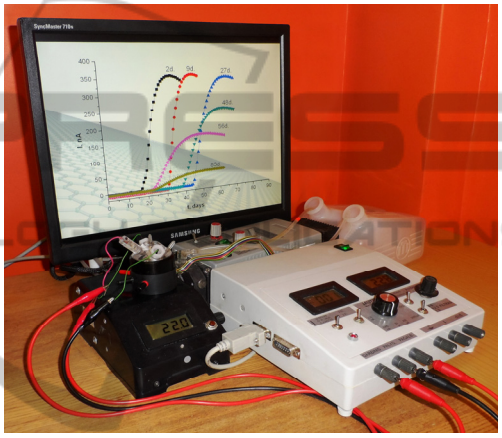

Figure 4: The prototype of urea analyser based on amperometric biosensor.

The urea measuring system consists of electrochemical three-electrode cell, a home-made potentiostat, peristaltic pump, stirrer and thermostat and response recorder.

3.5 Urea Determination in Dialysate

Amperometric type of sensors beside other well-known advantages such as comparable instrumental sensitivity and amenability to miniaturization also have one of very important feature – acceptability for functioning in turbid media. Thus, in this report, we present simple approach of the biosensor for determination of urea in dialysate. The measurements have been carried out by investigating dialysate leaked out from patients during haemodialysis. The samples were taken each hour of blood filtration procedure and in parallel they were examined at the hospital laboratory. Urea concentration data for two patients obtained by both methods are presented in Figure 5.

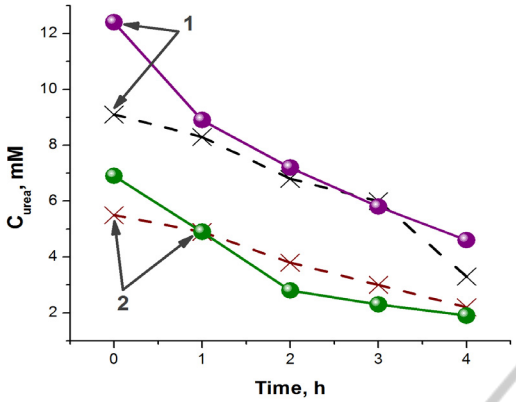

Figure 5: Comparison of urea concentration of two patients (1 and 2) obtained in dialysate using proposed analyser (solid line) and in the hospital laboratory (dashed line).

As can be seen in Figure 5, it was observed good correlation between data obtained by using analyser and in the hospital laboratory.

3.6 Computation

Numerical simulations were performed in order to validate the model, to investigate the biosensor ageing process as well as impact of the MG paste. The following parameter values were used in all the simulations:

$$x_1 = 5,\ x_2 - x_1 = 12,\ x_3 - x_2 = 150\mu m, \quad (10)$$

$$D_1 = 1.5\times10^{-10},\ D_2 = 3.7\times10^{-10}\, m^2 s^{-1}, \quad (11)$$

$$D_3 = 6.7\times10^{-10}\, m^2 s^{-1}, S_0 = 3mM, \quad (12)$$

$$V_{\max} = 1.5 mM s^{-1},\ K_M = 25mM, \quad (13)$$

$$A = 3mm^2. \quad (14)$$

The heterogeneous oxidation rate constant k was not known during this investigation therefore the product $\gamma \times k$ was used as a single parameter when performing numerical simulations.

The proposed model does not consider the decrease of a biosensor response. It will be analysed in the further investigation. In order to compare simulation results with the experimental ones, maximal response current of the experiment was used.

Initial simulations were performed in order to characterise the biosensor ageing process and impact of the MG paste on its response. For the first attempt, an assumption was made, that the inactivation of the urease decreases linearly over the time,

$$V_{\max} = k_{cat} e_0 (T_{\max} - T)/T_{\max}, \quad (15)$$

where T is an age of the biosensor, $T_{\max}$ is assumed to be time, during which the enzyme is inactivated completely, $k_{cat} = 10^4 s^{-1}$ is the catalytical constant of the urease, e_0 is the effective concentration of the urease in the layer between the electrode and the terylene membrane. Its value was theoretically estimated to be $e_0 < 0.02$ mM and derived by fitting simulation results to be 0.01 mM. Dependence of the biosensor response on the ageing is shown in Figure 6.

As can be seen in Figure 6, simulated results are close to the experimentally obtained values only in some cases. The results show, that increase of the active area of the electrode surface can have similar impact as the addition of the MG paste to the response of the biosensor, although further investigation is needed to check, if the impact remains the same at different urea concentrations and other parameter variations.

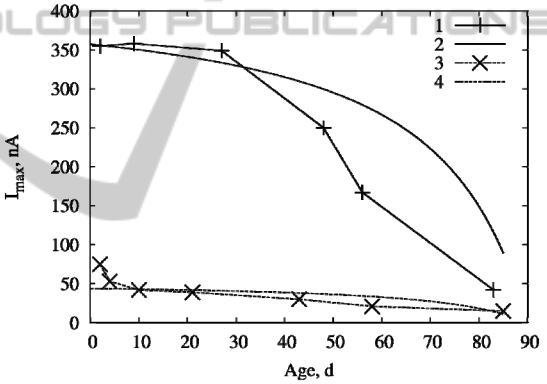

Figure 6: Dependence of the biosensor response on the age on the biosensor. Curves (1, 3) represent experimental measurements and (2, 4) stand for simulations for the biosensor with addition of MG paste (1, 2) and without it (3, 4).

The simulations show, that the inactivation of the biosensor is not linear as the simulated results do not fit with the simulations when changing biosensor's age. Exponential inactivation rate was also considered, although no close fit with experimental data was found.

4 CONCLUSIONS

The biosensor comprised of especially devoted electrode material MG and adjustable membrane containing immobilized urease can be applied for urea analyser.

Good data correlation with certified method confirmed that the proposed analyser can be used for rapid and simple detection of urea in dialysate. Besides proposed application, the focus of our future research will be adjustment of the analyser for veterinary or environment.

Preliminary simulations show, that the sensitivity of the urea biosensor can be increased by applying the MG paste to the urease-MG electrode.

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