Carbon Electrode based Urea Sensor
Modification of Graphite and New Polymeric Carriers for Enzyme Immobilization

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Keywords: Urea Biosensor, Modified Graphite, Polymeric Carrier, Enzyme, Amperometry.

Abstract: The amperometric biosensor for urea determination was designed based on the electrochemical oxidation of urea decomposition products produced by urease. The enzyme electrode, made of a specially developed modified graphite (MG) paste, was produced by covering the electrode surface with new polymeric carriers poly(urethane-urea) (PUU) microparticles containing immobilized urease from *Canavalia ensiformis* (E.C. 3.5.1.5.).

1 INTRODUCTION

Urea is a final product of metabolism of aliphatic nitrogen in organisms. Generally, abnormal urea concentration indicates kidney disease. Rapid determination of urea is important not only in clinical analysis but urea detection is great problem in fertilizes industry and in agriculture as well. This small molecule yet is a very important part for milk component. A high concentration of milk urea shows dietary disbalance, potential milk losses and risk of infertility (Miglior et al., 2007). Milk urea nitrogen levels are known to vary with the amount of protein in the diet, amount of urine excreted, amount of water intake, dry matter intake, sampling methods, breed, parity, and days in milk, season and herd management (Renny et al., 2005). These levels increase with adulteration and are detrimental to human health. Therefore, it is essential to test urea level in milk in all stages - from producing to consuming.

The most enzymatic methods of urea determination are based on enzymatic hydrolysis of urea in presence of urease with following determination of hydrolysis products. A number of photometric methods for the determination of NH^{4+} (Patton and Crouch, 1977) and using a piezo-electric sensor (Miglior et al., 2007) were created. However obviously, their application for express analysis, and especially in turbid media, is rather complicated. In a field of amperometric biosensors the most critical issues impeding their large-scale application is still the inefficient and power-demanding signal mediation from the biological sensing element to the transducer as well as poor stability of the employed biocatalyst (Muti et al., 2011). Thus, development of new electrode materials promising for effective electron transfer and immobilization of enzymes still is in a growing interest. Carbon nanomaterials and various polymeric carriers (PC) have been extensively studied and proved to be ideal materials for these purposes (Agui et al., 2008; Wang et al., 2004; Fan et al., 2006; Qureshi et al., 2009).

The goal of this work was to design urea biosensor based on specially developed MG electrode and newly proposed method of urease immobilization onto polymeric carriers and implement such electrochemical system for detection of urea in milk or other biological liquids.

2 EXPERIMENTAL

2.1 Preparation of Polymeric Carriers and Immobilization of Urease

Poly(urethane-urea) (PUU) microparticles from

Razumiene J., Sakinyte I., Kochane T., Maciulyte S., Straksys A., Budriene S. and Barkauskas J..
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DOI: 10.5220/0004326901970201
In *Proceedings of the International Conference on Biomedical Electronics and Devices* (BIODEVICES-2013), pages 197-201
ISBN: 978-989-8565-34-1

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. SEM image of lyophilized PC microparticles is shown in Fig. 1.

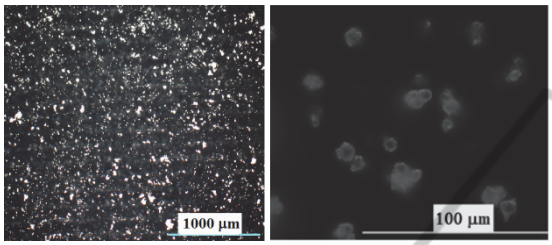

Figure 1: Optical microscopy images of PC.

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 different ratios) was stirred at 25 °C for 30 min (immediately after synthesis) and then left at 4 °C overnight. It was prepared and investigated folowing ratios: using 1540 U of urease for 0.5 g of PC (1 PC); using of 770 U for 0.5 g of PC (2 PC); using of 389 U for 0.5 g of PC (3 PC) and using of 112 U for 0.5 g of PC (4 PC). Next day the immobilized enzyme was thoroughly washed with buffer.

2.2 Preparation of MG and Amperometric 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. The obtained batches of MG were examined by titration and AFM analysis methods (Fig. 2). Titration analysis revealed the presence of small amount (0.14 – 0.17 mmol/g) of basic surface functional groups. AFM images show that sonication procedure causes the formation of finely dispersed MG particles (Fig. 2 A and B).

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 polymeric carriers containing immobilized enzyme urease to the surface of MG. Further the constructed biosensor was protected by using semipermeable terylene film.

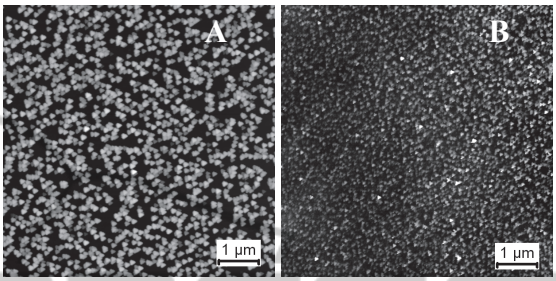

Figure 2: AFM images of MG. (A) batches prepared without sonication, and (B) bathes prepared including a sonication procedure.

2.3 Electrochemical Measurements

Electrochemical measurements were performed using an electrochemical system "PARSTAT 2273" (Princeton Applied Reasearch, USA) with a conventional 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.

The response of the prepared enzyme electrode to the addition of substrate was investigated under potentiostatic conditions at 0.4 V (vs. Ag/AgCl) in a stirred buffer solution. As a substrate was used phosphate buffer solution, pH 7.2, containing 1 M of urea. The program Origin Pro 8.0 (free trial version from http://www.originlab.com, OriginLab Corporation, US) was used for data analysis.

2.3.1 Measurements in Milk

Commercial milk was analysed using the developed biosensor. Taking into account that the concentration of urea in dairy products is 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, 1 M of urea solution was prepared in milk. For each measurement 2, 3, 5, 7 and 10 µl of the dairy product were added into electrochemical cell containing of 1 ml of buffer solution. Thus, the final dilution factor was from 50 to 500. Analogous experiments were carried out by adding in the

electrochemical cell of 1 M urea prepared in buffer solution.

3 RESULTS

3.1 Principle of Urea Detection

The urea biosensor and the amperometric detection principle based on the urease-catalyzed hydrolysis of urea are shown in Fig. 3. Carbamic acid and ammonia are the initial enzymatic reaction products of urea, and the carbamic acid is further hydrolyzed to ammonia and carbon dioxide. The final products namely, ammonia and carbon dioxide are electroinactive, thus, oxidation current observed during the enzymatic reaction must be attributed to the intermediate product. It can be assumed that the carbamic acid undergoes electrooxidation by forming nitrogen and carbon dioxide.

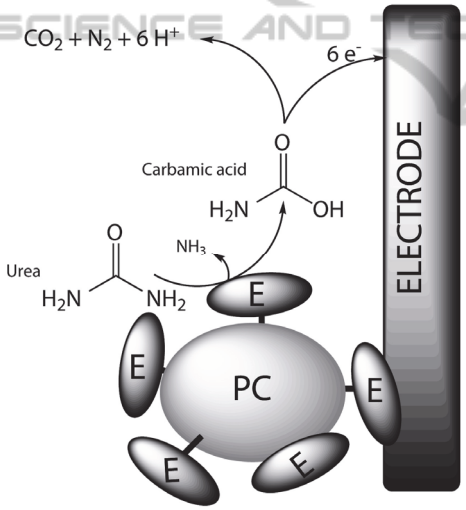

Figure 3: The biosensor and the amperometric detection principle based on the urease-catalyzed hydrolysis of urea.

Aiming to obtain easy reproducible, sensitive and stable biosensing system the enzyme was immobilized onto polymeric carriers. Enzymes, which have amino and hydroxyl groups, may be covalently immobilized by attachment to PUU microparticles, which have unreacted NCO groups and urea or urethane linkages are formed (Fig. 4). NCO groups of PUU at low temperature react faster with amino than with primary alcohol groups or water (Randall and Lee, 2002). Whereas immobilization procedure followed in aqueous media remained free NCO groups react with water by formation of CO_2 and they do not have any inactivation effect on enzyme latter (Budriene et al., 2007).

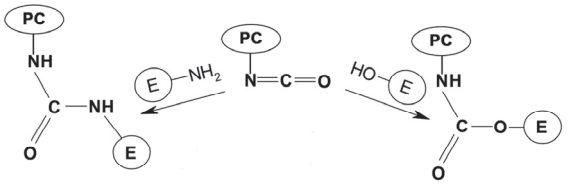

Figure 4: Immobilization scheme of the enzyme onto PC.

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 shows fast response (90 % of steady state current achieved in 1 min) and this feature is desirable for analytical instruments. The urea calibration curves obtained using biosensors based on different amount of enzyme immobilized onto polymeric carriers at applied electrode potential 0.4 V are shown in Fig. 5.

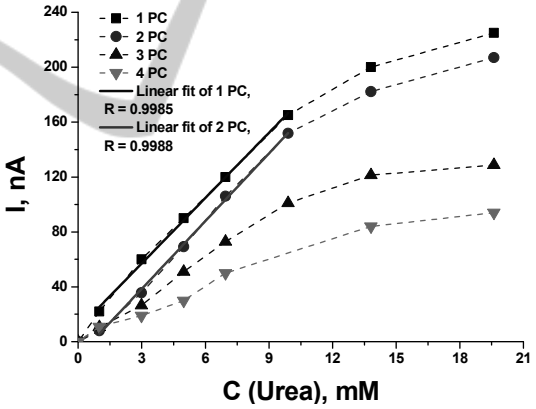

Figure 5: The urea calibration curves and linear range obtained using different amount of enzyme immobilized onto polymeric carriers. Applied electrode potential 0.4 V, phosphate buffer solution, pH 7.2.

The efficiency of electron transfer expressed by sensitivity of the biosensor depends on amount of immobilized enzyme. The best results were obtained by using 1540 U of urease for 0.5 g of PC (1 PC in Fig. 5). This enzyme and PC ratio was taken as optimal and used for measurements in milk. Linear response of this type of urea biosensor lies in the range of 1 – 10 mM (Fig. 5). The obtained results show that the method of immobilization of enzyme plays crucial role in biosensor design. Doubtless, the proper immobilization avoids the enzyme from fast inactivation and affords an effective electron transfer

from the active centre of the enzyme via intermediate products toward the electrode surface.

3.3 Urea Biosensor Stability

Stability of the biosensor designed using MG and immobilized onto polymeric carriers urease was investigated during one week (Fig. 6). The responses to the standard urea solution (5 mM) were periodically recorded at 20 °C. The residual response of the best biosensor operated at potential of 0.4 V was not less than about 50 % of initial magnitude over the period of one week. Highest rate of inactivation of the 4 PC biosensor activity indicates that in this case the stability of the biosensor was determined not by the inactivation process of urease but just desorption of not cross-linked to the PC enzyme (4 PC in Fig. 6).

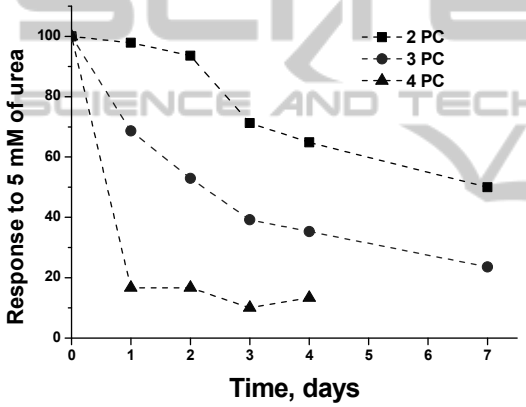

Figure 6: Stability of the biosensors designed using MG and different amounts of urease immobilized onto polymeric carriers. Applied electrode potential 0.4 V, phosphate buffer solution, pH 7.2.

3.4 Urea Determination in Milk

Amperometric type of sensors beside other well known advantages such as comparable instrumental sensitivity and amenability to miniaturization also has 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 diluted milk. The measurements have been carried out in conventional electrochemical cell by adding urea spiked both buffer solution and milk. The anodic current was registered and the urea concentration was calculated using urea calibration curve. The data are presented in Table 1.

It was observed good correlation between data obtained in buffer solution and in media containing different concentrations of urea as well as amount of milk (Table 1). The results encouraged us to carry on further experiments concerning analysis of other biological liquids.

Table 1: Comparison of urea concentration obtained in buffer solution and in diluted milk using proposed biosensor.

Added urea concentration, mM	Detected urea concentration in milk, mM	Detected urea concentration in buffer solution, mM
1.99	2.10	2.08
2.99	3.04	3.00
4.98	4.94	4.89
6.95	6.77	6.70
9.90	9.52	9.50

4 CONCLUSIONS

For this research especially devoted MG and polymeric carriers for enzymes have been fabricated and tested as the electrode materials for the amperometric urea biosensor.

It was revealed that the proposed biosensor can be used for rapid and simple detection of urea in diluted milk.

The biosensors exhibited fast response and sensitivity dependent on amount of immobilized enzyme. Although, synthesis of PC and enzyme immobilization methodology was not optimized properly yet, new polymeric carriers seems are very promising for biosensors design. Thus, our future investigations will be focused on improving of synthesis of PC by using different initial molar ratios of PVA and HMDI and immobilization of other important biocatalysts.

ACKNOWLEDGEMENTS

This work was funded by the European Social Fund under National Integrated Programme Biotechnology and Biopharmacy, grant VP1-3.1-SMM- 08-K01-005.

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