

Immobilization of urease on nanostructured polymer membrane and preparation of urea amperometric biosensor

Katya Gabrovska, Javor Ivanov, Ioana Vasileva, Nedyalka Dimova, Tzonka Godjevargova*

University "Prof. Dr. A. Zlatarov", Department of Biotechnology, Prof. Y. Yakimov Str. 1, 8010 Bourgas, Bulgaria

ARTICLE INFO

Article history:

Received 25 October 2010

Received in revised form 1 February 2011

Accepted 2 February 2011

Available online 16 February 2011

Keywords:

Modification

Membrane

Chitosan

Rhodium

Immobilization

Urease

Urea biosensor

ABSTRACT

A new matrix for enzyme immobilization of urease was obtained by incorporating rhodium nanoparticles (5% on activated charcoal) and chemical bonding of chitosan with different concentration (0.15%; 0.3%; 0.5%; 1.0%; 1.5%) in previously chemically modified AN copolymer membrane. The basic characteristics of the chitosan modified membranes were investigated. The SEM analyses were shown essential morphology change in the different modified membranes. Both the amount of bound protein and relative activity of immobilized enzyme were measured. A higher activity (about 77.44%) was measured for urease bound to AN copolymer membrane coated with 1.0% chitosan and containing rhodium nanoparticles. The basic characteristics (pH_{opt} , T_{opt} , thermal, storage and operation stability) of immobilized enzyme on this optimized modified membrane were also determined.

The prepared enzyme membrane was used for the construction of amperometric biosensor for urea detection. Its basic amperometric characteristics were investigated. A calibration plot was obtained for urea concentration ranging from 1.6 to 23 mM. A linear interval was detected along the calibration curve from 1.6 to 8.2 mM. The sensitivity of the constructed biosensor was calculated to be $3.1927 \mu A mM^{-1} cm^{-2}$. The correlation coefficient for this concentration range was 0.998. The detection limit with regard to urea was calculated to be 0.5 mM at a signal-to-noise ratio of 3. The biosensor was employed for 10 days while the maximum response to urea retained 86.8%.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Immobilization technology is becoming an important field in the biomedical science and biotechnology. A large number of bioactive materials such as drugs, proteins, plant and animal cells, and microorganisms of various classes were successfully immobilized with very high yields on appropriate supports [1–3]. As support matrices, polymeric membranes have attracted much attention because they can be produced easily in a wide variety of compositions and can be modified for immobilization of biomolecules by introducing a wide variety of ligand molecules. Amongst them, acrylonitrile (AN) copolymer membranes are very versatile and convenient for enzyme immobilization due to its hydrophilic nature, high chemical and mechanical stability and resistance toward microbial and enzymatic attacks [4,5].

Urease is most extensively studied for immobilizations and practical applications. This is because of the significance of the processes in which urease takes part and of their possible exploitation in practical applications. The immobilized urease was intended for

use in the construction of artificial organ systems, biosensors or bioreactors [6–8]. The foremost analytical application of urease is for quantification of urea in aqueous solutions [9]. Even though the major interest has been on its medical application, there is a growing demand for sound, reliable, and fast urea analytical procedures in other areas, such as environmental, food and industrial. The food industry too has the requirement of real time and accurate analysis of dairy products during manufacture and quality control. In this regard, urea biosensor could prove to be a valuable tool for monitoring the urea content of milk [10].

The concentration of urea is measured by monitoring the liberated NH_4^+ and HCO_3^- ions using a transducer such as amperometric, potentiometric, optical, thermal, or piezoelectric [11–17]. Although various urea biosensors that use a range of transducers have been studied extensively, the urease-based amperometric urea biosensor is considered one of the most promising approaches because it offers fast, simple, and low-cost detection. The NH_4^+ ions are not electroactive and it was oxidized to nitrogen molecule by two ways – using a second enzyme or catalytic metal [18,19]. In our case the product of enzyme reaction (ammonia) was catalytically and electrochemically oxidized by rhodium present in the modified polymer membrane assembled with Pt working electrode. The construction of our urea amperometric biosensor with replaceable urease rhodinized membrane replaces the necessity of changing

* Corresponding author. Tel.: +359 56 858 353; fax: +359 56 820 249.

E-mail address: godjevargova@yahoo.com (T. Godjevargova).

the electrode thereby decreasing the cost of the urea electrode and avoiding the use of a second enzyme.

The aim of the present work is to study the immobilization of urease on modified AN copolymer membrane with chitosan and rhodium nanoparticles. The immobilized urease was used for preparation of urea amperometric biosensors.

2. Materials and methods

2.1. Materials

Acrylonitrile – methylmethacrylate – sodium vinylsulfonate (AN) copolymer membranes were prepared without support according to a methodology described in [20]. The ternary copolymer (acrylonitrile – 91.3%; methylmethacrylate – 7.3%, and sodium vinylsulfonate – 1.4%) was a product of Lukoil Neftochim, Bourgas. Ultrafiltration membranes of acrylonitrile copolymer were measured to be 4 µm thick and could retain substances with molecular weight higher than 60,000 Da.

The chemical modification of these membranes was conducted by NaOH and ethylenediamine (EDA), chitosan (Fluka, Switzerland) and incorporated rhodium nanoparticles (5% on activated charcoal, with size 100 nm) in the membrane pores (Sigma–Aldrich, USA).

Immobilization of urease (E.C. 3.5.1.5, 270 U mg⁻¹, Merck, Germany) onto the membranes was carried out with glutaraldehyde (Fluka, Switzerland).

All reagents were of analytical grade.

2.2. Instruments

Electrochemical measurements were performed on a Palm-Sense Electrochemical Instrument (Palm Instruments BV, The Netherlands) with a conventional three-electrode system comprising of platinum wire as auxiliary electrode, Ag/AgCl electrode as reference and urease-immobilized membrane attached to a platinum electrode ($d = 10$ mm) as working electrode.

2.3. Chemical modification of AN copolymer membranes

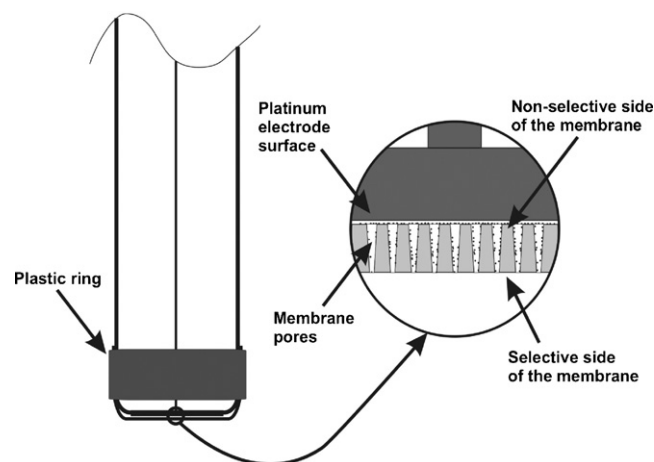
A membrane with area 10 cm² was dipped into 10 ml 10% solution of NaOH for 20 min at 40 °C. After that, it was washed with distilled water and remained in a 0.1 M solution of HCl for 120 min. Then the membrane was immersed in a 10% solution of EDA at room temperature for 60 min. The membrane was washed thoroughly with distilled water and impregnated with a 2.5% solution of glutaraldehyde at room temperature for 1 h. After that, the membrane was washed thoroughly with distilled water. The obtained chemically modified membrane was impregnated with a 1% chitosan solution with molecular weight 10,000 Da in acetic acid under continuously stirring conditions for 24 h and then washed with distilled water.

2.4. Immobilization of urease

The urease solution 1 ml 0.1% (0.06 M PBS, pH 5.8), containing 70 mg ml⁻¹ activated charcoal with 5% rhodium, was passed through the chemical modified membrane under pressure 3×10^5 Pa. After that the membrane was placed in 1 ml 0.1% urease solution (0.06 M PBS, pH 5.8) at 4 °C for 20 h. Finally the membrane was placed in 1 ml 2.5% glutaraldehyde solution for 1 h and then washed with distilled water and 0.1 M PBS, pH 7.0.

2.5. Electrochemical measurements of the urease biosensor

Each enzyme membrane was attached to a platinum working electrode, using a plastic ring, with the non-selective side



Scheme 1. Enzyme biosensor – an assembly of a platinum working electrode and an enzyme membrane.

of the membranes facing the platinum surface of the electrode (Scheme 1). Then this electrode was placed in an electrochemical cell containing 30 ml Tris buffer solution with pH 8.1 under stirring at 30 °C. A potential of 0.8 V was applied to the working electrode and the electrochemical current was awaited to become stationary. Then a series of 100 µL from a 500 mM solution of urea were added to the cell and the resulting current was recorded.

2.6. Determination of the apparent K_m

Value of K_m for urease was calculated from the Lineweaver–Burk plots using electrochemical data extracted from the calibration curve of the constructed urease biosensor with urea concentrations ranging from 1.6 mM to 23 mM and 30 °C.

2.7. Analytical methods

The amounts of functional (NH₂–) groups in the modified membranes were measured by residual titration in heterogeneous medium [21].

Hydrophilicity (χ) of the membrane was determined by the weight difference between the wet and the dry membrane. The hydrophilicity was calculated by following equation:

$$\chi\% = \frac{G_m - G_c}{G_m} 100$$

where G_m is the mass of membrane before drying, g; G_c is the mass of membrane after drying, g.

The amount of bound protein to modified membranes was determined by the modified method of Lowry. The method is based on spectrophotometric measurement of the blue colour resulting from the cupric ions binding to peptide bonds in alkali medium and from the reaction of the amino acid residues with Folin reactant [22].

The activities of the free and immobilized urease were determined using Nessler's method. The amount of NH₃, product of the enzyme reaction was determined spectrophotometrically at 480 nm [23].

2.8. SEM

The morphologies of the modified membranes have been investigated by scanning electron microscopy (SEM), using a JEOL JMS-6700F microscope (Japan).

Table 1

Amount of active groups and hydrophilicity of the initial and modified AN copolymer membranes with chitosan.

No. of membrane	Chitosan concentration, %	Amount of amino groups, mg eq g ⁻¹	Hydrophilicity, %
1	0.15	1.17	73
2	0.3	1.40	75
3	0.5	1.62	81
4	1.0	1.87	96
5	1.5	2.10	98
6	Without chitosan	–	65

2.9. Treatment of experimental data

Each experimental point in the figures for studying of properties of immobilized urease is the average of 4 independent experiments carried out under the same conditions. The experimental error never exceeded 4.4%.

3. Results and discussion

3.1. Modification of AN copolymer membrane

A new matrix for enzyme immobilization of urease was obtained by chemical bonding of chitosan and incorporating rhodium particles in previously chemically modified AN copolymer membrane in order to develop a high sensitive amperometric biosensor for determination of low concentration of urea.

In the initial AN copolymer membrane active groups for covalent binding of enzyme were absent. Thus its additional treatment was necessary. The chemical modification of AN copolymer membranes in order to incorporate more amino groups were obtained by three consecutive treatments with NaOH, EDA and binding chitosan via glutaraldehyde (Scheme 2).

The aim of the additional chemical binding of chitosan (CHI-NH₂) to AN copolymer membrane by glutaraldehyde was to receive more amino groups on the membrane surface and in its pores, to improve the biocompatibility and to create a better environment for the enzyme molecules.

As a result of these many-staged chemical treatments, the modified membrane became more hydrophilic than the initial one and contained a great amount of amino groups. Solutions of chitosan with different concentration (0.15%; 0.3%; 0.5%; 1.0%; 1.5%) were used for third modification. The aim was to be chosen which chitosan modified membrane was more suitable as a carrier for urease immobilization. The amount of amino groups and the degree of hydrophilicity of the modified with chitosan membranes was presented in Table 1 and they were compared with those of the initial membrane. As it can be expected, with the increase of the concentration of chitosan, the amount of the amino groups and the hydrophilicity of the initial membrane were increasing (Table 1). It is known that, not always the greatest amount of active groups provided the greatest activity of the immobilized enzyme. To study the most suitable matrix for enzyme immobilization the activity of immobilized urease on different chitosan membrane was determined.

3.2. Immobilization of urease

The obtained chitosan modified membranes were firstly treated with a solution of urease and rhodium nanoparticles through an ultrafiltration. The incorporation of the rhodium nanoparticles into the pore structure of the membrane through ultrafiltration had two important targets:

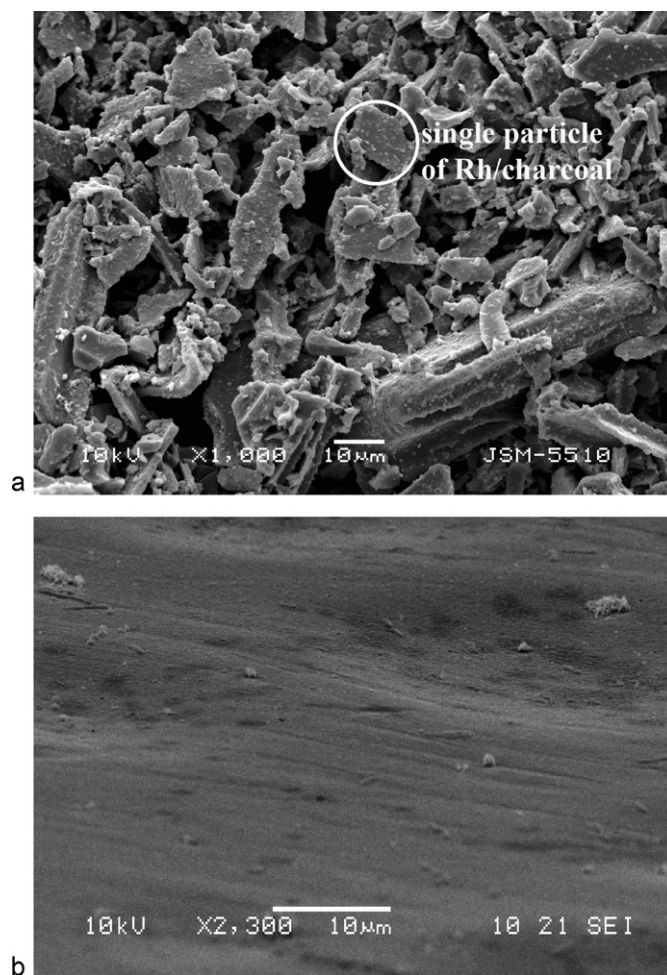
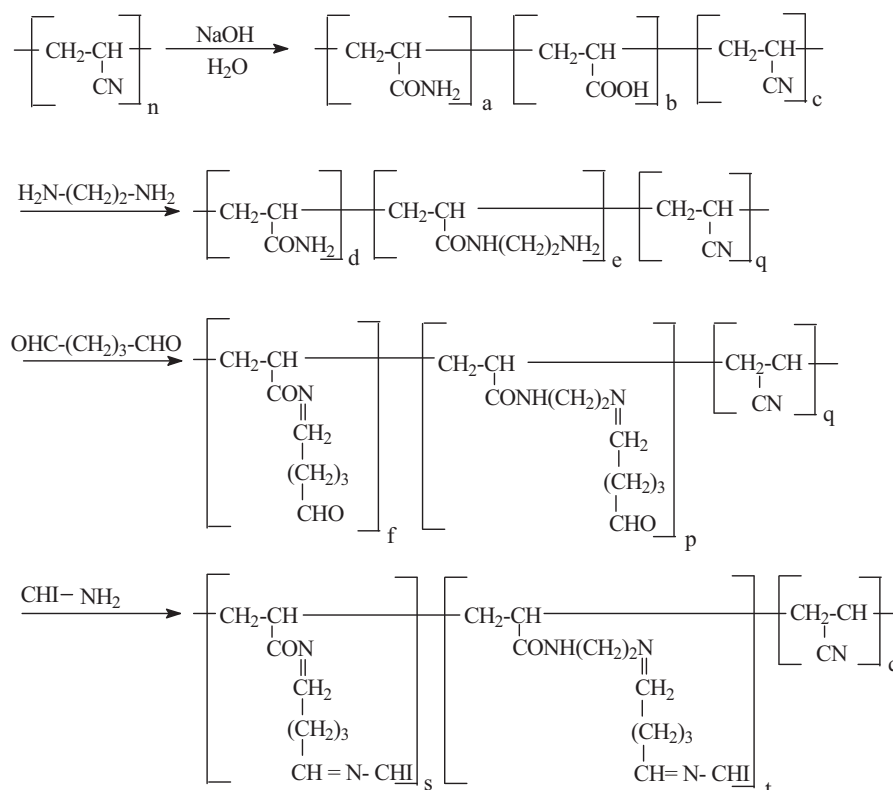


Fig. 1. SEM image of surface of modified membrane with chitosan and mixture of enzyme and rhodium nanoparticles (5% on activated charcoal) (a) and SEM cross-section of the same one without rhodium nanoparticles (5% on activated charcoal) (b).

1. The rhodium nanoparticles are an effective catalyst for ammonia oxidation (a product of the enzyme hydrolysis of urea) to nitrogen through a direct electrolytic reaction which indicated by the work platinum electrode. Both products of the enzyme reaction carbon dioxide and ammonia are not electroactive and they cannot be directly registered (without rhodium particles) from the amperometric transducer.
2. The rhodium nanoparticles give electroconductive properties of membrane which can realize an effective electron transport from the enzyme membrane to the transducer of the amperometric biosensor.

The modified membrane with chitosan and mixture of enzyme and rhodium nanoparticles (5% on activated charcoal) was proved by SEM analysis (Fig. 1a). It can be seen, that the enzyme molecules and the activated charcoal with rhodium nanoparticles were accumulated on the membrane surface. In comparison with that membrane, in Fig. 1b is presented the same membrane modified with chitosan, but it has a smooth surface, because of absence of charcoal and rhodium nanoparticles.

After that the membrane was additionally immersed in a solution of urease and after that in glutaraldehyde. The aim of this additional immobilization was the binding of greater amount of enzyme by crosslinking on the membrane surface. The activity of the immobilized urease onto the obtained carriers with different



Scheme 2. Chemical reactions for modification of AN copolymer membrane.

concentration of chitosan and the amount of the bound protein were defined in order to understand which one of the obtained immobilized systems is the most effective. It can be seen from Table 2 that the amount of the bound protein was highest at membrane 5, treated with 1.5% chitosan, at which the high amount of amino groups was posed (Table 1). There was the lowest amount of amino groups at membranes treated with 0.1% chitosan and respectively the quantity of the bound urease was the lowest.

The relative activity is very important characteristic of the immobilized enzyme. While comparing the results for the relative activity of the immobilized urease onto the modified membranes (Table 2), it was obvious that the highest activity was studied of the urease onto membrane which was covered with 1.0% chitosan (77.44%). Therefore, the most suitable membrane for the immobilization of urease amongst the five modified types, giving the maximum activity of the immobilized enzyme is AN copolymer membrane coated with 1.0% chitosan and containing rhodium nanoparticles (5% on activated charcoal). It was observed for the membrane 5 (Table 2), that larger amount of protein was bounded but the relative activity of the immobilized urease was lower. This is due to the local accumulation of protein, resulting in steric impediments for the substrate and it penetrated more dif-

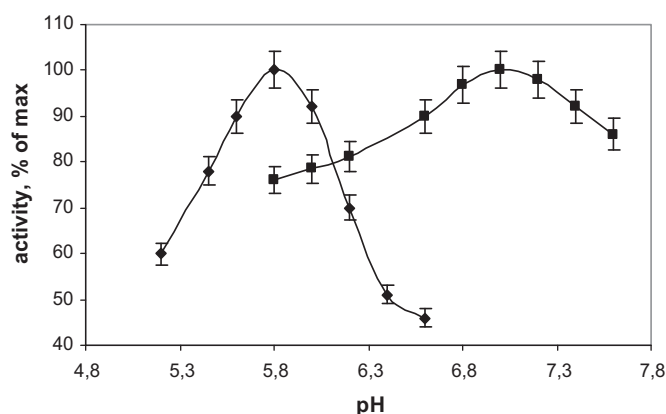


Fig. 2. pH optimum of the free (◆) and immobilized (■) urease onto AN copolymer membrane, treated with 1.0% chitosan and rhodium nanoparticles. The activity of the immobilized urease was fixed at temperature 30 °C and 0.1 M urea solution.

ficultly to the active site of the enzyme. That is why all other experiments were carried out with membranes modified with 1% chitosan.

3.3. The properties of immobilized urease

The immobilization of enzymes influences the pH optimum of the enzyme and depends on the nature of the support. The pH profiles of the free and immobilized urease on membrane modified with 1% chitosan are shown in Fig. 2. The reactions were carried out at different pH values (from 5.2 to 7.6) by the addition of suitable buffers. As shown in Fig. 2, optimum pH for free urease was found to be 5.8. It should be noted that pH_{opt} of immobilized enzyme changed depending on the type of the modified membrane [24]. In

Table 2

Relative activity of the immobilized urease and the amount of bound protein on the modified AN copolymer membranes with chitosan and rhodium nanoparticles.

No. of membrane	Chitosan concentration, %	Relative activity, %	Bound enzyme, $\mu\text{g cm}^{-1}$
1	0.15	27.69	18
2	0.3	32.15	25
3	0.5	55.53	45
4	1.0	77.44	50
5	1.5	73.83	69

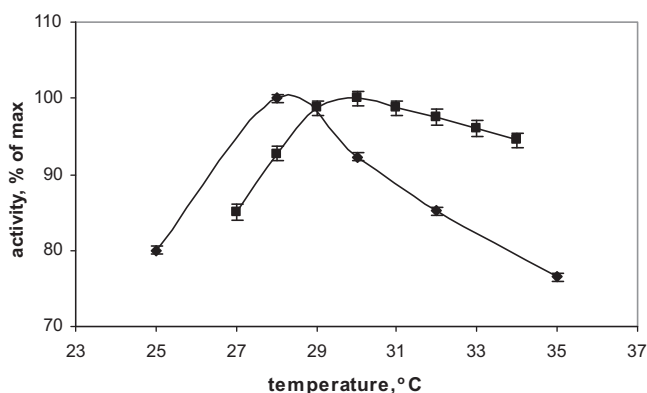


Fig. 3. Temperature optimum of the free (♦) and immobilized (■) urease onto AN copolymer membrane treated with 1.0% chitosan and rhodium nanoparticles.

our case the pH_{opt} of immobilized urease was shifted toward higher pH value ($pH_{opt} = 7.0$).

The temperature optima curves for the free and immobilized urease on membrane modified with 1% chitosan are shown in Fig. 3. The free urease had an optimum temperature of approximately 28 °C, whereas the temperature optimum of the immobilized urease was shifted to 30 °C. The increase in optimum temperature may be caused by the changing enzyme conformational structure upon immobilization, largely affect the observed changes in enzyme activity [25,26].

The thermal inactivation of the free and immobilized urease on membrane modified with 1% chitosan has been studied at 70 °C and is presented in Fig. 4. The bound enzyme was stable with time – 20% of the enzyme activity was lost for 180 min, while the free urease was totally inactivated. The increase in the thermal stability is mainly due to multipoint attachment of the urease on the surface of the support by covalent linkage. A very good thermal stability of the studied immobilized urease was obtained as compared to the results reported by other authors. For example Chen et al. [27] had obtained residual activity of 50% of the immobilized urease onto chitosan granules at 70 °C, at the 175-th min. The high thermal stability in our case is probably due to the strong crosslinking of the enzyme to the modified membranes supported by the glutaraldehyde.

For industrial applications, reusability of immobilized enzymes is one of the significant indexes to evaluate the properties of the enzyme, which can make the immobilized enzyme superior to the free one. Reusability was carried out by measuring the activity of the immobilized successive times. The maximum activity

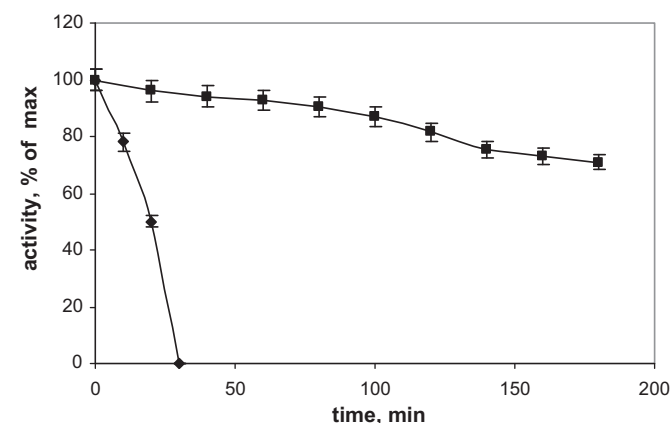


Fig. 4. Thermal inactivation at 70 °C of the free (♦) and immobilized (■) urease onto AN copolymer membrane treated with 1.0% chitosan and rhodium nanoparticles.

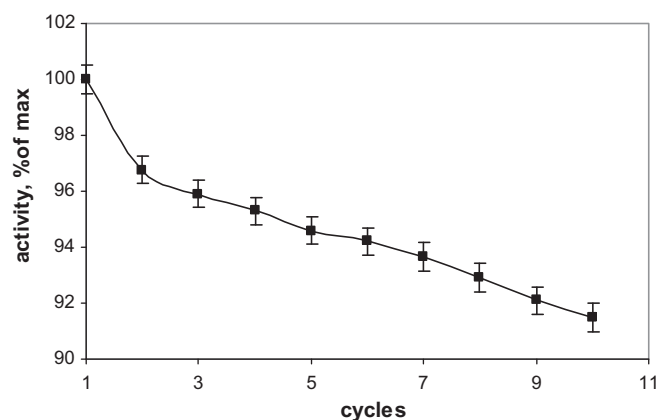


Fig. 5. Operation stability of immobilized (■) urease onto AN copolymer membrane treated with 1.0% chitosan and rhodium nanoparticles.

in the range of 100% was obtained at the beginning of reusability experiments. As shown in Fig. 5 the activity of bound urease on modified membranes showed good operation stability, and the enzyme activity loss was about 9% after 10 times. These results can be explained by the inactivation of the enzyme as a result of its denaturation and loss of enzyme from the membrane surface. Comparing the obtained results for operational stability, it can be seen that our results are better than these reported by other authors (Liang et al. [27] – 40% activity after 10 cycles), but are lower than Chen and Chiu [28] – 100% activity after 10 cycles.

The storage of the free and immobilized urease on modified membrane with 1% chitosan at 4 °C in phosphate buffer with optimum pH (pH 7) was also investigated. It was found that the immobilized urease retained 93% of its initial activity after 50 days when stored at 4 °C, while the free urease retained 50% of its initial activity. The higher stability at lower temperature can be ascribed to the protection of denaturation as a result of the attachment of urease onto the modified membranes.

3.4. The urea biosensor

In the recent years the interest to the immobilized urease has increased because of the intensive developing of biosensors on their base [29]. In this connection the possibility for application of the obtained nanostructured membrane with immobilized urease for developing of amperometric biosensor for detection of low concentration of urea was investigated.

The obtained nanostructured membrane without immobilized enzyme was attached to the platinum electrode and it was assembled in three electrode amperometric apparatus with special software program. Cyclic voltammetry (CV) was employed to study the behavior of the sensor in ammonia solution. The CV curves of the sensor are represented in Fig. 6. Curve 1 represents the cyclic voltammogram of sensor in a Tris buffer with pH 8.1 (in the absence of ammonia) and a curve 2 depicted the CV behavior of sensor in a buffer solution in which was added 100 μ L of 5 mM NH_3 solution. An anodic oxidation peak was observed at 0.8 V and a cathodic reduction peak at potential of 0.75 V. This indicated that the oxidation peak of ammonia to nitrogen occurs of 0.8 V. Therefore, the chronoamperometric curve of the urease sensor was registered at constant potential of 0.8 V. When the amperometric current became stationary series of 1.6 mM urea (final concentration) were successively added to Tris buffer with pH 8.1, placed in an electrochemical cell (Fig. 7 inside). The buffer solution was with higher pH than the pH optimum of the immobilized urease, because the rhodium catalyses the ammonium oxidation in alkaline medium [30]. By adding definite series of urea to the Tris buffer pH 8.1, the

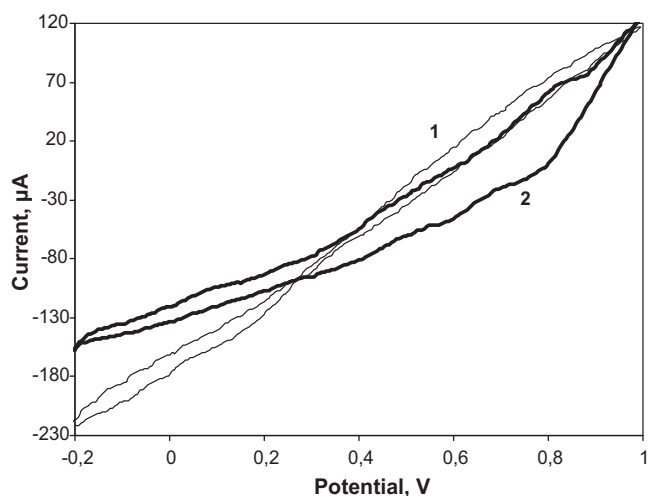


Fig. 6. Cyclic voltammograms of the rhodinized electrode in the absence of ammonia (1) and in the presence of 5 mM ammonia (2).

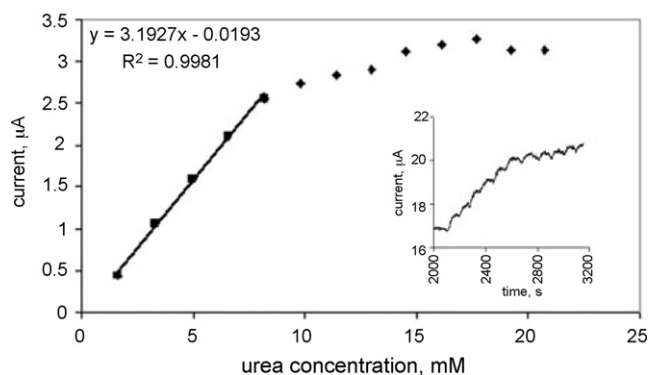


Fig. 7. Calibration plot for urea biosensor; inside – chronoamperometric curve of urease biosensor by continuously addition of 1.6 mM urea (final concentration) at applied potential 800 mV.

current was increasing as a result of the oxidation of ammonia to nitrogen, thankful to the catalytic effect of the rhodium particles and reached to a certain stationary value. After that a new series of substrate was added.

A calibration plot about urea was made on the base of the data of the chronoamperometric curve (Fig. 7). With the increase of the urea concentration, the amperometric respond increased linearly in the range: from 1.6 to 8.2 mM. The linear regression equation is $I (\mu\text{A}) = 3.1927x - 0.0193$ with higher correlation coefficient (R^2) of 0.998 ($n=5$). The sensitivity in the linear was higher $3.1927 \mu\text{A mM}^{-1} \text{cm}^{-2}$ than other urea electrode [31,32], respectively $7.48 \text{ nA mM}^{-1} \text{cm}^{-2}$ and $980 \text{ nM mM}^{-1} \text{cm}^{-2}$. The modified membrane with chitosan and rhodium nanoparticles can provide efficient electron transfer between the active site of the enzyme and the electrode, thereby enhancing the urea sensing activity. The detection limit was 0.5 mM at a signal-to-noise ratio of 3. The response to the presence of urea was immediate and stable with time for the catalysis reaching the value of the stationary current around 20 s.

From the graphic plot of Lineweaver–Burk (Fig. 8), using electrochemical data extracted from the calibration curves of the constructed urea biosensors, the Michaelis–Menten constant of the immobilized urease was investigated (K_m). The value of obtained K_m was compared with this of the free one. The immobilized urease presented higher value of K_m (52.6 mM), compared with the free urease (16 mM). The higher value of K_m was due to both the

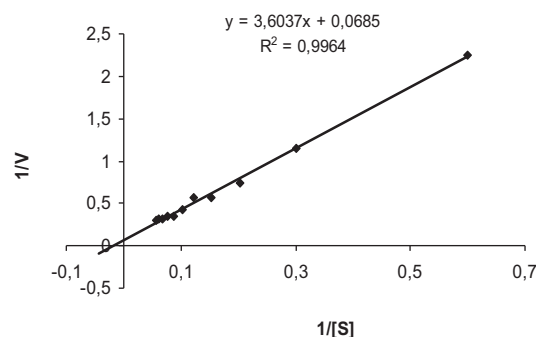


Fig. 8. Lineweaver–Burk plot for the immobilized urease onto AN copolymer membrane treated with 1.0% chitosan and rhodium particles.

occurred conformational changes in the enzyme as a result of the formed enzyme-substrate complex, and to the more difficult accessibility of the substrate to the active center of the immobilized enzyme.

3.5. Reproducibility and lifetime of urea biosensor

The reproducibility of successive tests using the same biosensor was investigated. Five successive measurements using the same biosensor were carried out at a 1.6 mM urea. The relative standard deviation (R.S.D.) of the potential responses was 5.9%. It was studied that after 4 operation cycles deactivation of electrode was occurred. This disadvantage was avoided by replacing the catalytic membrane with a fresh one. The biosensor was employed for 10 days while the maximum response to urea retained 86.8%.

4. Conclusions

It is obvious from the obtained results that the developed urease amperometric biosensor has good sensitivity and fast response toward low urea concentrations. This good characteristic of the biosensor is due to presence of chitosan and the rhodium nanoparticles in the polymer membrane, which provide high immobilization yield of urease, good biocompatibility and effective catalysis of the ammonia oxidation, which avoid using of a second enzyme. An important advantage, as believed, of the constructed biosensor, is that the enzyme membrane is a separate element and could be easily replaced when the catalytic membrane is deactivated, using a single working electrode. Another advantage of the electrode is that AN copolymer membrane possesses a selective and non-selective sides due to the asymmetry of the membrane pores. The enzyme molecules trapped into the pores of the non-selective membrane side cannot be washed away and are being protected from any electrochemical interference present in the solution during the measurement procedures.

Acknowledgements

The authors gratefully acknowledge to the Bulgarian Ministry of Education for financial support by grants from DOO2/125/2009 Project and NIH-218/2010 Project.

References

- [1] B. Krajewska, *Enzyme Microb. Technol.* 35 (2004) 126.
- [2] P. Linderholm, A. Bertsch, P. Renaud, *Physiol. Meas.* 23 (2004) 645.
- [3] S. Phadtare, V.P. Vinod, P.P. Wadgaonkar, M. Rao, M. Sastry, *Langmuir* 20 (2004) 3717.
- [4] P.R. Leriaio, L.J. Fonseca, M.A. Taipa, J.M. Cabral, M. Mateus, *Appl. Biochem. Biotechnol.* 110 (2003) 1.
- [5] T. Godjevargova, K. Gabrovska, *Macromol. Biosci.* 5 (2005) 459.

- [6] H. Zheng, H. Okada, S. Nojima, S. Suye, T. Hori, *Sci. Technol. Adv. Mater.* 5 (2004) 371.
- [7] K.F. Fernandes, C.S. Lima, F.M. Lopes, C.H. Collins, *Process Biochem.* 39 (2004) 957.
- [8] G. Pozniak, B. Krajewska, W. Trochimczuk, *Biomaterials* 16 (1995) 129.
- [9] R. Rajesh, V. Bisht, W. Takashima, K. Kanelo, *Biomaterials* 26 (2005) 3683.
- [10] J.H.T. Luong, P. Bouvrette, K.B. Male, *Trends Biotechnol.* 15 (1997) 369.
- [11] W. Frenzel, C.Y. Liu, *Fres. J. Anal. Chem.* 342 (1992) 276.
- [12] H. Hara, S. Matsumoto, *Analyst* 119 (1994) 1839.
- [13] S. Fan, H. Muller, B. Schweizer, W. Bohme, *Fres. J. Anal. Chem.* 347 (1993) 103.
- [14] X. Hu, N. Takenaka, S. Takasuna, M. Kitano, H. Bandow, Y. Maeda, M. Hattori, *Anal. Chem.* 65 (1993) 3489.
- [15] U. Oesch, S. Caras, J. Janata, *Anal. Chem.* 53 (1981) 1983.
- [16] Y. Miyahara, K. Tsukada, M. Miyagi, W. Simon, *Sens. Actuators B* 3 (1991) 287.
- [17] F.J. Saez de Viteri, D. Diamond, *Electroanalysis* 6 (1994) 9.
- [18] B.B. Rodriguez, J.A. Bolbot, I.E. Tothill, *Anal. Lett.* 37 (3) (2004) 415.
- [19] B.A. Lopez de Mishima, D. Lescano, T. Molina Holgado, H.T. Mishima, *Electrochim. Acta* 41 (3–4) (1998) 395.
- [20] S. Petrov, *J. Appl. Polym. Sci.* 62 (1996) 267.
- [21] K. Dimov, V. Sarmadjieva, P. Pavlov, In *Laboratory Practices on Technology of Synthetic Fibres*, Technica, Sofia, 1983, p. 145.
- [22] H. Lowry, N. Rosenbough, H. Farr, *J. Chem.* 193 (1951) 265.
- [23] A. Steffen, E. Straube, *Biotechnol. Bioeng.* 39 (1992) 725.
- [24] A. Fahmy, V. Bagos, T. Mohammed, *Bioresour. Technol.* 64 (1998) 121.
- [25] J. Leaka, J. Włodarczyk, W. Zaborska, *J. Mol. Catal. B: Enzyme* 6 (1999) 549.
- [26] I. Gancarz, J. Bryjak, M. Bryjak, G. Poniak, W. Tylus, *Eur. Polym. J.* 39 (2003) 1615.
- [27] J. Chen, S. Chiu, *Enzyme Microb. Technol.* 26 (2000) 359.
- [28] J.F. Liang, Y.T. Li, V.C. Yang, *J. Pharm. Sci.* 89 (2000) 979.
- [29] M. Singh, N. Verma, A.K. Garg, N. Redhu, *Sens. Actuators B* 134 (2008) 345.
- [30] A.C.A. de Vooys, M.T.M. Koper, R.A. van Santen, J.A.R. van Veen, *J. Electroanal. Chem.* 506 (2) (2001) 127.
- [31] A. Tiwari, S. Aryal, S. Pilla, S. Gong, *Talanta* 78 (2009) 1401.
- [32] I. Vostiar, J. Tkac, E. Sturdik, P. Gemeiner, *Bioelectrochemistry* 56 (2002) 113.