Digest Journal of Nanomaterials and Biostructures Vol. 7, No. 2, April - June 2012, p. 785 - 794

ELECTROCHEMICALLY DEPOSITED NANO FIBROUS POLYANILNE FOR AMPEROMETRIC DETERMINATION OF GLUCOSE

DALIBORKA JAMBREC, MILICA GVOZDENOVIĆ[a*], MIRJANA ANTOV[b], BRANIMIR GRGUR[a], BOJAN JOKIĆ[a], JASMINA STEVANOVIĆ[c], BRANIMIR JUGOVIĆ[d]

Innovation centre, Faculty of Technology & Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Belgrade, Serbia

[a]Faculty of Technology & Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Belgrade, Serbia

[b]Faculty of Technology, University of Novi Sad, Blvd. Cara Lazara 1, 21000 Novi Sad, Serbia

[c]Institute of Chemistry Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia

[d]Institute of Technical Science, Serbian Academy of Science and Arts, Knez Mihajlova 35, 11000 Belgrade, Serbia

Electrochemical deposition of polyaniline (PANI) on graphite electrode was performed galvanostaticaly at constant current density in the range of 1.0 – 5.0 mA cm^{-2} from aqueous acidic electrolyte containing aniline monomer. Based on ratio of doping/dedoping charge capacities, it was estimated that current density of 2.0 mA cm^{-2} was optimal. The structure of the electrochemically synthesized PANI was fibrous, uniform and three dimensional with highly developed surface. Immobilization of glucose oxidise (GOx) was achieved by cross linking via glutaraldehyde and the efficiency of the immobilization was determined spectrophotometrically. Chronoamperometric curves were recorded at different glucose concentrations and used to estimate the apparent Michaelis constant, which was shown to be 0.27 mM. The storage stability of the PANI enzyme electrode was also estimated.

(Received March 19, 2012; Accepted June 8, 2012)

Keywords: Electrochemical synthesis, Polyaniline, Immobilization, Michaelis constant

1. Introduction

The determination of glucose is a well-known application of biosensors, and it is very popular for research because of the significance glucose has in the human metabolic process. Diabetes mellitus is a widespread disease which is characterized by blood glucose concentration higher or lower than normal (80-120 mg/dl) due to the body's inability to produce or properly respond to insulin, the hormone that signals cells to take up and use glucose [1,2]. Diabetics represent about 6.4 % of the world's population, therefore it is socially important to develop low cost, sensitive, reliable, selective glucose sensors [3].

Beside their important role in the improvement of public health, glucose biosensors are also present in environmental monitoring, food and beverage industry (e.g. for detection of toxins [4,5] and insecticides [6]), bioprocess monitoring [1,7], etc.

Unlike thin-layer chromatography (TLC) and high-performance-liquid-chromatography (HPLC), which are time consuming, lab-based, unsuitable for regulatory fast monitoring [4] and costly [5,8], biosensors provide fast analysis and selective, sensitive and accurate measurements.

*Corresponding author: popovic@tmf.bg.ac.rs

A biosensor has three major components: a bio receptor (enzyme, antibody, DNA) for the recognition of an analyte, an immobilization surface (conducting polymers [9], nanomaterials [10], sol-gel films [11]) for the immobilization of the bio molecule and a transducer unit for the conversion of the biochemical reaction product into a recognizable signal [1]. An electrochemical biosensor is based on the conversion of the analyte concentration into an electrochemical signal. Depending on what is measured, it can be potentiometric, amperometric or conductometric. Because of their simplicity, selectivity and short response times, amperometric glucose biosensors are the most commonly used for glucose detection [12]. Amperometric biosensors based on conducting polymers provide many advantages and new possibilities to detect biologically active compounds [13-15].

The sensitivity of the biosensors depends on the characteristics of the enzyme used. One of the most widely used enzymes in the field of biosensors is glucose oxidise (GOx) because of its high specificity for a commercially important analyte, turnover number and stability [16].

Generally, it is difficult to exchange an electron between the enzyme and the solid surface of the electrodes directly, because of the loss of bioactivity of the enzyme due to the conformational changes in the process of adsorption on the electrode. Hence, in order to have a stable and sensitive response, the surface of the electrode should be modified [17]. That can be done by conductive polymers, because of a number of favourable characteristics, among which is the direct and easy deposition on the sensor electrode by the electrochemical oxidation of the monomer [18]. They provide a stable and porous matrix for the immobilization of the bio component and act as transducers to convert the chemical signal into an electrical one [1,19]. Conducting polymers are also known for their ability to be compatible with biological molecules in neutral aqueous solutions [9]. Biosensors prepared using a conducting polymer as a support material has fast response times with a high storage and operational stability [20]. They can be obtained by using both the chemical and electrochemical synthesis, but the latter is favourable. The advantage of the electrochemical synthesis is that the doping reaction occurs simultaneously with the growth of the polymer chain, and hence the polymer is obtained in its conductive form. Among the various techniques for the electrochemical synthesis, galvanostatic method has the advantage over other methods as the film thickness can be easily controlled by reaction time.

The most widely used conductive polymers for enzyme immobilization are polyaniline (PANI), polypyrrole (PPy) and polythiophene (PT) [1,7,21]. PANI has attracted much attention due to its high conductivity, ease of preparation and its environmental, thermal and electrochemical stability [22-26]. It is recognized as the only conducting polymer that is stable in air [27]. PANI is compatible with most enzymes and can be easily synthesized from aniline monomer in an aqueous solution [7]. It acts as an immobilization platform and an electron mediator in the same time.

A general requirement in electrochemical biosensors is to reproducibly immobilize the bio molecules onto the biosensor while keeping their biological activity [28]. The porosity of the conducting polymer is an important factor for the facile immobilization of an enzyme [19]. There are many methods for enzyme immobilization: physical entrapment, adsorption, covalent binding, cross linking and doping [29]. The weak point of many immobilization methods is enzyme leakage. However, this problem can be significantly reduced by using the chemical cross-linking method for immobilization via glutaraldehyde [19,20].

The principle of the glucose biosensor is based on the reaction between GOx and glucose shown in the following equation [30]:

$$\text{Glucose} + O_2 \xrightarrow{GOx} \text{Gluconic acid} + H_2O_2 \quad (1)$$

The amount of hydrogen peroxide which is directly proportional to the amount of glucose, can be electrochemically determined at the anode according to equation:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

In this paper we formed the biosensor by immobilizing the glucose oxidise on polyaniline, electrochemically synthesised on graphite, using cross linking method via glutaraldehyde.

Recently [31], we have investigated an enzyme electrode obtained by the immobilization of the GOx into the electrochemically formed polyaniline, using different electrochemical technique and the obtained results, namely very low value of the apparent Michaelis constant, had pointed to the high affinity of the enzyme towards substrate. Bearing in mind the practical importance of the amperometric determination, the aim of this paper was to obtain the optimal condition for the electrochemical synthesis of the polyaniline that would lead to the highest extent of its conductive form, and to confirm the previously obtained enzyme affinity [31] using amperometric procedure.

2. Experimental

Electrochemical synthesis of PANI on graphite electrode (S=0.64 cm^2) was performed from aqueous solution of 1 M HCl (p.a. Merck) containing 0.25 M of aniline (p.a. Fluka), at constant following anodic current densities: 1.0, 1.5, 2.0, 3.0 and 5.0 mA cm^{-2}. Dedoping (discharge), performed at cathodic current density of 1.0 mA cm^{-2} served for estimation of the amount of electrochemically active form of PANI.

Prior to the polymerization, graphite electrode was mechanically polished with fine emery papers (2/0, 3/0 and 4/0 in the increasing order) and then with polishing alumina (1 μm, Banner Scientific Ltd.) on polishing cloths (Buehler Ltd.). The surface of the electrode was cleaned ultrasonically (Bandelin Sonorex) for 5 minutes to remove traces of the polishing alumina.

The scanning electron microscopy (SEM) micrographs of the PANI electrode surface before and after immobilization of Gox were taken by (SEM, MIRA3 TESCAN).

The immobilization of GOx from *Aspergillus niger* (Sigma–Aldrich) was performed via glutaraldehyde (1.2% (w/w), Fluka). The PANI electrode was first left in glutaraldehyde for 1 h, and then immersed in phosphate buffer solution (pH 5.6; 0.1 M) containing 15 mg ml^{-1} of GOx for 24 h.

The determination of the amount of the immobilized proteins in PANI enzyme electrode was investigated by measurement of the protein concentration before and after immobilization using Bradford method [32] with BSA as the standard.

The PANI enzyme electrode was investigated at constant potential of 0.7 V (vs. standard calomel electrode, SCE) in solutions containing different glucose concentration. Prior to this, glucose stock solution (D-(+) glucose monohydrate for biochemistry, Merck) was left for 24 h in order to complete the mutarotation.

Electrochemical measurements were performed in standard three compartment electrochemical glass cell at ambient temperature (22 °C). Saturated calomel electrode (SCE) was used as reference and platinum wire as counter electrode. All electrochemical experiments were performed using PAR 273A potentiostat/galvanostat connected to PC.

3. Results and discussion

3.1 Electrochemical synthesis of PANI

It is well known that PANI can exist in different oxidation forms of which only emeraldine salt is electrochemically active and conductive [33]. Electrochemical polymerization of aniline proceeds simultaneously with insertion (doping) of chloride ions according to:

$$(PANI)_n + nyCl^- \rightarrow [PANI^{y+}(Cl^-)_y]_n + nye^- \quad (3)$$

where y is doping degree (ratio between the number of charges in the polymer and the number of monomer units). Different doping degrees refer to different oxidation levels, as schematically presented in Fig. 1 [34].

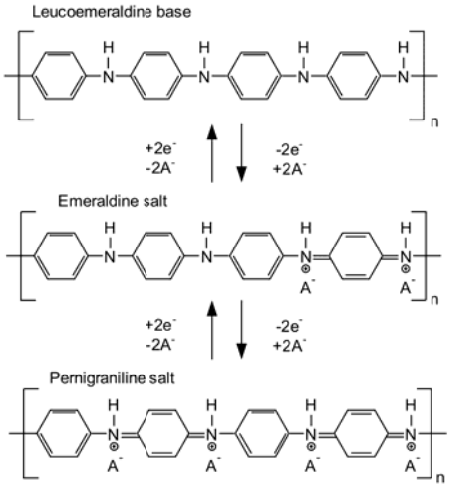

Fig. 1. Schematic presentation of different oxidation forms of PANI.

Leucemeraldine, a completely reduced form of PANI has doping degree of $y = 0$, while emeraldine salt with $y = 0.5$ is half oxidized form, and finally pernigraniline salt refers to fully oxidized form ($y = 1$). Since only emeralidne salt is conductive and capable of charge exchange, its amount can be estimated from dedoping charge capacities. Therefore in order to determine optimal current density for electrochemical synthesis of PANI, experiments were performed with different anodic current density, after which cathodic dedoping curves were recorded at constant current density of 1.0 mA cm^{-2}.

Since practically there were no difference in the shape of chronoamperometric curves of PANI electrochemical synthesis at different anodic current densities only curve obtained at 2.0 mA cm^{-2} is given in Fig. 2, while chronoamperometric dedoping curves at constant cathodic density of 1.0 mA cm^{-2}, for all samples synthesized at different current densities (as marked on figure), are shown in the insert of Fig. 2.

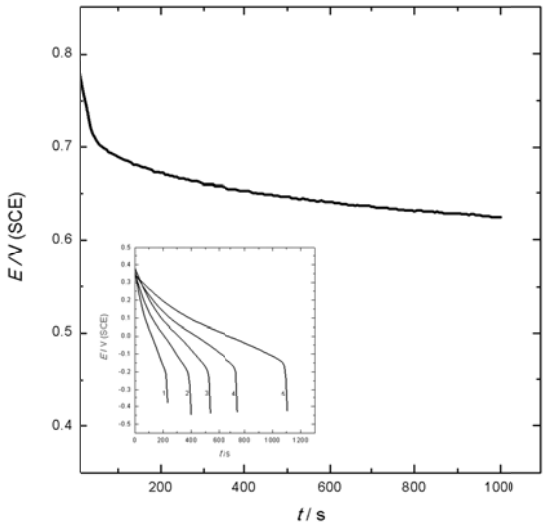

Fig. 2. Electrochemical polymerization of PANI from aqueous solution of 1.0 M HCl and 0.25 M of aniline at constant anodic current density of 2.0 mA cm^{-1}. Insert: dedoping of PANI at constant cathodic current density of 1.0 mA cm^{-2} for PANI electrodes obtained with different current densities: (1) – 1.0 mA cm^{-2}; (2) – 1.5 mA cm^{-2}; (3) – 2.0 mA cm^{-2}; (4) – 3.0 mA cm^{-2} and (5) – 5.0 mA cm^{-2}.

As it can be seen in Fig. 2 the initial increase of the potential is connected to formation of active sites on electrode surface on which further electrochemical polymerization of aniline proceeds at potential plateau of around 0.6 V (SCE). As expected, PANI obtained with different current densities i.e. with different charge capacities, had different discharge (dedoping) times. Charge and discharge (dedoping) capacities, and then their ratio, were calculated using data from Fig. 3, and the results are presented in Fig. 3.

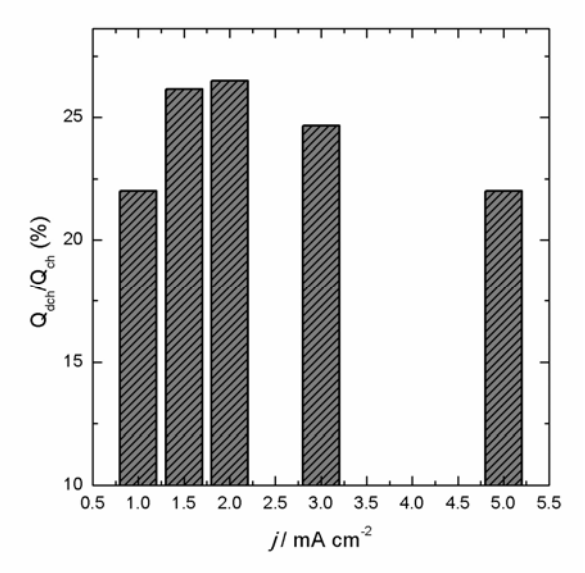

Fig. 3. Charge/discharge capacities ratio.

As it can be seen in Fig. 3 the best results, i.e. the greater amount of the conductive PANI form was obtained at current density of 2.0 mA cm^{-2}, and that current density was used for formation of PANI enzyme electrode.

3.2. Morphology of PANI

SEM micrograph of the PANI electrochemically deposited on graphite electrode is shown in Fig. 4.

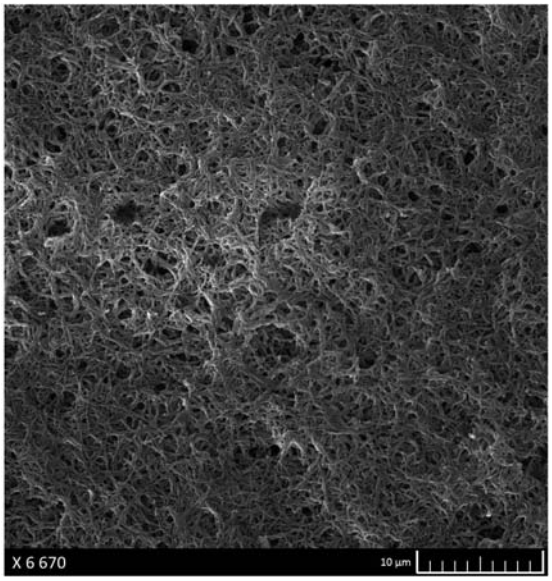

Fig. 4. SEM micrographs of the PANI electrochemically deposited onto graphite electrode.

As seen in Fig. 4, electrochemically deposited PANI is uniform, forming nano fibrous network with highly developed surface suitable for enzyme immobilization.

Fig. 5 shows the comparison of the electrode surface before and after the immobilization. It is shown that the diameter of the fibers after the immobilization is approximately two times larger than before the immobilization, and it can be concluded that the difference in the diameter derives from the immobilized GOx via glutaraldehyde.

3.3. Determination of the amount of the immobilized enzyme

The amount of the immobilized enzyme in the PANI electrode was determined as the difference of the amount of proteins in solution before and after the immobilization. The amount of proteins in the solution before immobilization was 1.7 mg ml^{-1}. It was estimated that after immobilization there was 1.2 mg ml^{-1} of proteins left in the solution, meaning that 0.5 mg ml^{-1} of the proteins were immobilized in the PANI electrode, which is 29%. Since the volume of the immobilization solution was 4.0 ml, it could be estimated that 2.0 mg (3.1 mg cm^{-2}) was immobilized in the PANI enzyme electrode.

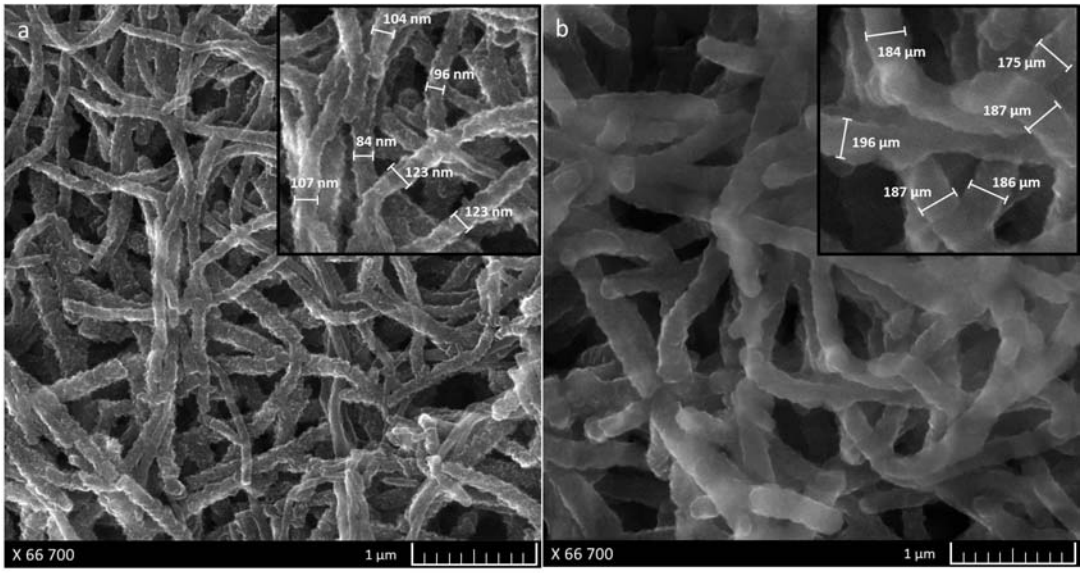

Fig. 5. a) SEM micrograph of the electrode surface a) before and b) after the immobilization. Inserts: enlarged part of the image for better visibility.

3.4. Kinetic parameters

The PANI electrode was formed by the immobilization of GOx via glutaraldehyde on PANI synthesized on the graphite electrode. The chronoamperometric curves for different glucose concentrations made at constant potential of 0.7 V (vs. SCE) are given in Fig. 6. For improved readability, only five concentrations (1, 2, 5, 10 and 25 mM)

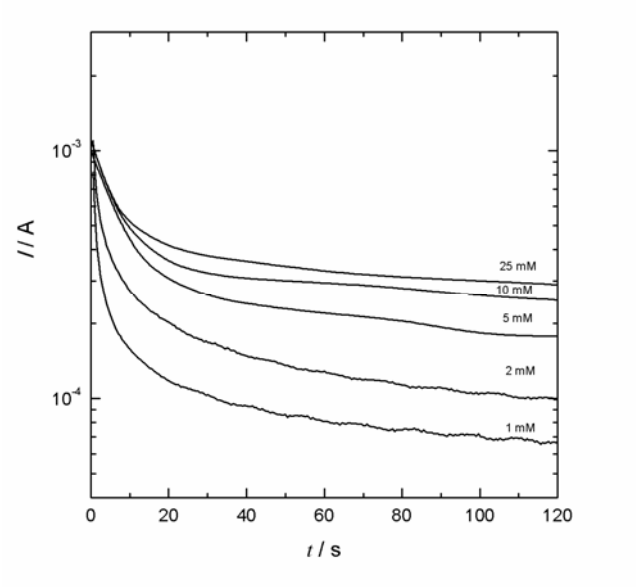

Fig. 6. Chronoamperometric curves of PANI enzyme electrode in glucose solution of 1, 2, 5, 10 and 25 mM.

The current dependence on glucose concentration was is given shown in insert of the Fig. 7., while linearization of the dependence is given in Fig. 7.

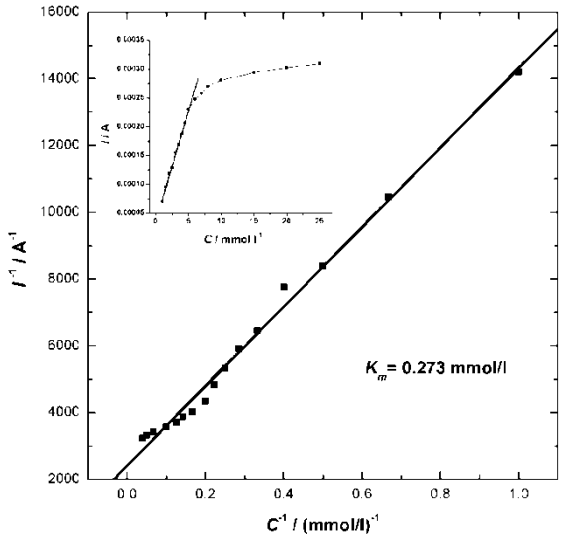

Fig. 7. Dependence of the reciprocal values of current on reciprocal values of glucose concentration, for the determination of apparent Michaelis constant. Insert: dependence of current on glucose concentration and linearity region.

As seen in the insert of Fig. 7, the current increases with the increase in glucose concentration over the entire range of the examined glucose concentration, while the linear region is observed below 1mM. The obtained dependence in the form of rectangular hyperbola is typical for enzyme kinetics. If it is assumed that the enzyme had been uniformly immobilized into PANI film, for lower glucose concentrations the reaction would occur only on the polymer surface, corresponding to the linear region in insert of the Fig. 7. For higher glucose concentrations, delay of the response time is observed, as a result of diffusion limitation.

Linearization of the current dependence on glucose concentration is given in Fig. 7, and it was used to estimate of the apparent Michaelis constant.

The apparent Michaelis constant was determined as the ratio between the intercept and the slope of the linear dependence at Fig.7, and it was found to be 0.273 mmol l^{-1}. That value is significantly lower than the results for free GOx from *Aspergillus niger* [34], which means that enzyme shows higher affinity for substrate after immobilization. Also, K_m from this experiment is lower than the reports from similar studies with gold nanoparticles [35] ZnO nanotubes [36], $CoFe_2O_4/SiO_2$ [37], and polyaniline as an immobilization platform [19,30,38]. Moreover, the K_m is practically the same as the one from our previous work [31], suggesting that the amperometric biosensor gives results as good as the potenciometric. This low value for the apparent Michaelis constant is probably the result of the developed three-dimensional structure of PANI that prevents diffusion limitations and allows a favorable orientation of the bounded enzyme, leading to high accessibility to the substrate.

3.5. Storage stability

In order to determine the storage stability of the PANI enzyme electrode the electrode was left in phosphate buffer (0.1 M, pH=5.6) at 8 °C. Current-time curves for glucose concentration of 3.0 mM were recorded after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days. Results are given in Fig. 7 in terms of the relative signal intensity.

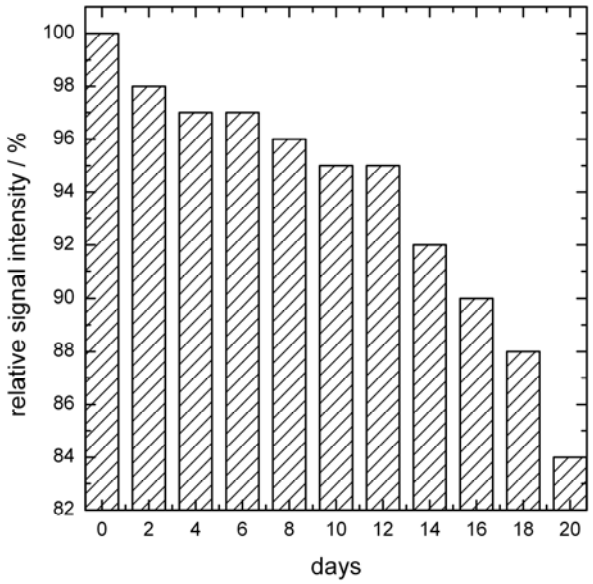

Fig. 8. Storage stability of PANI/GOx electrode .

After 6 days the PANI enzyme electrode lost 3% of its signal and after 20 days the electrode retained 84% of its initial signal, which is comparable with the results from other studies [39,40]. This value is a little lower compared to our previous work [31], where the electrode kept 88% of its initial signal after 20 days of examination.

The loss of the signal intensity could be the result of enzyme leaking and PANI degradation during storage at pH favorable for biochemical purposes, leading to the decrease of polymer conductivity.

4. Conclusions

Amperometric electrode for glucose sensing was formed by immobilization of the glucose oxidase (GOx) on the polyaniline (PANI) modified graphite electrode. It was shown that the most suitable current density for the electrochemical polymerization among five different current densities is the one of 2 mA cm^{-2}. Morphology of the galvanostatically deposited polyaniline was fibrous, uniform, three dimensional and porous, and thus suitable for the enzyme immobilization. It was estimated that 29% of proteins were immobilized in PANI. From current-glucose concentration dependence apparent Michaelis constant of 0.273 mmol l^{-1} was determined. This low value for apparent Michaelis constant is probably the result of an excellent three-dimensional structure of PANI that prevents diffusion limitations and allows a favorable orientation of the bound enzyme, leading to high accessibility to the substrate. Loss of the electrode signal could be a result of enzyme leakage and degradation of PANI, due to the conditions favorable for biochemical purposes, which led to the decrease of the polymer conductivity. Compared to the same system obtained by the potentiometric technique [31], this biosensor shows somewhat lower storage stability.

Acknowledgments

This work was supported by The Ministry of Science, Republic of Serbia, No. 172046.

References

[1] C. Dhand, M. Das, M. Datta and B. D. Malhotra, Biosens. Bioelectron., **26**, 2811 (1994).
[2] M. F. Whites and C. R. Kahn, J. Biol. Chem., **269**, 1 (1994).
[3] E-H Yoo and S-Y Lee, Sens., **10**, 4558 (2010).
[4] S. C. Li, J. H. Chen, H. Cao, D. S. Yao and D. L. Liu, Food Control, **22**, 43 (2011).
[5] B. Van der Gaag, S. Spath, H. Dietrich, E. Stigter, G. Boonzaaijer, T. Van Osenbruggen and K.Koopal, Food Control, **14**, 251 (2003).
[6] C. B. S. Roepcke, S. B. Muench, H. Schulze, T. T. Bachmann, R. D. Schmid and B. Hauer, Food Control, **22**, 1061 (2011).
[7] A. Kausaite-Minkstimienea, V. Mazeikoa, A. Ramanaviciene and A. Ramanavicius, Biosens.Bioelectron.,**26**, 790 (2010).
[8] N. Nagatani, A. Takeuchi, M. A. Hossain, T. Yuhi, T. Endo, K. Kerman, Y. Takamura E. Tamiya, Food Control, **18**, 914 (2007).
[9] M. Gerard, A. Chaubey and B. D. Malhotra, Biosens. Bioelectron., **17**, 345 (2002).
[10] X. Luo, A. Morrin, A. J. Killard and M. R. Smyth, Anal. Chim. Acta, **575**, 39 (2006).
[11] A. A. Ansari, A. Kaushik, P. R. Solanki and B. D. Malhotra, Electrochem. Commun. **10**, 1246 (2008).
[12] H. Wang, C. Zhou, J. Liang, H. Yu and F. Peng, Int. J. Electrochem. Sci., **3**, 1180 (2008).
[13] A. Ramanavicius, A. Kausaite and A. Ramanaviciene, Sens.Actuators, B, **111–112**, 532 (2005).
[14] A. Rahman, P. Kumar, D-S Park and Y-B Shim, Sens., **8**, 118 (2008).
[15] E. M. I. Mala Ekanayake, D. M. G. Preethichandra and K. Kaneto, Sens. Actuators, B,**132**, 166 (2008).
[16] R. Wilson and A. P. F. Turner, Biosens. Bioelectron., **7**, 165 (1992).
[17] P. Norouzi, F. Faridbod, B. Larijani and M. R. Ganjali, Int. J. Electrochem. Sci., **5**, 1213 (2010).
[18] P. D. Gaikwad, D. J.Shirale, V. K. Gade, P. A. Savale, H. J. Kharat, K. P. Kakde M. D. Shirsat, Int. J. Electrochem. Sci., **1**, 425 (2006).
[19] P. D. Gaikwad, D. J. Shirale, P. A. Savale, K. Datta, P. Ghosh, A. J. Pathan, G. Rabbaniand M. D. Shirsat, Int. J. Electrochem. Sci., **2**, 488 (2007).
[20] M. Shaolin, J. Electroanal.Chem., **370**, 135 (1994).

[21] P. N. Barlett and J. M. Cooper, J. Electroanal.Chem., **362**, 1 (1993).
[22] Y. Kang, S. K. Kim and C. Lee, Mater.Sci. Eng., *C*, **24**, 39-41 (2004).
[23] S. Palaniappan, *Eur. Polym. J.*, **37**, 975-981 (2001).
[24] A. N. Ivanov, L. V. Lukachova, G. A. Evtugyn, E. E. Karyakina, S. G. Kiseleva, H. C. Budnikov, A. V. Orlov, G. P. Karpacheva and A. A. Karyakin, Bioelectrochemistry, **55**, 75 (2002).
[25] C. Chiang and A. Q. G. MacDiarmid, Synth. Met., **13**, 193 (1986).
[26] A. Eftekhari, Synth. Met., **125**, 295–300 (2002).
[27] T. A. Sergeyeva, N. V. Lavrik, S. A. Piletsky, A. E. Rachkov and A. V. El`skaya, Sens. Actuators, B, **34**, 283 (1996).
[28] W. Jin, G. Yang, L. Wu, Q. Wang, H. Shao, A. Qin, B. Yu, D. Li and B. Cai, Food Control, **22**, 1609 (2011).
[29] D. Wei and A. Ivaska, Chem. Anal. (Warsaw), **51**, 839 (2006).
[30] X. Pan, J. Kan and L. Yuan, Sens. Actuators, B, **102**, 325 (2004).
[31] M. M. Gvozdenović, B. Z. Jugović, D. I. Bezbradica, M. G. Antov, Z. D. Knežević-Jugović B. N. Grgur, Food Chemistry, **124**, 396 (2011).
[32] M. M. Bradford, Anal. Biochem.,**72**, 248 (2004).
[33] N. Gospodinova and L. Terlemezyan, Prog. Polym. Sci., **23**, 1443 (1998).
[34] M. Swoboda and V. Massey, J. Biol. Chem., **240**, 2290 (1965).
[35] J. Wang, L. Wang, J. Di and Y. Tu, Sens. Actuators, B, **135**, 283 (2008).
[36] T. Kong, Y. Chen, Y. Ye, K. Zhang, Z. Wang and X. Wang, Sens. Actuators, B, **138**, 344 (2009).
[37] H. Wang, J. Huang, C. Wang, D. Li, L. Ding and Y. Han, Appl. Surf. Sci., **257**, 5739 (2010).
[38] H. Xue, Z. Shen and C. Li, Biosens. Bioelectron., **20**, 2330 (2005).
[39] L. Xu, Y. Zhu, X. Yang and C. Li, Mater. Sci. Eng.,C, **29**, 1306 (2009).
[40] Y. Zou, L. Sun, F. Xu, Biosens. Bioelectron., **22**, 2669 (2007)