AMPEROMETRIC GLUCOSE BIOSENSORS BASED ON FUNCTIONALIZED ELECTROCHEMICALLY REDUCED GRAPHENE OXIDE

Matei RAICOPOL[1], Ioana VLĂSCEANU[2], Iulian LUPULESCU[3], Ana Maria Brezoiu[4], Luisa PILAN[5]*

In the present work we report a new strategy to create glucose amperometric biosensors with improved analytical characteristics using graphene as support for glucose oxidase. First, a reduced graphene oxide film was obtained on the glassy carbon electrode surface by direct electrochemical reduction of graphene oxide from a suspension in water. For facilitating the oxidation of the enzymatically generated H_2O_2, in a second step, Pt nanoparticles were electrodeposited on the graphene modified electrodes. The obtained biosensors showed good analytical performances in terms of high sensitivity and wide linear range.

Keywords: glucose amperometric biosensor, electrochemically reduced graphene oxide, glucose oxidase, aryl diazonium salts

1. Introduction

The recent trend in the biosensors field is the use of modified electrodes to enable wider detection ranges, increased detection sensitivity and to eliminate the influence of interfering chemical species. In this context, electrochemical biosensors based on nanomaterials have attracted much attention lately.
Graphene is now positioned among the top-ranked novel materials which show potential to open new perspectives in the field of biosensing devices. The application of graphen in electrochemical biosensors proved to be a great success, both because of the inherent properties of graphene, but also due to recent improvements in the synthesis and manufacture of functional graphene. Unlike

[1] Lecturer, Dept. of Organic Chemistry, University POLITEHNICA of Bucharest, Romania, e-mail: matei_raicopol@yahoo.com
[2] Student, Dept. of Inorganic Chemistry, Physical Chemistry and Electrochemistry, University POLITEHNICA of Bucharest, Romania, e-mail: ioananicole.vlasceanu@gmail.com
[3] Student, Dept. of Inorganic Chemistry, Physical Chemistry and Electrochemistry, University POLITEHNICA of Bucharest, Romania, e-mail: iulian.lupulescu@gmail.com
[4] Student, Dept. of Organic Chemistry, University POLITEHNICA of Bucharest, Romania, e-mail: anamaria_brezoiu@yahoo.com
[5]* Associate Prof., Dept. of Inorganic Chemistry, Physical Chemistry and Electrochemistry, University POLITEHNICA of Bucharest, Romania, corresponding author, e-mail: luisa_pilan@yahoo.com

other carbon nanomaterials (e.g. carbon nanotubes) the superior performance of graphene in this field can be attributed to [1-3]: surface availability for a higher degree of functionalization; higher chemical reactivity; higher electrical conductivity, good biocompatibility and the possibility of synthesis with minimal cost using graphite as raw material and without introducing metallic impurities. In addition, its electrocatalytic action diminishes the overpotential associated to the reduction and/or oxidation of many electroactive compounds, minimizing the interferences that occur in real samples [4]. As electrode modifiers both graphene oxide (GO) and reduced graphene oxide (RGO) have been used. There are numerous studies stating that the GO, having a large specific surface area and numerous functional groups, is an ideal substrate for immobilizing enzymes [5,6]. However, recent studies have shown that the immobilization of enzymes using the electrostatic interaction as the driving force for enzyme binding to GO severely affected the activity of the enzyme and also the stability of enzymatic electrode is not good enough for practical applications. Also, an inherent disadvantage is the lack of GO electrical conductivity. Most of the electrochemical biosensors based on graphene materials reported in the literature have been developed for the glucose detection and used chemically reduced graphene oxide as a nanomaterial. The chemical reduction method has some disadvantages, such as contamination of the resulting product and the difficulty to obtain stable aqueous dispersions [7].

Electrochemically reduced graphene oxide (ERGO) is a promising candidate for enzyme immobilization. It was proved that the interaction between the enzyme and immobilization matrix is a critical factor for the amount of surface-bound enzyme, enzyme activity and stability [8]. The electrochemical method is a fast and environmentally friendly process that allows both preparation of a product without contaminants, but also a good control over its degree of reduction and its direct deposition on the surface of the electrode.

Biosensors based on glucose oxidase (GOx) for glucose sensing continue to be the primary model system in the development of new sensing materials and methods [13,14]. GOx contains a flavin group (FAD) redox center, which catalyzes the oxidation of glucose to glucolactone. The glucose concentration can be determined by measuring the oxidation current of hydrogen peroxide (H_2O_2), a side product of the enzymatic reaction.

In most graphene-based electrochemical sensors, GO-modified electrodes have been fabricated by drop casting GO dispersions onto the surface of various electrode materials such as Pt [9], Au [10] and glassy carbon (GC) [11]. In our approach, a reduced graphene oxide (RGO) film is first deposited on the electrode, by direct electrochemical reduction of graphene oxide at the glassy carbon surface. In order to facilitate the oxidation of the enzymatically generated H_2O_2, a second step consists in the electrochemical deposition of Pt nanoparticles on the ERGO/GC electrodes, as previous studies have shown that the oxidation of

H_2O_2 is favored on oxidized Pt surfaces [12]. The third step consists of the functionalization of Pt/ERGO/GC with carboxyphenyl groups by the electrochemical reduction of the corresponding aryl diazonium salt. The carboxyl functionalities allow the covalent attachment of GOx using standard carbodiimide chemistry.

The present work describes the construction and application of ERGO-based glucose biosensors which combine the advantageous features of graphene and Pt nanoparticles in order to achieve a high sensitivity. Furthermore, the covalent attachment of glucose oxidase is employed as a means to increase the stability of the biosensors.

2. Experimental

Chemicals and reagents

Graphene oxide (aqueous dispersion, 2 mg/mL) was purchased from Aldrich. Potassium hexacyanoferrate (III), potassium chloride, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma–Aldrich. Anhydrous acetonitrile (99.8%, noted MeCN) and tetra-*n*-butylammonium tetrafluoroborate (99%, noted $TBABF_4^-$) were obtained from Aldrich and were used as received. Glucose oxidase from *Aspergillus Niger*, 200U/mg (GOx) was purchased from Sigma.

4-carboxyphenyl diazonium tetrafluoroborate (HOOC-ArTFB) was prepared by standard diazotation of the corresponding amine with $NaNO_2$ in fluoroboric acid medium as described in our previous studies [15,16].

Ferrocenemethylamine hydrochloride was synthesized according to Ref. [17].

Phosphate buffer solutions (0.1 M) of pH 7.4 were employed as supporting electrolyte in the biosensor testing.

Instrumentation

The electrochemical experiments were performed at room temperature and were controlled by an Autolab 128N potentiostat/galvanostat (Eco-Chemie). A three-electrode configuration consisting of bare or modified glassy carbon (GC) (Metrohm, disks, diameter *2* mm) as working electrodes, and a Pt wire as counter electrode were used. As reference electrode, a saturated calomel electrode (SCE) was employed in aqueous solutions and the Ag/10 mM $AgNO_3$, 0.1 M $TBABF_4^-$ reference electrode in non-aqueous solutions, respectively.

Preparation of the modified electrodes

Prior to modification, the electrode surface was polished in 0.05 μm alumina slurry on a microcloth pad. After polishing the electrodes were

thoroughly rinsed with water and sonicated for 5 min. in water and then rinsed again with water. Electrochemical reduction of GO at GC electrodes (usually from 0.5mg/mL GO aqueous solutions, in phosphate buffer pH = 7.4) was performed by cyclic voltammetry (scan rate of 100 mVs^{-1}). Best results in terms of sensor sensitivity were obtained for at least 20 cycles of GO electroreduction, but reproducible results have been obtained for 40 reduction cycles.
Pt nanoparticles deposition at ERGO/GC modified electrodes was done using the procedure reported in ref. [17], by pulse deposition (potential pulse sequence: −1.0 V/0.2 s; 0.0 V/1 s, 200 repetitions) from 1 mM hexachloroplatinic acid in water.

The functionalization of the ERGO and Pt/ERGO electrodes with carboxyphenyl groups was carried out in MeCN solutions containing 5 mM of diazonium salt and 0.1M $TBABF_4^-$ supporting electrolyte, by potential cycling between 0 and -1 V for 1÷3 cycles, at a scan rate of $0.1Vs^{-1}$. Afterwards, modified electrodes were removed from the grafting solution and rinsed with large volumes of MeCN and then water. The electrodes were then imersed in an aqueous solution containing 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 20 mM N-hydroxysuccinimide (NHS) for 3 h in 0.1M phosphate buffer pH 5.5, in order to activate –COOH groups. After the activation, electrodes were rinsed with water and incubated in a 2 mg/mL GOx solution in phosphate buffer pH 7.4 for 24 h. If not used immediately, the electrodes were stored at 4 °C in 0.1 M phosphate buffer solution (pH 7.4).

3. Results and Discussion

Electrochemical reduction of graphene oxide (GO) at glassy carbon (GC) electrodes

In Fig 1 is presented the electrochemical reduction of GO by potential cycling between 0 and −1 V ($0.1\ Vs^{-1}$) at GC electrodes from a 0.5mg/mL GO dispersion in phosphate buffer (pH = 7.4). In the first potential cycle, the reduction peaks of GO are clearly distinguishable at approx. −0.7 V and −1.0 V. The capacitive current increases after the first five cycles and stabilizes after the 40^{th} cycle. The increase of the capacitive current is due to the increase of the electrode's active area when a conducting reduced graphene oxide film is gradually deposited on the surface.

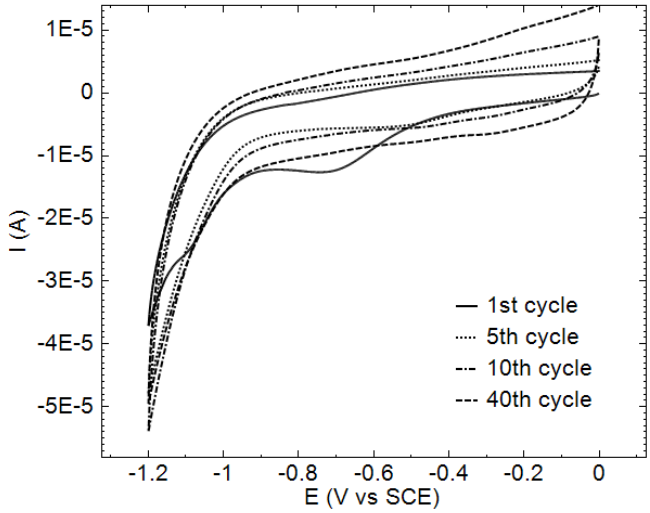

Fig. 1. CVs depicting the electrochemical reduction of GO at a GC electrode (40 cycles, scan rate 0.1V/s).

Electrochemical reduction of 4-carboxyphenyl diazonium tetrafluoroborate at ERGO/GC electrodes

The electrodes modified with the electrochemically reduced graphene oxide (ERGO) were further functionalized with carboxyphenyl (HOOC-Ar) groups *via* electrochemical reduction of 4-carboxyphenyl diazonium tetrafluoroborate (1 mM) in MeCN with 0.1M $TBABF_4^-$ as supporting electrolyte. The voltammograms (scan rate of 0.1 V s^{-1}) recorded for the surface derivatization are presented in Fig. 2. The first sweep showed two characteristic reduction peaks at −0.2 V and −0.55V with no associated oxidation peak indicating the loss of N_2 and the formation of aryl radicals which bind to the ERGO surface. Subsequent scans showed no electrochemistry, indicative of a passivated electrode. The partial blocking of the ERGO/GC surface after the modification with aryl diazonium salts was confirmed by CV using potassium ferricyanide as a redox probe (Fig. 3).

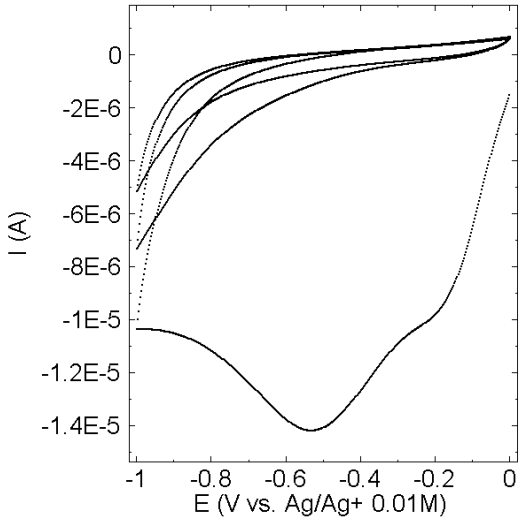

Fig. 2. Successive CVs depicting the electrochemical reduction of 4-carboxyphenyl diazonium tetrafluoroborate (5 mM in 0.1 M $TBABF_4^-$/MeCN) at ERGO electrodes, by cycling in the potential range of (0; -1) V, at a scan rate of 0.1 Vs^{-1}.

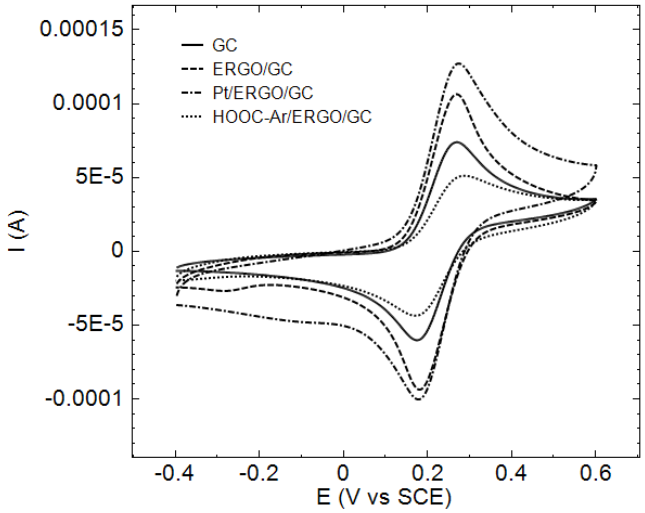

Fig. 3. CVs of bare GC, ERGO/GC, Pt/ERGO/GC and HOOC-Ar/ERGO/GC electrodes in 5 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl solution, at a scan rate of 0.1 Vs^{-1}.

Electrochemical impedance spectroscopy (EIS) measurements, performed at 0.21V vs. SCE, for the bare GC, ERGO/GC and carboxyphenyl-modified ERGO/GC electrodes are shown in Fig. 4. The Nyquist plot of electrochemical impedance spectra consists of two sections: a semicircle part in high frequency domain which reflects an electron transfer limited process at the electrode surface and a linear part in the low frequency domain corresponding to a diffusion limited

process. The electron transfer resistance R_{ct} can be fitted as the semicircle diameter. It is obvious that R_{ct} values of ERGO/GC modified electrodes were negligible when compared to those of bare GC electrode, suggesting that the conductivity of ERGO film is much better than that of GC. On the other hand, after the functionalization with carboxyphenyl groups was performed, the corresponding R_{ct} increased slightly indicating the presence of a grafted layer that lead to a slight hindrance of the electron transfer process. These tests gave strong evidence of a layer which partially blocked access of ferricyanide to the electrode surface.

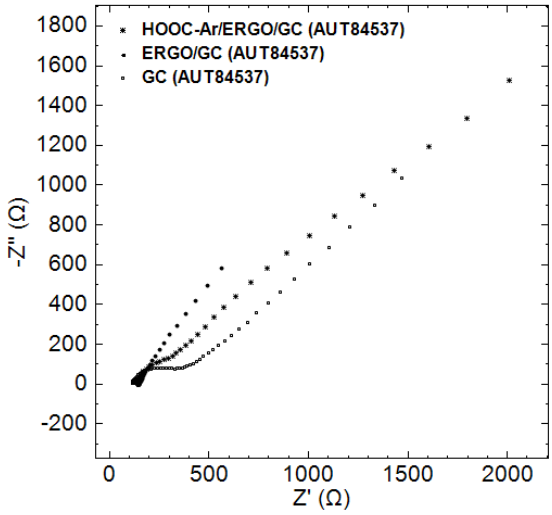

Fig 4. Nyquist plots of bare GC, ERGO/GC and HOOC-Ar/ERGO/GC modified electrodes in 5 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl solution. The frequency range is from 0.1 Hz to 100 kHz.

ERGO electrode modification with Pt nanoparticles

In order to improve the biosensor performance, Pt nanoparticles were deposited on the ERGO/GC electrode. Pt nanoparticles deposition was done by pulse deposition (potential pulse sequence: −1.0 V/0.2 s; 0.0 V/1 s, 200 repetitions) from a 1 mM hexachloroplatinic acid aqueous solution following the procedure reported by Andronescu et al. [18]. The CVs performed in a 5 mM ferricyanide solution on ERGO electrodes after the modification with Pt nanoparticles showed an increase of the redox peaks, suggesting a facilitated access of the ferricyanide redox couple to the electrode surface (Fig. 3). Performing the electrochemical grafting of carboxyphenyl groups at Pt/ERGO/GC electrodes as described above resulted in a shift of the characteristic diazonium reduction peak to −0.3 V (Fig. 5), probably due to the Pt nanoparticles deposition on the electrode surface.

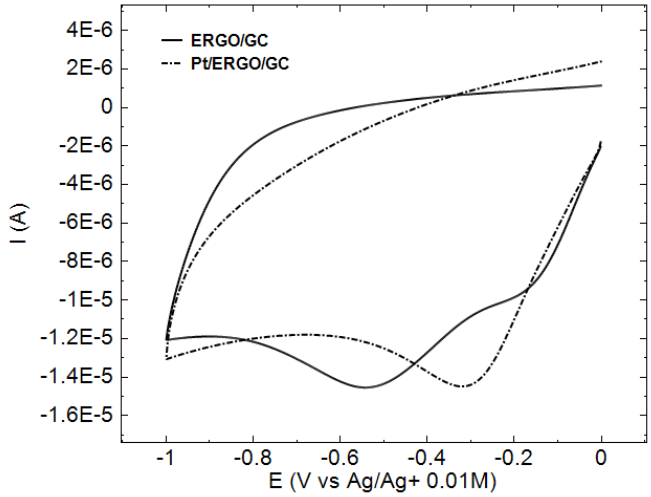

Fig. 5. The electrochemical reduction (1st cycle) of 4-carboxyphenyl diazonium tetrafluoroborate (5 mM in 0.1 M $TBABF_4^-$/MeCN) at ERGO and Pt/ERGO electrodes by cycling in the potential range of (0; -1) V, at a scan rate of 0.1 Vs^{-1}

Covalent attachment of GOx by EDC-NHS coupling

After the functionalization of ERGO and Pt/ERGO electrodes with carboxyphenyl groups, the next step consisted of the covalent attachment of glucose oxidase (GOx). The modified surfaces were immersed in an aqueous solution of 10 mM EDC and 20 mM NHS for 3 h in 0.1M phosphate buffer (pH 5.5) in order to activate –COOH groups. After the activation, the electrodes were rinsed with water and incubated in a 10mg/mL GOx solution in phosphate buffer pH 7.4 for 24 h.

In order to demonstrate the covalent linkage of amines by using EDC-NHS protocol at carboxyphenyl modified ERGO/GC electrodes, the attachment of ferrocene groups at these electrodes has been tested because their presence can be evidenced by a pronounced redox signal in CV experiments. Covalent attachment of ferrocenemethylamine to carboxylic acid terminated aryl groups followed the procedure described in the literature [19]. The carboxyphenyl modified ERGO electrodes were activated with EDC and NHS as above, followed by immersion in a 5 mM ferrocenemethylamine hydrochloride solution (phosphate buffer, pH 7.4) for 24 h. CVs measured in an aqueous solution of 0.1 M phosphate buffer at different scan rates ($10mVs^{-1}$ ÷ 200 mVs^{-1}) for the modified electrodes after the immobilization of ferrocenylmethyl groups (Fc/ERGO/GC) are shown in Fig. 6(A). The well defined redox peaks showing a linear variation in peak current with scan rate (Fig. 6(B)) indicate that the ferrocene was surface bound. Without activation by EDC and NHS, only very poor defined redox peaks due to physisorption were observed (results not shown). Also, the CVs of the Fc/ERGO/GC electrodes showed almost ideal behaviour with regards to peak

separation at slow scan rates (ΔEp = 20 mV) and a very small variation of the redox peak potentials with the scan rate.

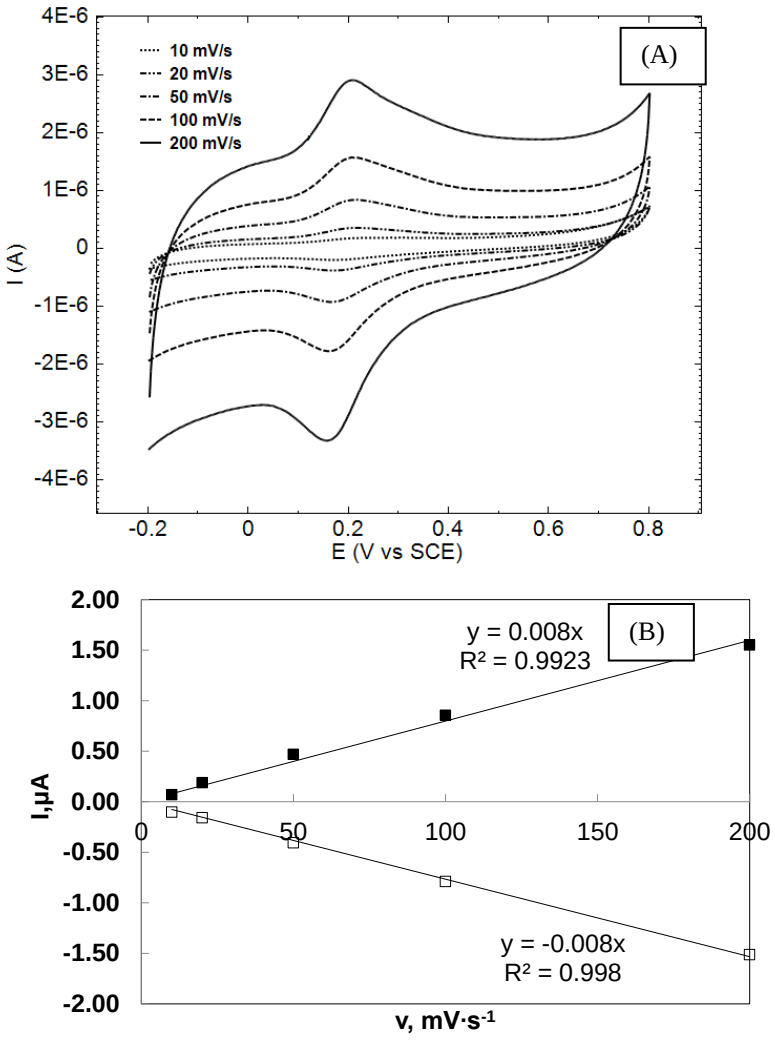

Fig. 6. (A) Cyclic voltammograms of Fc/ERGO/GC electrode in 0.1M phosphate buffer, pH 7.4, at different scan rates. Potentials are referred vs. SCE electrode. (B) Cathodic and anodic peak currents vs. scan rate.

Biosensor testing

Amperometric measurements were performed in stirred 0.1 M phosphate buffer solution (pH 7.4) by injecting different volumes of 10 mM, 0.1 M and 0.5 M glucose solution after baseline stabilization at the applied potential. The amperometric responses of the GOx-ERGO/GC and GOx-Pt/ERGO/GC electrodes were tested at a potential of 0.6V. The typical calibration curve of the

biosensor based on GOx-Pt/ERGO/GC electrode is linear with glucose concentration up to 6 mM and then a plateau is reached gradually at higher glucose concentration (up to 50mM). The GOx-Pt/ERGO/GC electrode has a detection limit of 0.02 mM, comparatively higher than the GOx-ERGO/GC biosensor (0.2 mM), and has a larger response current.

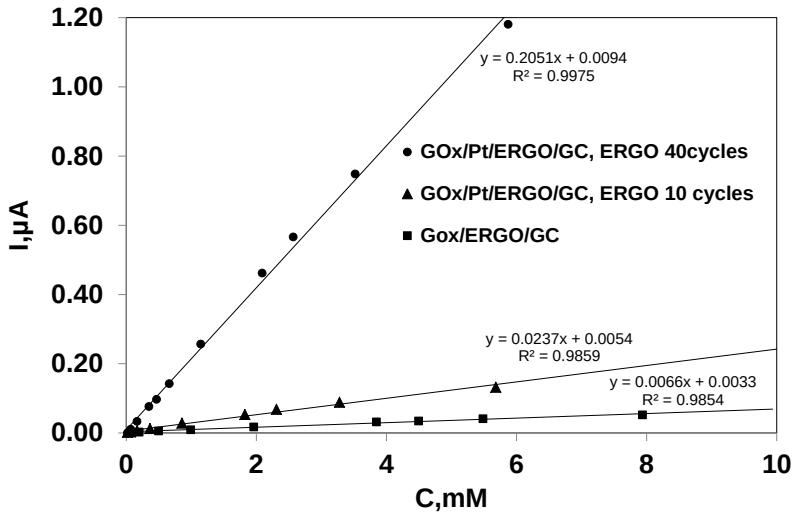

Fig. 7. Calibration plots (linear region) at 0.6V for GOx-ERGO/GC and GOx-Pt/ERGO/GC modified electrodes.

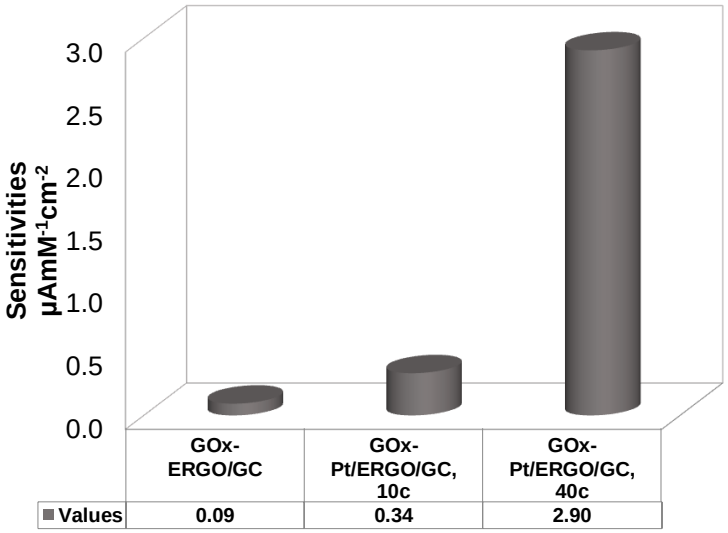

Fig. 8. Biosensor sensitivities at the operating potential of 0.6 V.

6. Conclusions

This study establishes a new approach for glucose detection and provides a general route for the fabrication of graphene-based biosensing platforms. The covalent attachment of GOx at the carboxyphenyl-functionalized ERGO surface has been successfully achieved using a carbodiimide protocol. The biosensor sensitivity was further enhanced after the deposition of Pt nanoparticles on the ERGO surface. By combining the advantageous features of Pt nanoparticles and graphene, we have developed glucose biosensors with good analytical performances in terms of high sensitivity, wide linear range and improved stability.

Acknowledgements

The work has been funded by the Sectorial Operational Program Human Resources Development 2007-2013 of the Ministry of European Funds through the Financial Agreement POSDRU/159/1.5/S/134398.
The authors gratefully acknowledge the financial support of the Romanian National Authority for Scientific Research, CNCSUEFISCDI, under grant PN-II-PT-PCCA-2013-4-2151 number 236/2014.

R E F E R E N C E S

[1]. *V. Georgakilas, M. Otyepka, A. B. Bourlinos, V. Chandra, N. Kim, K. C. Kemp, P. Hobza, R. Zboril, and K. S. Kim*, "Functionalization of Graphene: Covalent and Non-Covalent Approaches, Derivatives and Applications" Chem. Rev., **vol. 112**, 2012, pp. 6156–6214.
[2]. *C.K. Chua, and M. Pumera,* "Covalent chemistry on graphene", Chem. Soc. Rev., **vol. 42**, no. 8, 2013, pp. 3222– 3233.
[3]. *M. Pumera, A. Ambrosi, A. Bonanni, E.L.K.Chng, and H.L. Poh*, "Graphene for electrochemical sensing and biosensing", Trends Anal. Chem., **vol. 29**, 2010, pp. 954–965.
[4]. *T. Gan, S. Hu*, "Electrochemical sensors based on graphene materials", Microchim. Acta, **vol. 175**, 2011, pp. 1–19.
[5]. *J. Zhang, F. Zhang, H. Yang, X. Huang, H. Liu, J. Zhang, and S. Guo,* "Graphene Oxide as a Matrix for Enzyme Immobilization", Langmuir, **vol. 26**, 2010, pp.6083 – 6085.
[6]. *K. Wang, J. Ruan, H. Song, J. Zhang, Y. Wo, S. Guo, and D. Cui*, "Biocompatibility of graphene oxide", Nanoscale Res. Lett., **vol. 6**, no. 8, 2011, pp. 1-8.
[7]. *N. Behabtu, J. R. Lomeda, M. J. Green, A. L. Higginbotham, A. Sinitskii, D. V. Kosynkin, D. Tsentalovich, A. Parra-Vasquez, J. Schmidt, E. Kesselman, Y. Cohen, Y. Talmon, J. M. Tour and M. Pasquali*, "Spontaneous high-concentration dispersions and liquid crystals of graphene" Nature Nanotechnol., **vol. 5**, 2010, pp. 406-411.
[8]. *Y. Fang and E. Wang,* „Electrochemical biosensors on platforms of graphene", Chem. Commun., **vol. 49**, no. 83, 2013, pp. 9526-9539.
[9] *Y. Liu , D. S. Yu , C. Zeng , Z. C. Miao , and L. M. Dai*,"Biocompatible graphene oxide-based glucose biosensors" Langmuir, **vol. 26** , 2010 , pp. 6158–6160.

[10] *C.S. Shan, H.F. Yang, D.X. Han, Q.X. Zhang, A. Ivaska, and L. Niu*, "Graphene/AuNPs/chitosan nanocomposites film for glucose biosensing", Biosens. Bioelectron., **vol. 25** , 2010, pp. 1070-1074.

[11] *Z. J. Wang, J. Zhang, Z. Yin, S. X. Wu, D. Mandler, H. Zhang*, "Fabrication of Nanoelectrode Ensembles by Electrodepositon of Au Nanoparticles on Single-Layer Graphene Oxide Sheets", Nanoscale, **vol. 4**, 2012, pp. 2728-2733.

[12]. *S.B.Hall, E.A.Khudaish, A.L.Hart*, Electrochemical oxidation of hydrogen peroxide at platinum electrodes. Part 1. An adsorption-controlled mechanism, Electrochim. Acta, **vol. 43**, no. 5, 1998, pp. 579-588.

[13]. *J. Wang, M. Musameh, and J.W. Mo*, "Acid stability of carbon paste enzyme electrodes", Anal. Chem., **vol. 78**, 2006, pp. 7044–7047.

[14]. *M. Raicopol and L. Pilan*, "Highly selective and stable glucose biosensors based on polyaniline / carbon nanotubes composites**"** U.P.B. Sci. Bull., Series B, **vol. 76**, no. 1, 2014, pp. 155-166.

[15]. *M. Raicopol, L. Necula, M. Ioniță and L. Pilan,* "Electrochemical reduction of aryl diazonium salts: a versatile way for carbon nanotubes functionalisation", Surf. Interface Anal., **vol. 44**, 2012, pp. 1081–1085.

[16]. *L. Pilan, M. Raicopol, A. Prună, and V.Branzoi*, "Polyaniline/carbon nanotube composite films electrosynthesis through diazonium salts electroreduction and electrochemical polymerization", Surf. Interface Anal., **vol. 44**, 2012, pp. 1198–1202.

[17] *H.-B. Kraatz*, "Synthesis and electrochemistry of ferrocenemethylamine and its conjugated acid. Crystal structure of ferrocenemethylammonium chloride", J. Organomet. Chem. **vol. 579**, 1999, pp 222-226.

[18]. *C. Andronescu, S. Pöller, W. Schuhmann*, „Electrochemically induced deposition of poly(benzoxazine) precursors as immobilization matrix for enzymes", Electrochem. Commun., **vol. 41**, 2014, pp. 12–15.

[19]. *J. Liu, J.J. Gooding, M.N. Paddon-Row*, "Unusually rapid heterogeneous electron transfer through a saturated bridge 18 bonds in length" Chem. Commun., **vol. 5**, 2005, pp. 631-633.