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Investigation of carbon nanofibers as support for bioactive substances

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Abstract In this paper we have studied the adsorption properties of various bio-active systems onto the surface of carbon nanofibers (CNF) synthesized by chemical vapor deposition (CVD). Amino acids (alanine, aspartic acid, glutamic acid) and glucose oxidase (GOx) were adsorbed on CNF and the results were compared with those obtained when activated carbon (AC) was used as support. CNF and AC properties (hydrophilic or hydrophobic properties) were characterized by the pH value, the concentration of acidic/basic sites and by naphthalene adsorption. CNF with immobilized GOx was additionally investigated as a highly sensitive glucose biosensor. An amperometric method was used in an original manner to detect the changes in the specific activity of GOx, immobilized longer time on CNF. The method demonstrates that not the whole enzyme adsorbed onto CNF can catalyze the oxidation of glucose from the solution.

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1 Introduction

Carbon nanostructures [1–4] due to their unique properties have been attracting remarkable interests as potential adsorbents-supports [5], material for biosensors [6] and nanobiotechnologies [7, 8]. Carbon nanostructures, used as electrode materials, have many advantages that include good electrical conductivity, a wide potential range, chemical inertness, enhanced electron transfer in electrochemical reactions, and high sensitivity.

Carbon nanostructures were shown to allow functionalization with different bio-molecules such as peptide, protein, and DNA while protein immunosensors were previously made by attaching antibodies to the carboxylated ends of nanotube forests [9–14]. As support for bioactive substances carbon nanostructures have some advantages like small size with large surface area and easy protein immobilization with retention of enzymatic activity.

Moreover, previously other types of carbon nanostructures such as single and multi wall carbon nanotubes were used for biological conjugation with proteins, antibodies, and DNA. In this sense, a major development was reported when highly sensitive SWNT-FET devices, have been achieved for the detection of protein adsorptions and specific protein–protein interactions [15]. Externally, the carbon nanostructures were chemically derivatized to make them biocompatible and cell-specific through peptide and antibody targeting. Carbon nanotubes paste electrodes were shown to be suitable for adsorptive stripping potentiometric measurements of trace levels of nucleic acids [16].

The present contribution describes the investigation of the novel adsorption properties [17] of carbon nanofibers (CNF) produced in our laboratory, for some bio-molecules like: alanine, aspartic acid, glutamic acid, and glucose

oxidase. Alanine is a non-polar molecule while aspartic and glutamic acids are polar, acidic amino acids. All the adsorption experiments over the CNF surfaces were performed in parallel with activated carbon (AC) for comparison. For all the experiments the nanofibers were formed as a solid block material comprising an entangled network of CNF.

The work comprises two major parts. In the first one, the adsorption of bio-active substances (various enzymes) onto the surface of CNFs is presented, while the second one is a novel application of complex molecules GOx, which are composed of many aminoacids, adsorbed onto CNF materials for the detection of glucose. The novel results of the amino acids adsorption onto carbon nanomaterials can result in the development of advanced amperometric sensors for the detection of heavy metals.

Additionally, we have built a glucose biosensor by co-immobilization of GOx and redox mediator on CNFs [18]. The activity of immobilized GOx was determined by an original amperometric method.

2 Experimental

2.1 Chemicals and apparatus

Alanine, aspartic acid, glutamic acid, HCl, and NaOH were obtained from Merck; GOx, D(+)-glucose and ninhydrine were obtained from Sigma while Coomassie Blue G-250 was bought from Fluka; activated charcoal for data comparison was purchased from Pierce Chemical Company, Rockford, Illinois, USA.

Electrochemical experiments were performed with a three electrode system (Beckman potentiostat). An Ag/AgCl and a platinum electrode were used as reference and auxiliary electrode, respectively, while working electrode was made from CNF paste.

2.2 Preparation of CNFs

Carbon nanofibres were prepared by chemical vapor deposition method (CVD) using Fe/Ni/Cu (85:10:5) catalyst (Fig. 1) [19]. After preparation, they were purified in 37% HCl, carefully washed in double distilled water and dried at 120°C. Transmission electron microscopy showed a "herringbone" structures. The specific area was determined by BET method and was found to be 170 $m^2\ g^{-1}$.

The pH of carbon supports (CNF or AC) was measured with a Philips ion-meter, using the following procedure: about 0.01 g of dry CNF was added to 10 ml of distilled water, then the suspension was shaken overnight to reach equilibrium and the pH of the solution was measured.

Hydrophilic and hydrophobic properties of CNF were characterized by the concentration of acidic and basic sites of different strength on the surface of the support, respectively, by naphthalene adsorption from aqueous solution [5].

Acidic and basic sites were determined by Boehms titration method [20]. In the case of acidic values, about 0.01 g of CNF was added to a beaker containing 10 ml of 0.02 N NaOH solution. The beaker was shaken for 24 h under purging N_2 and then the excess of base was titrated with 0.02N HCl. The acidic value was calculated from the amount of HCl that reacted with NaOH. The basic value was determined by converse titration.

Hydrophobicity of the support was expressed in nmol of adsorbed naphthalene/m^2 of accessible surface area [5]. About 5.9 mg of CNF (specific area 1 m^2) was kept under the vacuum and then this amount was mixed with 2 ml of 0.1 mM naphthalene, for 2 h. The concentration of naphthalene was determined by UV spectroscopy at $\lambda = 225$ nm.

2.3 Adsorption of amino acids

Adsorption of amino acids was performed from solutions of 10^{-5} to 10^{-3} M amino acids in distilled water. The amino acids were adsorbed at ambient temperature for 24 h, under constant stirring.

The ratio between the adsorbent weight (in g) and the solution volume (in ml) was 1–100. The amino acids concentration was determined by ninhydrin method and measured spectrophotometrically at $\lambda = 575$ nm. The amount of adsorbed amino acids was calculated from the difference of its concentration in solution before and after adsorption and expressed in mg/g of support.

2.4 Adsorption of enzyme

The adsorption of enzyme was performed under similar conditions with those used for amino acids, but the pH of the enzyme solution was pH 7.4. The concentration of GOx was determined by Bradford method, using Coomassie Blue G-250 as reagent [21].

The amount of enzyme adsorbed on CNF surface was calculated from the difference of its concentration in solution before and after adsorption and expressed in mg/g of support.

2.5 Fabrication of glucose biosensor

Amperometric enzyme-based bioelectrodes [6] are suitable for in vitro monitoring of glucose concentration. The second-generation glucose biosensors have used redox

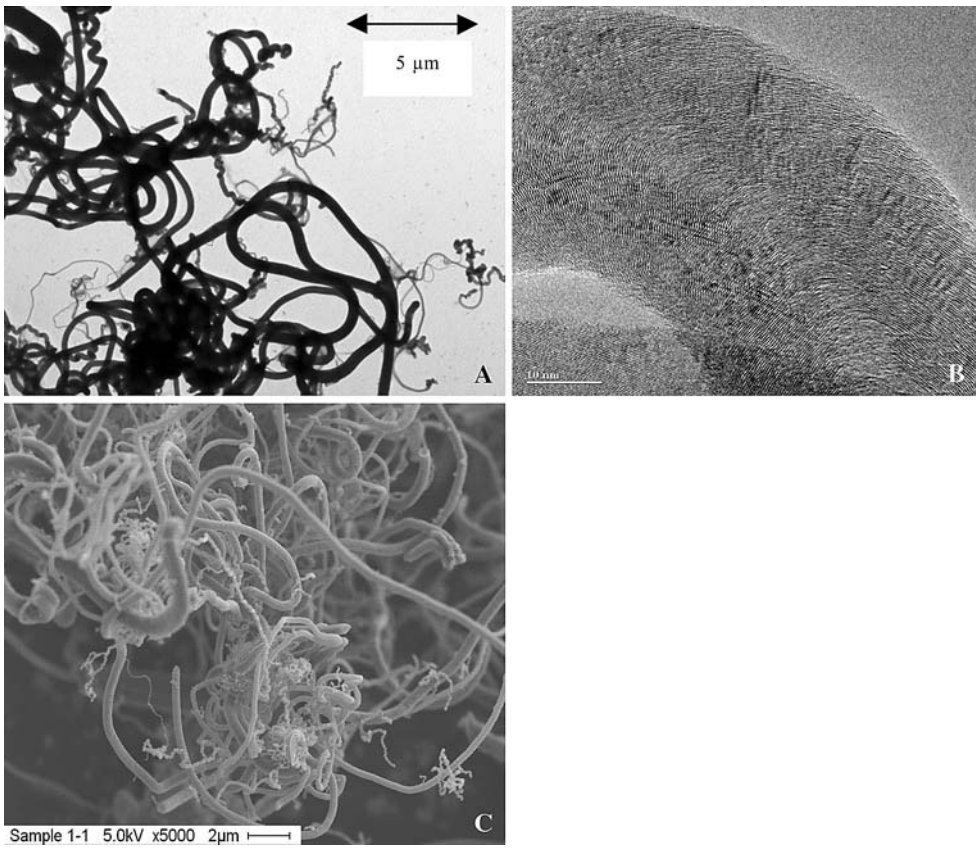

Fig. 1 (**a**) TEM images of CNF (from ethylene at 600°C on Fe/Ni/Cu (85:10:5) as catalyst; (**b**) HRTEM images of CNT; (**c**)-SEM images of CNT

mediators and which have a high insensitivity to oxygen fluctuations. By the method presented in this contribution, the amperometric experiments can be performed at lower potentials, where the interfering reactions do not take place. The enzymatic reaction is:

$$\text{Glucose} + \text{GOx}\,(\text{FAD}^+) \rightarrow \text{Gluconolactone} + \text{GOx}\,(\text{FADH}_2) \quad (1)$$

$$\text{FADH}_2 + 2\text{Med}_{(\text{ox})} \rightarrow \text{FAD}^+ + 2\text{Med}_{(\text{red})} + 2\text{H}^+ \quad (2)$$

$$2\text{Med}_{(\text{red})} \rightarrow 2\text{Med}_{(\text{ox})} + 2\text{e}^- \quad (3)$$

where FAD/$FADH_2$ is the cofactor of enzyme, while $Med_{(ox)}$ and $Med_{(red)}$ are the oxidized and reduced forms of the mediator, respectively.

A simple procedure was used for the fabrication of glucose biosensor. Typically 10 mg of CNFs were mixed with 1 mg of GOx and 3 μl of $K_4[Fe(CN)_6]$ (10^{-3} M dissolved in 0.1 M PBS pH 7.4). The paste was let to dry in the air, at room temperature, for about 15 min. After that, a drop of silicon oil was added and mixed with the paste that was introduced into a cylindrical cavity, at the end of a PVC rod. The electrical contact was ensured by a platinum wire. The surface was subsequently rinsed with deionised water, in order to remove the enzyme that was not properly adsorbed on CNF surface.

The amperometric experiments were performed at a potential of +0.4 V vs. Ag/AgCl (as the one presented in the cyclic voltammetry of Fig. 2) in solution of 0.1 M PBS pH 7 +0.5 M KCl. The mediator $K_4[Fe(CN)_6]$ was introduced in the sensor formed as a paste of CNF. The sensor has linearly responded to glucose, in the concentration range of 1.7–5 mM. The time required to reach 95% of the maximum steady-state current was around 30 s.

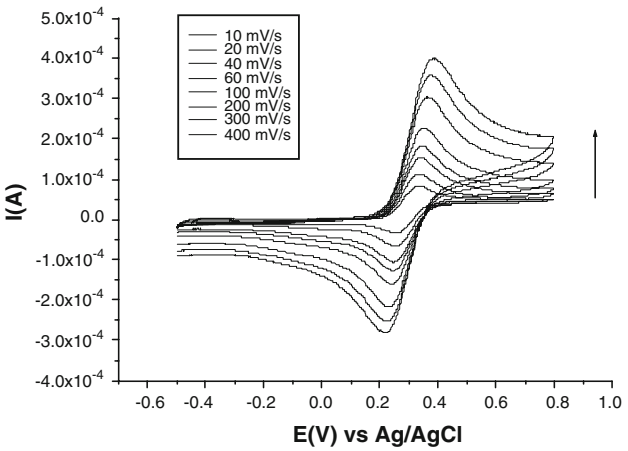

Fig. 2 Cyclic voltamogramme for glucose biosensor in 10^{-2} M $K_4[Fe(CN)_6]$ in 0.1 M phosphate buffer + 0.5 M KCl

3 Results and discussion

The surface of the samples (CNF and AC) was weakly acidic due to its origin, preparation, and method of activation. The results obtained from acid/base titration were in general good agreement with the pH values. The surface acidity/basicity of CNF is fourth/twice times greater than that of AC while the concentration of acidic sites on the CNF and AC is several times lower than that of basic sites. CNF is more hydrophobic than AC as can be seen from Table 1.

In Table 2 are presented the values of maximum theoretical monolayer coverage, n_m and affinity constant, K, which measures the affinity between interacting adsorbates and surface (calculated from Langmuir model of adsorption isotherms). The Langmuir equation is expressed as follows:

$$n = \frac{n_m K c}{1 + Kc}$$

where K = Langmuir equilibrium constant, c = aqueous concentration, n = amount adsorbed, and n_m = maximum amount adsorbed as c increases. The Langmuir equation can be optimized by linear regression and nonlinear regression methods.

Data obtained experimentally were fitted into the Langmuir adsorption isotherm. The correlation coefficients (R^2) obtained for Langmuir equations were in the range of 0.84–0.98. From the adsorption isotherms (Figs. 3, 4) one can see that the adsorption of amino acids onto CNFs increases from alanine to aspartic acid (alanine < aspartic acid). Glutamic acid was not found to obey the Langmuir equation. Glutamic acid has been very weakly adsorbed due to the presence of an additional methylene group that increases its hydrophobicity. The adsorption isotherm is not a type I but a type III, case for which the latent heat (the binding energy between the molecules of the adsorbent) is close to the adsorption energy of the first monolayer. For small concentrations the adsorption is very weak bit for large concentrations several layers are formed.

When activated carbon was used as substrate, the adsorption of amino acids increased from aspartic acid to alanine and to glutamic acid (aspartic acid < alanine < glutamic acid). AC support is less hydrophobic compared with CNT. Therefore, the supplementary methylene group in glutamic acid do not present such a strong influence anymore and its adsorption takes place hydrophilically, being better adsorbed comparing with alanine because it has more hydrophilic groups.

GOx was also adsorbed on CNF and AC. In comparison with CNF, the adsorption process on AC does not obey the Langmuir equation. This means that the intermolecular interactions between adsorbat molecules are stronger than interaction between the adsorbat molecules and support.

In Table 2 are presented maximum theoretical coverage (n_m) and affinity constant (K) calculated from Langmuir equation (exceptions are glutamic acid and GOx, whose adsorption on CNF and AC, respectively, do not obey the Langmuir equation—Figs. 3c, 4b).

The filamentous morphology of CNF is responsible for the greater stability of adsorbed enzyme compared with the enzyme used directly in solution.

3.1 Detection of enzyme activity immobilized on CNF

Our purpose was to highlight the evolution of the glucose biosensor, due to the fact that GOx's enzymatic activity usually decreases in time. We have prepared six biosensors using the same amount of GOx (1 mg). For the first series of three biosensors the calibration function was recorded immediately after preparation while for the second series of three biosensors it was recorded a year later (Fig. 5). The initial enzymatic activity was found to be 157 U/mg.

The decrease in time of the enzymatic activity was determined with the second series of biosensors, using the following procedure. First the calibration curve was recorded until the electrical signal reached saturation (the glucose concentration was about 0.8 M—Fig. 6a). In that moment no glucose was added to the solution and the electrical signal decreased in time, due to glucose consumption by GOx (Fig. 6b).

Using the data in Figs. 6a, b we have determined the glucose variation in concentration and the glucose consumption, in 1 min. From these data, we have calculated the enzymatic activity. A current decrease from 16.5 to

Table 1 pH, hydrophilic, and hydrophobic properties of CNF and AC

Sample	BET surface m^2/g	pH	Acidic values meq/g	Basic values meq/g	Naphthalene adsorption $nmol/m^2$
CNF	170	6.20	0.15	0.6	51.17
AC	1400	6.52	0.04	0.28	27.8

Table 2 Parameters n_m and K calculated from Langmuir adsorption isotherms

Adsorbent	Alanine n_m, mg g^{-1}	K	Aspartic acid n_m, mg g^{-1}	K	Glutamic acid n_m, mg g^{-1}	K	GOx n_m, mg g^{-1}	K
CNF	0.095	150	1.04	8	–	–	10	0.83
AC	0.088	56.5	0.037	382	0.17	44.6	–	–

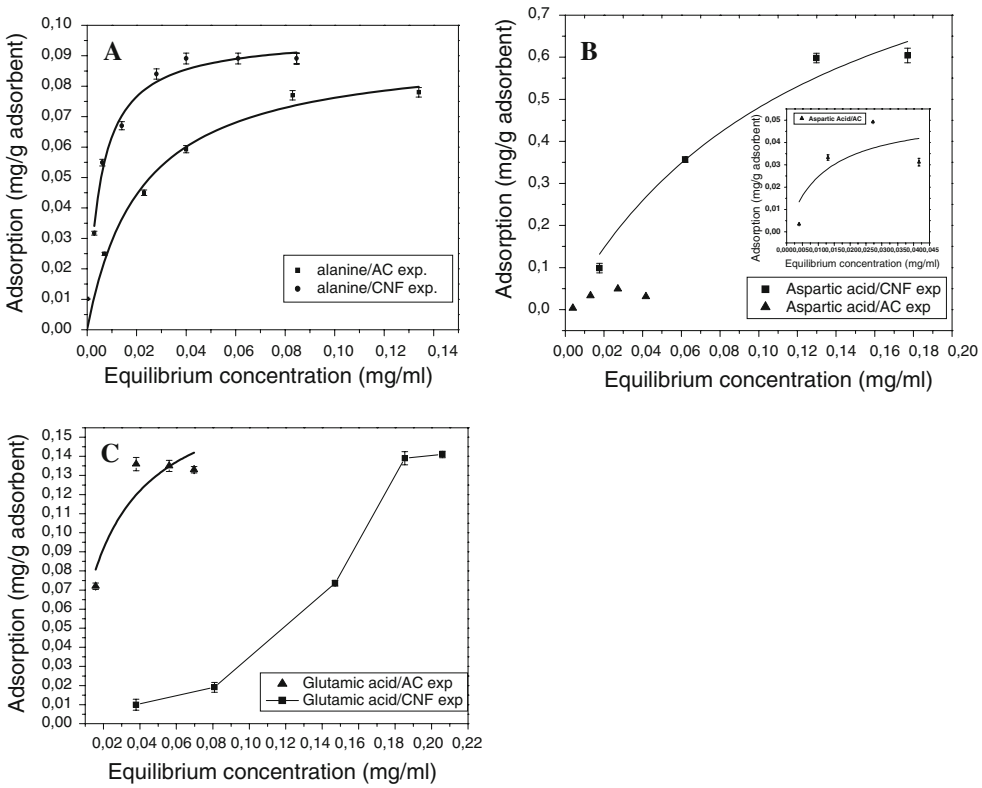

Fig. 3 The adsorption isotherms of alanine (**a**), aspartic acid (**b**), and glutamic acid (**c**) on CNF and AC (error bars represent the standard deviation of the mean for five samples)

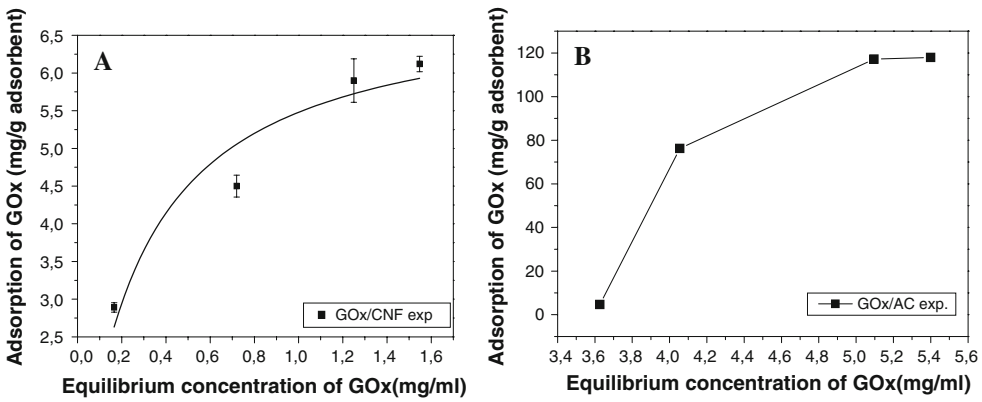

Fig. 4 The adsorption isotherms of GOx on CNF (**a**) and AC (**b**) (error bars represent the standard deviation of the mean for five samples)

12.21 μA (Fig. 6b) is equivalent to a concentration decrease from 0.8 to 0.6 mM (Fig. 6a). This means that an amount of 10^{-5} mole of glucose was oxidized in 1 min (in 50 ml solution). The activity was determined using the enzymatic activity definition. In our case, the specific activity was 5 IU/mg for the enzyme immobilised on CNF.

We have used the second series of biosensors in another experience. After the electrical signal reached saturation (Figs. 5, 6a) we have added in the reaction cell a certain amount of enzyme (11 mg). The specific activity was determined by taking into consideration the decrease of the current, from 30 to 14.85 μA. From these graphs we have determined the number of moles that correspond to the current decrease (86 micromoles). The enzymatic activity was therefore 86/11 = 7.81 IU/mg. This enzymatic activity was 1.5 times higher than that of the enzyme immobilized on CNF.

The biosensor has a linear range between 1.7 and 5 mM and a sensitivity of 8.6 μA/mM. After 1 year, these characteristics have changed (linear range 1–3 mM and sensitivity 1.5 μA/mM).

From the experimental results one can see that the enzymatic activity of GOx decreases in time. In Table 3 we have presented Michaelis–Menten constants and also the reduction of enzymatic activity (in percent).

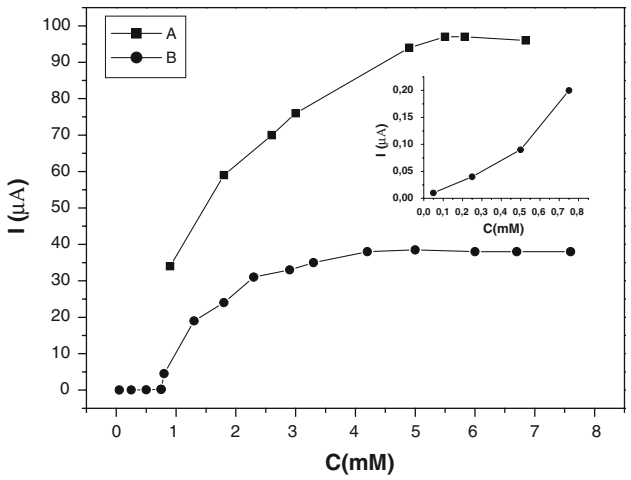

Fig. 5 Calibration curves of glucose biosensors: (**a**) first biosensor; (**b**) second biosensor (the points represent the media of determinations for three sensors)

We have prepared a new series of three biosensors, using 6 mg GOx/1 g CNF (the exact absorption quantity found in adsorption experiments) and we have obtained the calibration curve presented in Fig. 7.

One can see that in this case the response is very weak (the saturation current is 3.33 μA) and we suppose that this is due to the fact that not all the enzyme immobilized on CNF is accessible for the enzymatic reaction that takes place at electrode surface. Therefore, the quantity of the enzyme required to obtain a strong enough signal of the biosensor has to be larger than the adsorbed quantity on the CNFs because part of the enzyme is blocked in the mass of the carbon paste and does not participate to the reaction.

4 Conclusions

We have investigated two types of carbon structures (CNF and AC) and we have found that they were weakly acidic

Table 3 The decrease of GOx activity in time

Time	Current (μA)	*K* (mM)	Enzyme activity (U/mg)	Enzymatic activity decreased (%)	Figure 5
After preparation	96	0.15	157	0	a
After 12 months	38	0.12	64	59	b

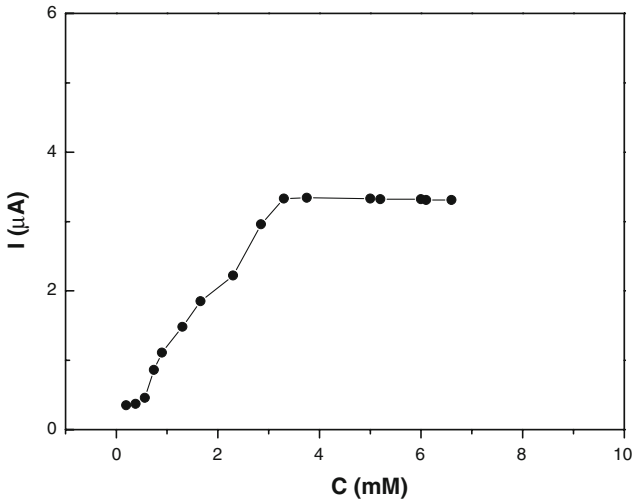

Fig. 7 Calibration curve of glucose biosensor prepared with 6 mg of GOx/g CNF (the points represent the media of determinations for three sensors)

mainly due to preparation and activation methods. The adsorption of amino acids on CNF increases from alanine to aspartic acid (alanine < aspartic acid) due to molecular weight and acid–base functionalities of amino acid molecules. Glutamic acid does not obey the Langmuir equation. When activated carbon was used as substrate the adsorption of amino acids increased from aspartic acid to alanine

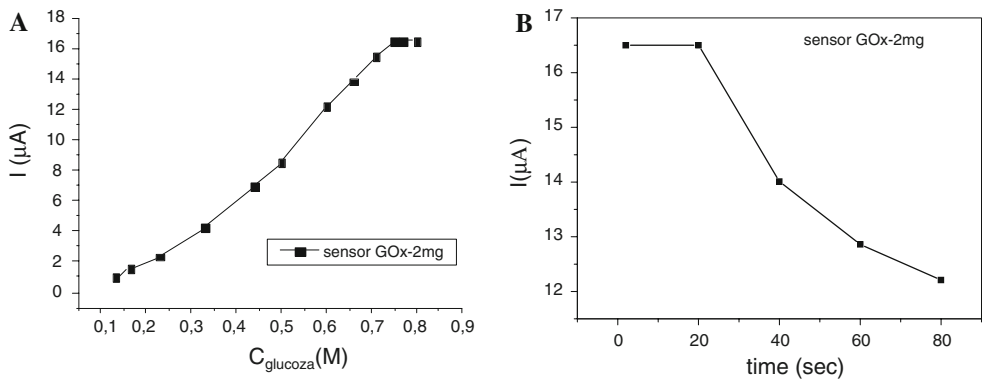

Fig. 6 (**a**) calibration curve of glucose biosensor; (**b**) biosensor response during glucose consumption (the points represent the media of determinations for three sensors)

and to glutamic acid (aspartic acid < alanine < glutamic acid). The interaction between GOx and CNF support was complex, depending on factors like steric hindrance or chemical groups attached to CNF surface. The filamentous morphology of CNF was responsible for the greater stability of adsorbed enzyme compared with the enzyme used directly in solution.

We have used the amperometric method to detect in an original manner the changes in specific activity of GOx immobilized on CNF. This study demonstrates that CNFs can be successfully used as support for enzyme immobilization. The specific activity was determined by taking into consideration the decrease of the current in time. The proposed method is fast and very simple and demonstrates that not all the enzyme immobilized on nanofibres can catalyze the oxidation of glucose.

References

1. Y.M. Volfkovich, A.Yu. Rychagov, O.N. Efimov, B.P. Tarasov, E.P. Krinichnaya, V.E. Sosenkin, N.F. Nikolskaya, A.P. Moravskii, Structural and electrochemical properties of carbon nanotubes and nanofibers. Russ. J. Electrochem. (Engl. Transl.) **38**, 663 (2002). doi:10.1023/A:1016019021741
2. F. Marken, M.L. Gerrard, I.M. Mellor, R.J. Mortimer, C.E. Madden, S. Fletcher et al., Voltammetry at carbon nanofiber electrodes. Electrochem. Commun. **3**, 177 (2001). doi:10.1016/S1388-2481(01)00132-1
3. T.E. Mcknight, A.V. Melechko, M.A. Guillorn, V.I. Merkulov, M.J. Doktycz, C.T. Culbertson et al., Effects of microprocessing on the electrochemistry of Carbon nanofiber electrodes. J. Phys. Chem. B. **107**, 10722 (2003). doi:10.1021/jp034872+
4. M.S. Kumar, S.H. Lee, T.Y. Kim, T.H. Kim, S.M. Song, J.W. Yang, K.S. Nahm, E.-K. Suh, DC electric field assisted alignment of carbon nanotubes on metal electrodes. Solid-State Electron. **47**, 2075 (2003). doi:10.1016/S0038-1101(03)00258-2
5. G.A. Kovalenko, E.V. Kuznetsova, Y.I. Mogilnykh, I.S. Andreeva, D.G. Kuvshinov, N.A. Rudina, Catalytic filamentous carbons for immobilization of biologically active substances and non-growing bacterial cells. Carbon **39**, 1033 (2001). doi:10.1016/S0008-6223(00)00216-5
6. L. Zajoncova, M. Jilek, V. Beranova, P. Pec, A biosensor for the determination of amylase activity. Biosens. Bioelectron. **20**, 240–245 (2004). doi:10.1016/j.bios.2004.01.006
7. J. Conyers, V. Moore, M. Lackey, R. Partha, P. Cherukuri, J. Hudson, A. Leonard, J. Tour, J. Huff, Biocompatibility of carbon nanostructures for therapeutic and diagnostic applications. Nanomedicine. **2**(4), 304 (2006)
8. J.A. Lee, S.S. Lee, K.-C. Lee, P. Seil, B.-C. Woo, J.-O. Lee, Biosensor utilizing resist-derived carbon nanostructures. Appl. Phys. Lett. **90**(26), 264103 (2007)
9. J.J. Davis, M.L.H. Green, H.A.O. Hill, Y.C. Leung, P.J. Sadler, J. Sloan, A.V. Xavier, S.C. Tsang, The immobilisation of proteins in carbon nanotubes. Inorg. Chim. Acta **272**, 261 (1998). doi:10.1016/S0020-1693(97)05926-4
10. R.J. Chen, Y. Zhang, D. Wang, H. Dai, Noncovalent sidewall functionalization of single-walled carbon nanotubes for protein immobilization. J. Am. Chem. Soc. **123**, 3838 (2001). doi:10.1021/ja010172b
11. S. Wang, E.S. Humphreys, S.-Y. Chung, D.F. Delduco, S.R. Lusting, H. Wang et al., Peptides with selective affinity for carbon nanotubes. Nat. Mater. **2**, 196 (2003). doi:10.1038/nmat833
12. V. Zorbas, A. Smith, A. Ortiz-Acevedo, H. Xie, G.R. Dieckmann, R.K. Draper et al., Importance of aromatic content for peptide/single-walled carbon nanotube interactions. J. Am. Chem. Soc. **127**(35), 12323 (2005). doi:10.1021/ja050747v
13. A.B. Dalton, A. Ortiz-Acevedo, V. Zorbas, E. Brunner, W.M. Sampson, S. Collins, J.M. Razal, M.M. Yoshida, R.H. Baughman, R.K. Draper, I.H. Musselman, M. Jose-Yacaman, G.R. Dieckmann, Hierarchical self-assembly of peptide coated carbon nanotubes. Adv. Funct. Mater. **14**(12), 1147 (2004). doi:10.1002/adfm.200400190
14. G.R. Dieckmann, A.B. Dalton, P.A. Johnson, J. Razal, J. Chen, G.M. Giordano, E. Munoz, I.H. Musselman, R.H. Baughman, R.K. Draper, Controlled assembly of carbon nanotubes by designed amphiphilic peptide helices. J. Am. Chem. Soc. **125**(7), 1770 (2003). doi:10.1021/ja029084x
15. H.R. Byon, H.C. Choi, Network single-walled carbon nanotube-field effect transistors (SWNT-FETs) with increased schottky contact area for highly sensitive biosensor applications. J. Am. Chem. Soc. **128**(7), 2188 (2006). doi:10.1021/ja056897n
16. C.V. Nguyen, L. Delzeit, A.M. Cassell, J. Li, J. Han, M. Meyyappan, Preparation of nucleic acid functionalized carbon nanotube arrays. Nano. Lett. **2**(10), 1079 (2002). doi:10.1021/nl025689f
17. L. Olenic, G.H. Mihăilescu, D. Lupu, A.L. Biriş, P. Mărgineanu, Carbon nanofibres-supports for adsorption of biologically active substances. Studia UBB. Phys XLIX **3**, 121 (2004)
18. D. Lupu, A.R. Biris, A. Jianu, C. Bunescu, E. Burkel, E. Indrea, G.H. Mihailescu, S. Pruneanu, L. Olenic, I. Misan, Carbon nanostructures produced by CCVD with induction heating. Carbon **42**, 503 (2004). doi:10.1016/j.carbon.2003.12.034
19. S. Pruneanu, Z. Ali, G. Watson, S.Q. Hu, D. Lupu, A.R. Biris, L. Olenic, G. Mihailescu, Investigation of electrochemical properties of carbon nanofibres prepared by CCVD method. Part. Sci. Technol. **24**, 311 (2006). doi:10.1080/02726350600840654
20. H.P. Boehms, Chemical identification of surface groups. Adv. Catal. **16**, 179 (1966). doi:10.1016/S0360-0564(08)60354-5
21. M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**, 248 (1976). doi:10.1016/0003-2697(76)90527-3