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ORIGINAL PAPER

Comparison of protein immobilisation methods onto oxidised and native carbon nanofibres for optimum biosensor development

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Abstract The properties of native and oxidised graphene layered carbon nanofibres are compared, and their utilisation in enzyme biosensor systems using different immobilisation methods are evaluated. The efficient oxidation of carbon nanofibres with concentrated H_2SO_4/HNO_3 is confirmed by Raman spectroscopy while the introduction of carboxylic acid groups on the surface of the fibres by titration studies. The oxidised fibres show enhanced oxidation efficiency to hydrogen peroxide, while at the same time they exhibit a more efficient and selective interaction with enzymes. The analytical characteristics of biosensor systems based on the adsorption or covalent immobilisation of the enzyme glucose oxidase on carbon nanofibres are compared. The study reveals that carbon nanofibres are excellent substrates for enzyme immobilisation allowing the development of highly stable biosensor systems.

Keywords Graphene layered carbon nanofibres · Oxidation · Covalent immobilisation · Adsorption · Biosensor

Introduction

There is a growing interest in the use of nanomaterials for the optimisation of bio-analytical systems and development of nanosystems [1–4]. In the field of biosensors, the most critical issues impeding their large-scale application is still the poor stability of the biocatalyst employed as well as the inefficient and power-demanding signal mediation from the biological sensing element to the transducer. The unique physical and chemical characteristics of certain nanomaterials, such as high surface-to-volume ratio, high catalytic activity and efficient electron transfer capabilities, make them ideal substrates for the development of advanced and reliable biosensor systems [4–6]. Nanomaterials can thus act as immobilisation matrices, transduction platforms as well as electron mediators.

Carbon nanomaterials [7–13] have been extensively studied and proved to be ideal materials for the development of biosensor systems with improved analytical characteristics. Most of the work has been focused on carbon nanotubes [14–19], while recently, carbon nanofibres [20, 21] and graphene [22] have also been shown to be excellent alternatives as matrices for the design of biosensors. Carbon nanofibres are cylindrical wire-shaped structures, composed of graphite layers stacked around the fibre's axis in different directions giving distinct, well-organised nanowires. The fact that they are composed of well-arranged graphite sheets results in wires with high conductivity. In addition, the exposed and chemically active edges allows for these nanowires to have a very large chemically active surface area. These characteristics make these carbon nanofibres very promising electrochemical transducers and ideal substrates for the immobilisation of biomolecules. Compared to carbon nanotubes, carbon nanofibres have many active sites throughout their surface [23] allowing the efficient and facile electron transfer of electroactive analytes [21, 24–26]. In addition, the physical and chemical properties of these carbon nanofibres can be precisely controlled by modification of their surface. Chemical treatment can be used to introduce surface functional groups in order to increase the solubility and facilitate the selective interaction with biomolecules. Oxi-

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dation of carbon nanofibres with concentrated acids is the most common procedure for their chemical modification that increases the oxygen-containing surface functional groups [27]. Many liquid-phase oxidising agents have been utilised for the oxidation of carbon nanofibres, including nitric acid (HNO_3), mixtures of nitric and sulphuric acid, hydrogen peroxide (H_2O_2), permanganate ($KMnO_4$), ruthenium tetroxide (RuO_4) as well as potassium hydroxide (KOH) [28–30]. During these oxidation processes, several oxygen-rich groups such as carboxylic acids, alcohols, aldehydes, ethers, lactones and epoxides are formed on the surface of the carbon nanofibres. These groups, native or modified, allow for the selective immobilisation of biomolecules such as proteins, enzymes and DNA. It has been shown that the chemical procedure based on a mixture of concentrated H_2SO_4/HNO_3 acids leads to very low yields of carbon nanofibres; however, it creates the maximum number of surface carboxylic acid groups [29], which are very valuable to be used for the covalent grafting of a variety of reagents onto the edges of the graphene layers of the nanofibre side surface.

The stabilisation of biomolecules when within or onto the biosensor transducer is of crucial importance for the development of stable analytical systems. Certain biomolecules, such as enzymes, are from their nature not very stable in a non-biological biosensor environment. When in vitro, proteins are sensitive to denaturation or inactivation by either fluctuations in the pH, temperature, as well by the presence of organic solvents and detergents which are usually employed for the construction of a biosensor. It has been shown that the introduction of functional groups onto the surface of the nanofibre increases the amount of the protein that can be immobilised onto its surface. It is suggested that this is due to the stronger interaction of the enzyme with the nanofibres. Both the adsorption [20, 31] as well as the covalent immobilisation [32–34] of different biomolecules onto the surface of the carbon nanofibres have been explored; however, a comparative study concerning the stability of the corresponding biosensors under the same experimental conditions has not been accomplished so far.

In this study, we compare both the type and amount of active sites generated onto the surface of the nanofibres together with the enzyme immobilisation method in order to elucidate the optimum methodology for the development of the most stable electrochemical biosensors based on carbon nanofibres. For this, the oxidation of carbon nanofibres is achieved using concentrated H_2SO_4/HNO_3 solution. The fibres are then fully characterised regarding the type and number of the surface functional groups as well as their transduction efficiency. Finally, the oxidised carbon nanofibres are utilised for the development of glucose biosensors using either adsorption or covalent immobilisation of the enzyme glucose oxidase, and the analytical characteristics of the developed biosensors are compared.

Experimental

Reagents

Carbon nanofibres (GFE) were purchased from Electrovac AG. The enzyme glucose oxidase (GOx) from *Aspergillus niger* was purchased from Fluka. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Sigma and Fluka, respectively. All other reagents were of analytical grade. In all experiments, nanopure water (18 MΩ, EASYpure model D7033, Barnstead) was used for the preparation of the solutions.

Surface oxidation of carbon nanofibres

The oxidation of carbon nanofibres (CNFs) was carried out using concentrated H_2SO_4/HNO_3 (3:1) solution. One gram of CNFs was dispersed in 75 mL concentrated H_2SO_4 and 25 mL concentrated HNO_3. The dispersion was sonicated for 6 h, diluted in 1,000 mL deionised water and subsequently filtered through filter paper. The oxidised CNFs were washed with NaOH (10 mM), HNO_3 (10 mM) and finally with deionised water until pH7.0. To remove the amorphous

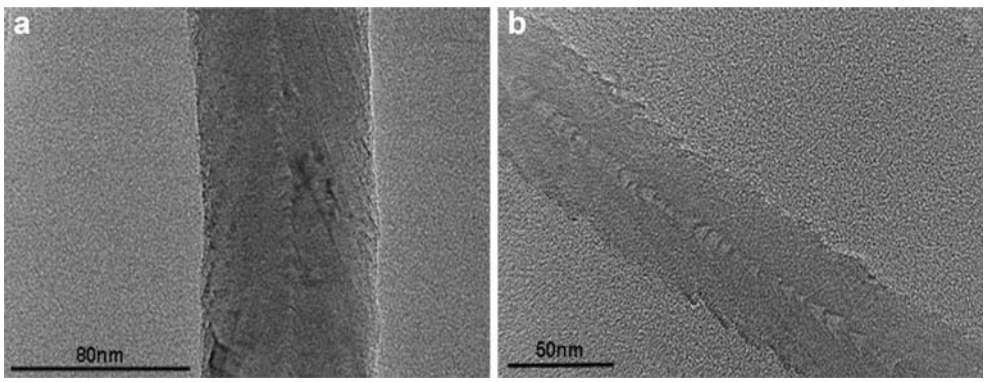

Fig. 1 TEM images of the **a** native and **b** oxidised carbon nanofibre

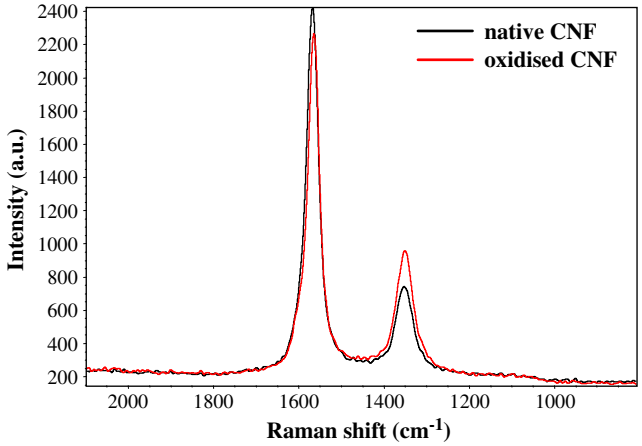

Fig. 2 Raman spectra of the native and oxidised carbon nanofibres

carbon, which was probably formed during the above procedure, CNFs were added to 20 mL of piranha solution (H_2SO_4/H_2O_2 4:1) for 1 h at 70°C. The piranha-treated nanofibres were diluted in deionised water, filtered and rinsed with deionised water until pH neutral. The acid-treated CNFs were dried under vacuum at 100°C overnight. The total yield of carbon nanofibres was determined after drying, and it was found to be approximately 15% of the original amount.

Characterisation of carbon nanofibres

High-resolution transmission electron microscopy (TEM) images of the samples were obtained using a JEM-2100 LaB_6 Transmission Microscope. CNFs were characterised using Raman spectroscopy. The Raman measurements were performed at room temperature using a Nicolet Almega XR Raman spectrometer with a 473 nm blue laser as an excitation source, and the beam was focused on the sample through a microscope equipped with a ×50 objective. The determination of the functional groups (total acidity and basicity) of native and oxidised carbon nanofibres was performed by direct titration with either NaOH or HCl [35, 36]. The determination of carboxylic acid groups was performed by titration with $NaHCO_3$. In each case, 0.5 g of the sample was placed in 25 mL of deaerated, nanopure water and left overnight under argon. Following this, additions of either 0.01 M HCl, NaOH or $NaHCO_3$ were made, and the pH of the suspension was monitored using a pH glass electrode (81-72 BN, Thermo Orion).

Biosensor construction

The enzyme glucose oxidase was immobilised on the native and on the acid-treated CNFs by adsorption. Five hundred milligrams of each CNF were suspended in 2 mL of a solution with the desired amount of glucose oxidase (10 mg/mL) in 10 mM phosphate buffer at pH7.5. The stabilisation of the enzyme on the CNFs was performed at 4°C for 12 h under stirring. Subsequently, the suspensions were filtered through 0.2-μm filters, washed with phosphate buffer, and finally dried at room temperature. In addition, GOx was covalently immobilised on the acidic treated CNFs via carbodiimide chemistry. For this, 50 mg of acidic treated CNFs were added in 3 mL dimethyl sulphoxide (DMSO) solution with EDC (15 mg/mL). One hundred fifty milligrams of sulfo-NHS were added under stirring, and the reaction was allowed to occur at room temperature for 3 h. Then, the activated CNFs were filtered through 0.2-μm filters, washed with DMSO solution and cold deionised water. In a short period of time, the filtered CNFs were immersed in a 10 mM phosphate solution (pH7.5) of GOx (10 mg/mL) at room temperature for 3 h under stirring. The resulting CNFs were treated as previously. The GOx-loaded nanofibres were placed into platinum-backed electrode cells, which were coated with dialysis membrane. The diameter of the electrode was 5.6 mm, while the thickness of the nanofibre film was 0.5 mm.

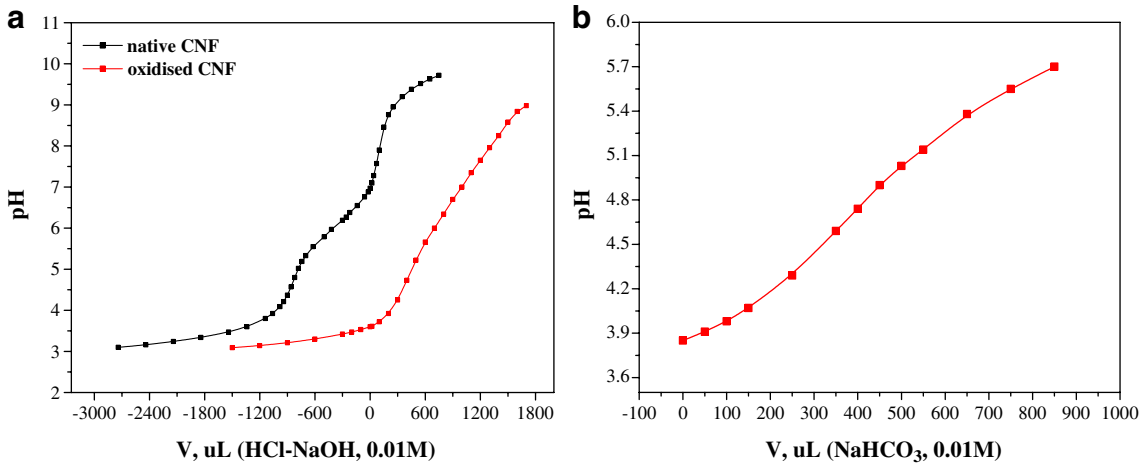

Fig. 3 Titration curves of **a** the native and oxidised carbon nanofibres to HCl and NaOH and **b** of the oxidised carbon nanofibres to $NaHCO_3$

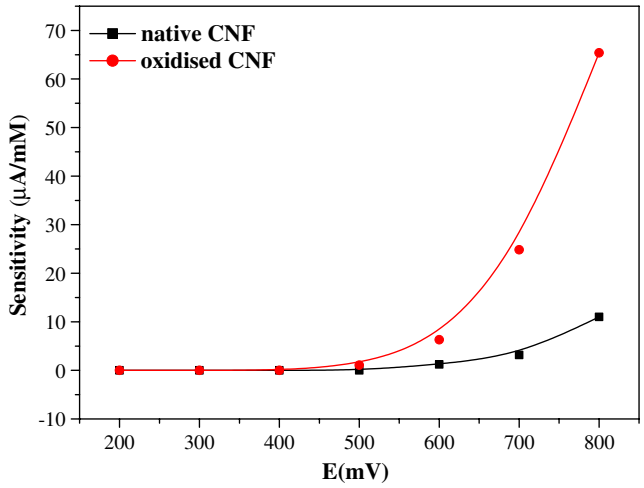

Fig. 4 Hydrodynamic voltammogram of the native and oxidised carbon nanofibre sensors to hydrogen peroxide

Electrochemical measurements

Electrochemical experiments were carried out using a three-electrode system with a silver/silver chloride double-junction reference electrode and a platinum counter electrode. All measurements were performed in 10 mM phosphate buffer adjusted at pH7.5. Hydrodynamic voltammograms of the sensors for hydrogen peroxide concentrations from 0.2 to 1.2 mM were obtained using an Autolab PGSTAT 30 potentiostat, applying different potentials ranging from +0.2 to +0.8 V. The sensitivity and the stability of the biosensors were monitored amperometrically at the working potential of +0.7 V with additions of glucose solution (0.5 M). The potentiostat used was a Metrohm 791 VA detector.

Results and discussion

Transmission electron microscopy

The native and oxidised carbon nanofibres were characterised by transmission electron microscopy in order to determine their morphology and size distribution. In Fig. 1a, we observe that the carbon nanofibres have a mean diameter of 80 nm and that the graphitic platelets are arranged in a "herringbone" structure. After the oxidation treatment, the diameter of the carbon nanofibres decreases to approximately 50 nm as it is shown in Fig. 1b. Also, the surface of the oxidised nanofibres presents a more intense saw-toothed morphology as a result of the oxidation treatment.

Raman study-amount of functional groups

The degree of carbon nanofibre oxidation was determined using Raman spectroscopy. The treatment of carbon nanofibres with strong oxidising agents induces defect sites at the graphitic structure, converting some of the sp^2 hybridised carbons to sp^3. At the Raman spectra of the native and oxidised carbon nanofibres shown in Fig. 2, the peak at 1,350 cm^{-1} corresponds to the disorder-induced D band (sp^3 hybridised carbons) and the peak at 1,580 cm^{-1} to the graphitic-induced G band (sp^2 hybridised carbons) [37]. The ratio of the intensity of the D band to the intensity of the G band is an indicator of the degree of carbon nanofibres oxidation [38]. From the Raman spectrum of the native carbon nanofibres, it is calculated that the value of the ratio of the intensity of D band to G band is 0.30. On the other hand, the ratio of the intensity of D band to G band in the case of the H_2SO_4/HNO_3 treated carbon nanofibres changes to 0.42. This increase is attributed to the appearance of more defect sites upon carbon nanofibre treatment, confirming the efficient oxidation of carbon nanofibre surface.

Titration-type of functional groups

The determination of the surface functional groups of the native and oxidised carbon nanofibres was performed by direct acid–base titration. The number of the basic sites is calculated from the amount of HCl required to titrate the solution of the fibres, while the number of acidic sites is derived from the amount of NaOH required. The acidic properties of the surface of the fibres are attributed to the presence of carboxyl, lactone and phenol groups, while the basic character is an indication for the presence of pyrones, ethers and carbonyl groups. As shown in Fig. 3a, the titration curve of the native carbon nanofibres shows two equivalent

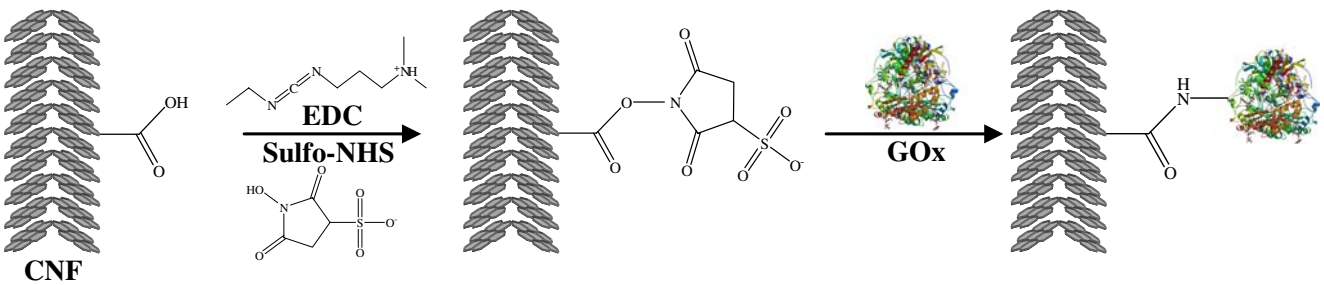

Fig. 5 Schematic representation of the covalent immobilisation of GOx on the oxidised carbon nanofibres

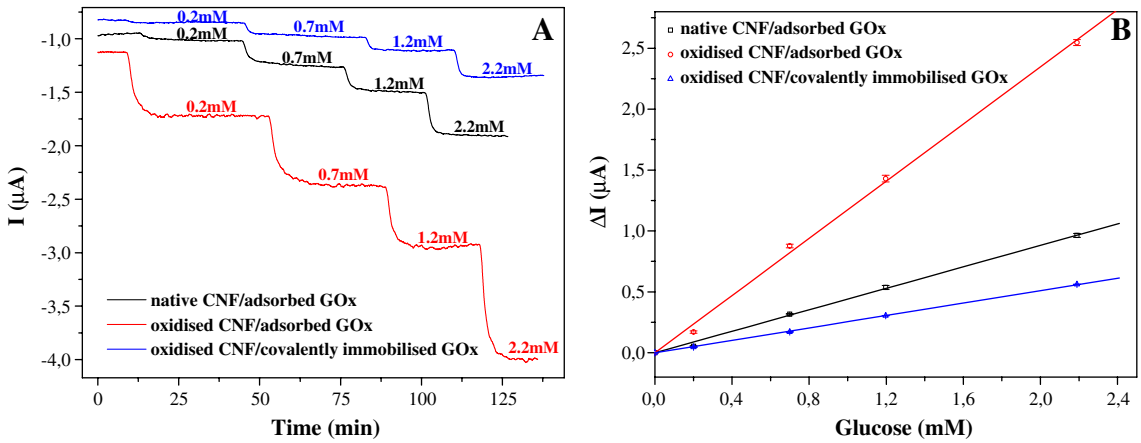

Fig. 6 **a** Amperometric curves and **b** calibration curves of the GOx biosensors based on carbon nanofibres to glucose. The working potential was +0.7 V vs Ag/AgCl (N=3)

points indicating the presence of both acidic and basic groups on their surface. More precisely, it is calculated that the native carbon nanofibres contain 26.2 μmol/g acidic functional groups and 163.8 μmol/g basic functional groups. On the other hand, the oxidation of carbon nanofibres with concentrated H_2SO_4/HNO_3 increases the amount of the acidic functional groups to 345.0 μmol/g, while the concentration of basic functional groups on the surface of the nanofibres is below the limit of detection of the method.

In order to determine the number of carboxylic acid groups on the surface of the nanofibres selectively, a titration of the carboxylic groups was performed using $NaHCO_3$ [39]. Initially, the amount of carboxylic acid groups of the native nanofibres was attempted. Due to the very small amount of these groups in the native fibres, this determination was not possible since their concentration is below the detection limit of the method. On the other hand, from the data from the titration of the oxidised carbon nanofibres (Fig. 3b), it is calculated that the oxidised carbon nanofibres contain 81.4 μmol/g carboxylic acid groups. This large number of carboxylic acid groups on the surface of the nanofibres is subsequently used for the grafting of coupling reagents or direct protein adsorption.

Hydrodynamic voltammogram-mediation efficiency

The transduction efficiency of oxidised and native nanofibres was explored by measuring the amperometric response of the corresponding sensors to hydrogen peroxide in a wide range of potentials. Since hydrogen peroxide is produced during the enzymatic reaction of oxidases, it is the reagent that is further oxidised on the surface of the working electrode for signal generation, and it is thus a fundamental value for the evaluation of oxidase-based biosensors. Sensors using either native as well as oxidised nanofibres were developed, and their response to hydrogen peroxide was monitored over the potential range of +0.2 to +0.8 V. The hydrodynamic voltammograms of the sensors are shown in Fig. 4. From these data, it is clear that the sensor with the oxidised carbon nanofibres shows higher anodic currents compared to the sensor with the native carbon nanofibres. The differentiation in the response of the sensors starts at 0.5 V and increases as the potential increases. At 0.8 V, the sensitivities to hydrogen peroxide of the biosensor based on the oxidised carbon nanofibres is six times higher (65.4 vs 11.0 μA/mM). These results confirm the fact that the oxidation of carbon nanofibre surface increases significantly the sensitivity of the biosensor.

Enzyme stabilisation on carbon nanofibres

Subsequently, the immobilisation and stabilisation of the enzyme glucose oxidase on the native and oxidised carbon nanofibres was examined using two different immobilisa-

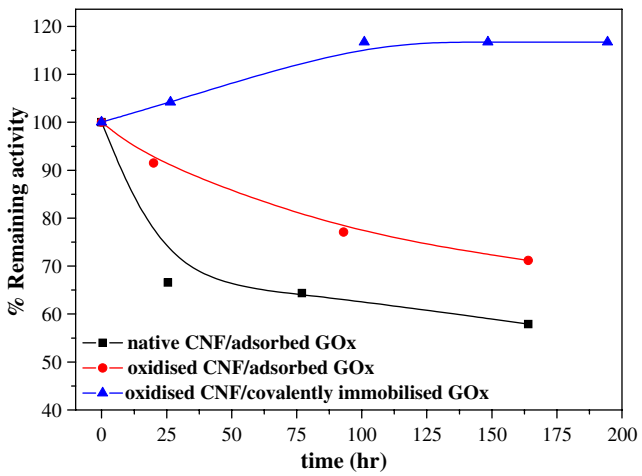

Fig. 7 Stability under continuous polarisation of GOx biosensors based on carbon nanofibres (+0.7 V, 25 °C)

tion methods of the enzyme: adsorption and covalent bonding via carbodiimide chemistry (Fig. 5). The sensitivity and operational stability of the corresponding biosensors was monitored under continuous polarisation at +0.7 V. Successive additions of glucose lead to an increase of the anodic current, which is attributed to the oxidation of glucose to gluconic acid and the subsequent oxidation of the produced hydrogen peroxide on the surface of the electrode [40]. Through this reaction sequence, enzymatic activity can be directly related to the biosensor sensitivity. At frequent intervals, the calibration curves of the biosensors were obtained, while between measurements, the biosensors were kept in buffer solution poised at +0.7 V. The amperometric curves and the corresponding calibration curves of the GOx biosensors are presented in Fig. 6a, b, respectively. The response of all biosensors is linear to glucose concentrations from 0.2 to 2.2 mM, the response time is between 0.5 to 1 min, and the sensor-to-sensor reproducibility is excellent (<2% RSD, n=3). The sensitivity of the biosensors based on adsorption of GOx on both the native and oxidised carbon nanofibres were calculated to be 0.45 and 1.18 μA/mM, respectively. The increased sensitivity of the GOx biosensor based on the oxidised nanofibres compared to the biosensor with the native nanofibres can be attributed to the higher enzyme loadings on the oxidised nanofibres. Also, it could be ascribed to the enhanced transduction efficiency of the oxidised nanofibres due to the presence of more functional groups on their surface. The sensitivity of the biosensor based on the covalent immobilisation of GOx on the oxidised carbon nanofibres was calculated to be 0.24 μA/mM. This lower response can be attributed to the known fact that the covalent immobilisation of the enzyme on the nanofibres is selectively performed only through the carboxylic acid groups and not all the surface functional groups of the nanofibres, resulting in lower enzyme loadings.

The stability of all the biosensors was monitored under continuous polarisation for 164 h at 25 °C, poised at +0.7 V vs Ag/AgCl electrode. As it is shown in Fig. 7, the GOx biosensors based on the adsorption of the enzyme on the nanofibres present a gradual decrease in their sensitivity during time. For the GOx biosensors based on the native and oxidised carbon nanofibres, the remaining activities after 164 h of continuous polarisation were 58% and 71%, respectively. On the other hand, the GOx biosensor based on the covalent immobilisation of the enzyme on the oxidised carbon nanofibres retains all its initial activity even after 194 h of continuous operation. The activity of the biosensor based on covalent immobilisation onto the oxidised nanofibres started to decrease after this time, reaching 50% of its initial value after 600 h of continuous polarisation.

From these results, it is concluded that for the development of a long-lasting biosensor based on carbon nanofibres, the optimum procedure requires the oxidation of the surface in order to increase the number of carboxylic functional groups, with subsequent covalent immobilisation of the protein. Even though the initial sensitivity of the biosensor based on adsorption of the enzyme onto the oxidised nanofibres is higher than that of the biosensor based on the covalent immobilised enzymes, the later one shows a much more stable response over time, and it is thus more suitable for biosensor devices.

Conclusions

In this study, the electrochemical properties of oxidised carbon nanofibres compared to native carbon nanofibres as well as the ability of carbon nanofibres to stabilise enzymes were examined. It is shown that the oxidation of the nanofibres with concentrated H_2SO_4/HNO_3 increases the active sites on their surface as it is confirmed by Raman spectroscopy and introduces acidic functional groups along the fibre surface. The acidic treatment leads to a metamaterial that presents higher response to hydrogen peroxide and favours enzyme adsorption and stabilisation. In addition, the large number of carboxylic acid groups on the surface of the oxidised carbon nanofibres allows the covalent immobilisation of enzymes that enhances their stability and prevents their leaching to solution. These results reveal that oxidised carbon nanofibres are excellent matrices for the development of biosensors with improved analytical characteristics.

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