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Single-Wall Carbon Nanotubes Covalently Functionalized with Polylysine: Synthesis, Characterization and Analytical Applications for the Development of Electrochemical (Bio)Sensors

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Abstract: This work reports the synthesis of single-wall carbon nanotubes (SWCNT) covalently functionalized with polylysine (Plys) and the analytical performance of glassy carbon electrodes (GCE) modified with this material (GCE/SWCNT-Plys). The resulting electrodes showed an important decrease in the overvoltages for the oxidation of ascorbic acid, uric acid and hydrogen peroxide as well as for the reduction of hydrogen peroxide. The favorable interaction of glucose oxidase (GOx) with SWCNT-Plys allowed the sensitive and selective glucose biosensing at −0.100 V without any permselective membrane. The proposed sensor was challenged with different real samples without pretreatment showing an excellent correlation with the reported values.

Keywords: Carbon nanotubes · Single-wall carbon nanotubes · Glassy carbon electrode · Enzymatic biosensors · Glucose oxidase · Glucose biosensors · Polylysine · Covalent functionalization · Biosensors

1 Introduction

Since the pioneering work of Britto et al. [1], carbon nanotubes (CNT) have fueled two decades of innovations in the field of electrochemical sensors [2–8]. The major drawbacks of these nanostructures, that limit their application in the development of biosensors, are the trend to self-aggregation and the consequent extremely low solubility in most solvents [9]. Tremendous efforts have been made to avoid this difficulty without hindering benefits and to make possible the incorporation of CNTs in electrochemical transducers. In this sense, the use of solvents [10], surfactants [11,12], polymers [13–15], liquid binder [16,17], and ionic liquids [18] to disperse CNTs have demonstrated to be highly successful.

In particular, the presence of polymers facilitates the dispersion of CNTs assisted by ultrasonication, since π-stacking, hydrophobic interaction or thermodynamic entanglement promote the spontaneous formation of reversible nanotube-polymer supramolecular architectures easier to disperse in aqueous solvents [13,19,20]. This strategy, relying on non-covalent interactions, demonstrated to be very popular for its simplicity and effectiveness. In addition, once dry, the polymers act as a matrix, greatly enhancing the mechanical resistance of the deposit, facilitating the attachment of specific chemical functions to the CNTs without damaging their Csp^2 electronic structure [15,21].

CNTs functionalization was also successfully performed by covalent bonding [22]. Most of the protocols for CNTs covalent functionalization rely on their harsh initial oxidation to generate different oxygenated functions, mainly carboxylic residues. These oxygenated functions present the advantage of working as anchoring sites to further functionalize nanotubes taking advantages of the versatility offered by organic synthesis. This methodology is often viewed as a more "destructive" pathway than the non-covalent approach since the electronic conjugation of CNTs is greatly affected when grafting different molecules. Nevertheless, the loss of conjugation is partial and the oxidized nanotubes are conductive enough to obtain functional electrodes [23–25]. Furthermore, electrocatalytic properties of carbon allotropes are attributed to sidewall defects acting as pyrolytic graphite edge-plane [26,27]. In this sense, oxidized nanotubes can be considered as "defect-enriched" and what is lost in conduction is earned in reactivity.

Biopolymers have received special interest to functionalize CNTs. Peptide synthesis represents an effective tool to combine and tune their properties. Jalit et al. [28] demonstrated the efficient dispersion of multi-wall carbon

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nanotubes (MWCNTs) by non-covalent functionalization with polylysine (Plys) and the successful development of electrochemical biosensors for glucose quantification [28] and detection of the hybridization event [29]. The glucose biosensor was obtained by layer-by-layer self-assembling of glucose oxidase (GOx) at glassy carbon electrodes (GCE) modified with MWCNT-Plys (GCE/MWCNT-Plys) and the biosensing of glucose was performed by amperometry at 0.700 V from the oxidation of the hydrogen peroxide enzymatically generated. Nevertheless, this platform did not offer the possibility of the sensitive detection of glucose from the reduction of the hydrogen peroxide enzymatically generated. To the best of our knowledge, no attempts have been done to build equivalent electrochemical glucose biosensing platforms based on the use of Plys covalently-bonded to CNTs.

The goal of this work was the synthesis of single-wall carbon nanotubes (SWCNT) covalently functionalized with Plys (SWCNT-Plys) and the development of electrochemical (bio)sensors obtained by modification of GCE with SWCNT-Plys (GCE/SWCNT-Plys). In the following sections we discuss the synthesis and characterization of SWCNT-Plys, the selection of the conditions for an efficient dispersion and robust deposition of SWCNT-Plys at GCE, the electrochemical activity of GCE/SWCNT-Plys using common redox markers, and the sensitive and selective glucose biosensing at GCE modified with SWCNT-Plys and GOx at very low potential (−0.100 V) from the reduction of the hydrogen peroxide enzymatically generated in the presence of GOx incorporated to the SWCNT-Plys dispersion.

2 Experimental

2.1 Materials and Reagents

Single-wall carbon nanotubes (SWCNT) synthesized by the arc-discharge method using Ni/Y catalyst (AP-SWNT grade) were purchased from Carbon Solutions Inc. (Riverside, California). They were purified by air oxidation at 350 °C for 2 h and then refluxed in 3 M HCl for 2 h as described in [30]. Sodium dodecyl-benzenesulfonate (SDBS), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexa-fluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (EDIPA) were purchased from Sigma-Aldrich. Likewise, poly-L-lysine hydrochloride (Plys) (P9404, molecular weight > 30000), dopamine (Do), uric acid (UA), and glucose oxidase (GOx) were received from Sigma-Aldrich. Ascorbic acid (AA), hydrogen peroxide, and sodium phosphates were purchased from Baker. Ultrapure water ($\rho = 18$ MΩ cm) obtained from a Millipore-MilliQ system was used for preparing all the solutions. A 0.050 M phosphate buffer solution pH 7.40 (PBS) was employed as supporting electrolyte. Other chemicals were reagent grade and used without further purification. All the experiments were conducted at room temperature.

Pepsi, Gatorade and honey were obtained from a local supermarket, while Alenys was acquired from a pharmacy. These samples were used without any pretreatment.

2.2 Apparatus

The electrochemical experiments were performed with a TEQ 04 potentiostat. The electrodes were inserted into the cell (BAS, Model MF-1084) through holes in its Teflon cover. Glassy carbon electrodes (GCE) either bare or modified with a given dispersion were used as working electrodes, while platinum and Ag/AgCl, 3 M NaCl (BAS, Model RE-5B) were used as counter and reference electrodes, respectively. All potentials are referred to the latter. A magnetic stirrer (BASi Cell stand) set at 800 rpm and a stirring bar provided the convective transport during the amperometric measurements. Infrared spectroscopy (FTIR) measurements were carried out in a Bruker Vertex 70 spectrometer. The samples were prepared by mixing small amounts of the powder samples with spectroscopic grade KBr and pressing to form pellets. Thermogravimetric measurements (TGA) were carried out in a Setaram balance, model Setsys Evolution 16/18, under a nitrogen inert flow and a heating ramp of 10 °C min^{-1} in the range from room temperature to 1000 °C.

2.3 Synthesis of Functionalized SWCNT

Purified SWCNT were oxidized in a 3 M H_2SO_4/HNO_3 mixture (50 : 50 v/v) by refluxing for 3 h. The solid was filtered, rinsed with deionized water, oven-dried and functionalized with Plys. Figure 1 shows the scheme for SWCNT functionalization. In a typical experiment,

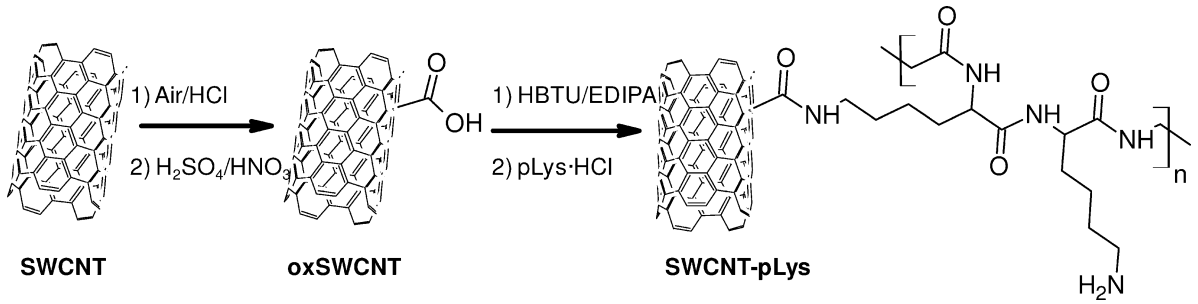

Fig. 1. Scheme of SWCNT stepwise Plys functionalization.

100 mg of oxidized SWCNTs (oxSWCNT) were placed in a round-bottom flask and bath sonicated for 1 h in a 0.5% w/v SDBS aqueous solution. After that, the suspension was transferred to a schlenk flask and purged with Ar under magnetic stirring. A gentle continuous flow of Ar and the magnetic stirring were kept along the whole functionalization process. The SWCNT dispersion was cooled with a water/ice bath. Afterwards, it was allowed to react for 45 min at 0 °C with 300 mg of HBTU and 2 mL of EDIPA. The next step was the reaction with 8 mg of Plys at room temperature. The reaction underwent for 48 h at room temperature. After that, the resulting solution was inserted in a dialysis sack (Sigma Aldrich cat. #D6066-25EA), tightly tied and immersed in a 5 L tank full of deionized water. The dialysis was performed for a week, with replacements of the water every 24 h. Finally, the dialyzed medium was lyophilized in a Telstar Cryodos freeze dryer until complete dryness. Covalently modified nanotubes (SWCNT-Plys) were used as obtained. Zhang et al. [31] reported the covalent modification of SWCNTs with Plys, although they used a different protocol to activate the carboxylic groups based on carbodiimide derivatives. The carboxylic coupling route by means of carbodiimide derivatives presents some drawbacks since carbodiimide-based esters or amides suffer hydrolysis in aqueous media. Recent publications [32,33] have highlighted the complexity of this process, with the possible formation of different byproducts. In our case, based on the efficiency of using benzotriazoles as coupling agents for peptide synthesis [34,35], the activation of the carboxylic groups was carried out with benzotriazoles. In addition, the synthesis we propose here is softer and the use of SDBS allows the CNT to be dispersed without over-oxidation. It is important to consider that the initial pretreatment employed by Zhang et al. [31] is extremely harsh (1 h sonication in concentrated HNO_3/H_2SO_4 3:1) and under these conditions the material they obtained is constituted by (very) short nanotubes as stated by the authors.

2.4 Working Electrodes Preparation

2.4.1 Preparation of SWCNT-Plys Dispersion

The SWCNT-Plys dispersion (1.0 mg/mL) was prepared by adding H_2O/EtOH/PBS (95:50:5 v/v) to the SWCNT-Plys powder, followed by ultrasonication for 15 minutes.

2.4.2 Preparation of GOx-SWCNT-Plys Dispersion

The GOx powder was added to a fresh SWCNT-Plys dispersion to reach 1.0 mg/mL GOx and mixed by vortex agitation for 1.0 min.

2.4.3 Preparation of GCE Modified with SWCNT-Plys (GCE/SWCNT-Plys) and SWCNT-Plys-GOx (GCE/SWCNT-Plys/GOx)

GCE was polished with alumina slurries of 1.0, 0.30, and 0.05 μm for 2 min each. Before functionalization, the electrode was cycled in a 0.050 M phosphate buffer solution pH 7.40 between −0.200 V and 0.800 V (10 cycles). Then, it was modified with the SWCNT-Plys dispersion in the following way: an aliquot of 20 μL was dropped on top of a polished GCE and the solvent was allowed to evaporate at room temperature. GCE/SWCNT-Plys/GOx was prepared in a similar way using SWCNT-Plys/GOx. Before starting the experiments, the modified electrodes were cycled ten times between −0.200 V and 0.800 V at 0.100 V s^{-1}.

3 Results and Discussion

3.1 Characterization of Functionalized Carbon Nanotubes

The identification of functional groups was conducted by FTIR. Figure 2A displays the FTIR spectra for purified SWCNTs, oxSWCNTs and Plys-SWCNTs. FTIR spectra for purified and oxidized SWCNTs show some characteristic bands of the oxygen functional groups, typically at ~1100 cm^{-1} (C–O stretching), ~1320 cm^{-1} (O–H stretching), ~1720 cm^{-1} (C=O stretching) and at ~3400 cm^{-1} and ~1600–1620 cm^{-1} (moisture). The carboxylation process (sample oxSWCNT) increases the bands corresponding to oxygen groups, specifically COOH, which is detected at 1722 cm^{-1}. The presence of the carboxylate ion can be also detected from the bands at 1580–1620 cm^{-1} and 1320 cm^{-1} [36].

After functionalization with Plys, the consumption of COOH groups is denoted by the increase in the ratio between the intensity of the band at 1723 cm^{-1} and the intensity of the rest of carbonyl-based bands (in the range of 1578–1624 cm^{-1}). This region becomes much more populated after reaction, exhibiting new contributions (1600–1624 cm^{-1}) corresponding to amide bond. This shoulder also includes the contribution of primary aliphatic amines of Plys. Additional proofs of the presence of free amines are the bands at 1042 and 1186 cm^{-1} due to C–N stretching vibrations in amines [37]. The attached moieties also cause a pronounced increase in the intensity of C_{sp3}-H vibrations in methylene groups (~2850–2950 cm^{-1}), consistent with the aliphatic segments of the functional groups.

Figure 2B shows TGA plots of purified SWCNT, oxSWCNT, and SWCNT-Plys. The thermograms show that purified SWCNTs possess a very low amount of functional groups since the oxygenated residues incorporated upon air oxidation are mostly removed during the HCl reflux. However, the remaining groups are not negligible, as can be seen by a slight weight loss (~6 wt%). OxSWCNTs present a large amount of surface groups, which are thermally desorbed. The amount of carboxylic

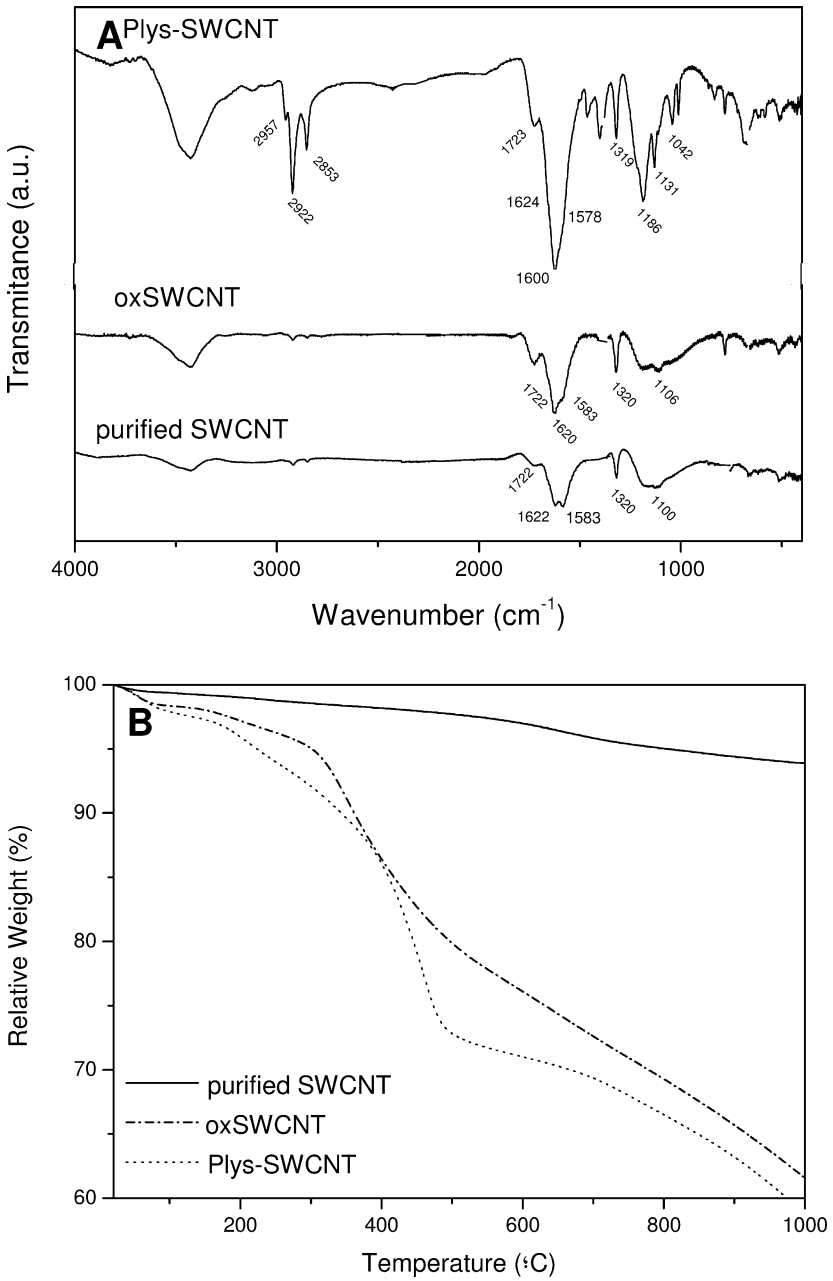

Fig. 2. FTIR (A) and TGA (B) characterization of purified SWCNT, oxidized SWCNT, and Plys-functionalized SWCNT.

functions, estimated from the release observed between 150 and 300 °C, was 0.66 mmol/g SWCNT. Between 300 and 500 °C, there is a clear weight loss corresponding to desorption of other oxygenated groups, mostly quinones, phenols and acid anhydrides [38]. It is important to mention that these latter groups are able to spontaneously hydrolyze in aqueous medium during Plys functionalization yielding carboxylic acid groups, so the effective content in COOH available for the functionalization is expected to be higher than the initially existing in the solid sample (the reagents for the covalent reactions were added in large excess to take into account this fact).

Regarding the weight loss profile in Plys-SWCNTs, it possesses similar moisture content as their non-functionalized predecessors (as judged by the weight loss between room temperature and 100–120 °C), and its thermal stability between 150–300 °C seems to be lower than that of oxSWCNTs. This could be explained considering that the decarboxylation taking place in this temperature range is more pronounced for Plys-functionalized SWCNTs since it suffers the loss of additional COOH groups coming from the attached Plys chains. Taking into account the weight loss difference observed in oxSWCNT and SWCNT-Plys between 150 and 300 °C it is possible to estimate the amount of functionalization, which is ~6 wt%.

The amount of free primary amine groups dangling from the SWCNT surface was quantified using the Kaiser test, through the standard procedure described in the lit-

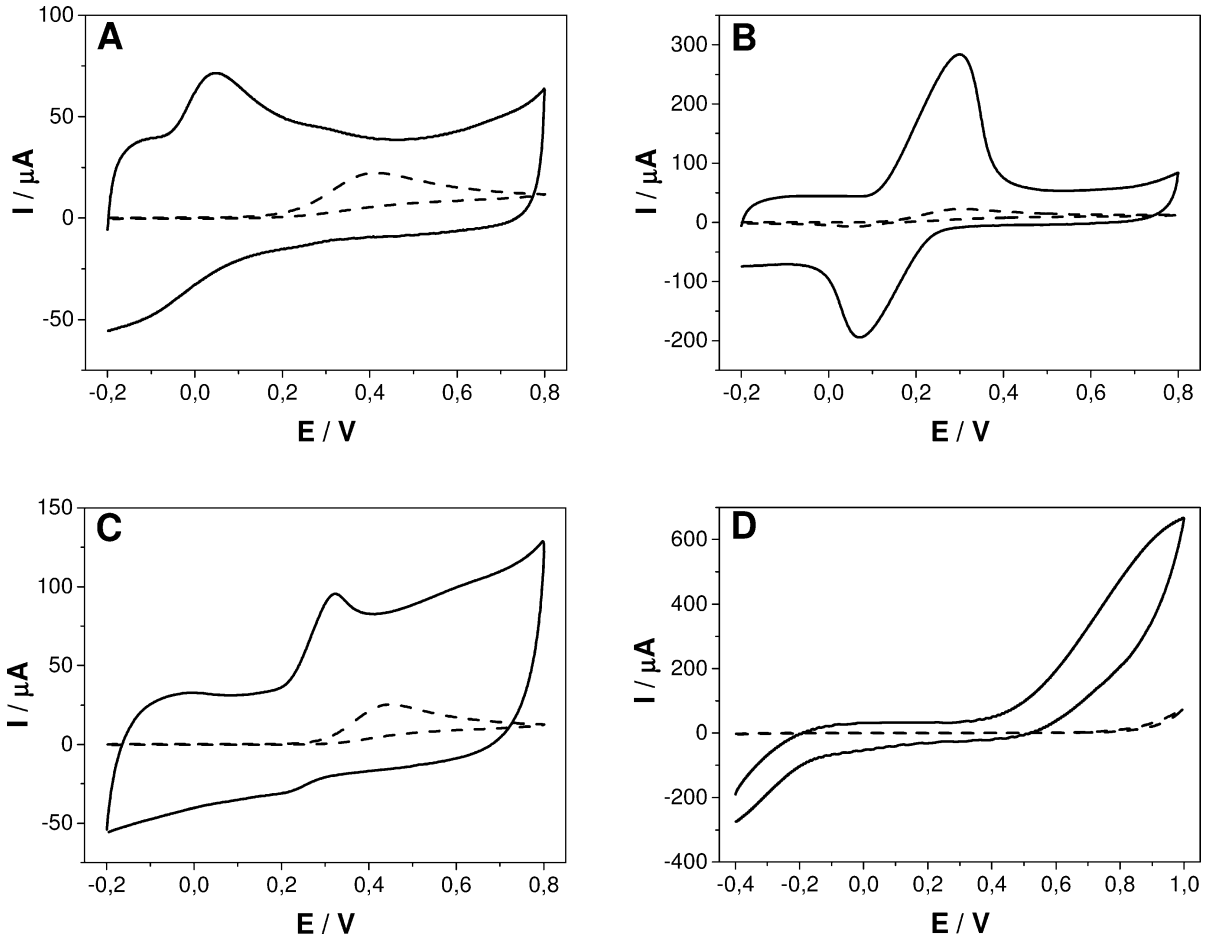

Fig. 3. Cyclic voltammetry obtained at GCE (dashed line) and GCE/SWCNT-Plys (solid line) in presence of 1.0×10^{-3} M AA (A), 1.0×10^{-3} M Do (B), 1.0×10^{-3} M UA (C), and 5.0×10^{-2} M H_2O_2 (D). Scan rate: 0.100 V s^{-1}.

erature [39,40]. The Kaiser test provided further insights into the functionalization outcome. A value of 164.1 µmol NH_2/g SWCNT-Plys, calculated as the difference between non functionalized and functionalized samples, was obtained as a neat average value.

3.2 Electrochemical Behavior of GCE Modified with SWCNT-Plys

Figure 3 shows voltammetric profiles obtained at 0.100 V s^{-1} at bare GCE (dashed line) and GCE/SWCNT-Plys (solid line) for 1.0×10^{-3} M AA (A), Do (B) and UA (C), and 5.0×10^{-2} M hydrogen peroxide (D). Compared to bare GCE, there is an important decrease in the overvoltages for AA (340 mV) and UA (110 mV) oxidation. This decrease is even more significant than the one obtained at GCE modified with MWCNT functionalized with Plys in a non-covalent way [27] (340 vs. 176, and 110 vs. 95 mV for AA and UA, respectively). In the case of Do, at GCE/SWCNT-Plys there is a dramatic increase (920%) in the oxidation peak current and a better definition of the reduction peak current ($I_{red}/I_{ox(GCE)}=0.33$; $I_{red}/I_{ox(GCE/SWCNT\text{-}Plys)}=0.76$).The presence of SWCNT-Plys at GCE also produces a significant decrease in the overvoltages for the oxidation and reduction of hydrogen peroxide, the oxidation starting at 0.350 V and the reduction at 0.00 V (Figure 3D). A well-defined amperometric response was obtained for successive additions of hydrogen peroxide at −0.100 V (not shown). The sensitivity obtained under these conditions was $(2.8\pm0.2)\times10^3$ µA M^{-1}, value highly competitive compared to other strategies based on the reduction of hydrogen peroxide at graphite composite electrodes modified with catalytic particles [41–46].

3.3 Analytical Performance of GCE/SWCNT-Plys-GOx for Glucose Quantification

The selectivity is one of the most critical aspects in the development of electrochemical glucose biosensors. In this sense, the detection of glucose from the reduction of enzymatically generated hydrogen peroxide is an interesting alternative, although it requires high overvoltages. Taking into account the catalytic activity of GCE/SWCNT-Plys towards the reduction of hydrogen peroxide previously discussed, we built a glucose biosensor based on the formation of a supramolecular architecture containing GOx. In order to prevent the enzyme denatura-

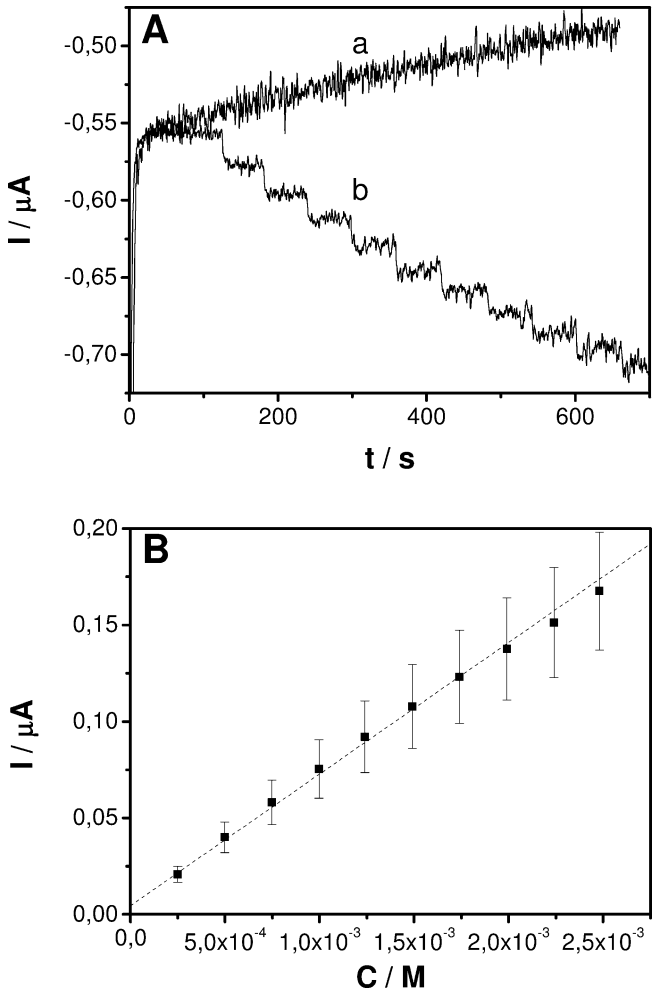

Fig. 4. (A) Amperometric response obtained at GCE/oxSWCNT/GOx (a) and GCE/SWCNT-Plys/GOx (b) for successive additions of 2.5×10^{-4} M glucose. (B) Calibration plot obtained from the recordings shown in A. b Working potential: −0.100 V.

tion, it was added to fresh SWCNT-Plys dispersion and mixed with vortex just before drop-coating the electrode.

The solvent composition for preparing this supramolecular architecture demonstrated to have a remarkable effect on the biosensor response, being the ratio 95 : 50 : 5 % v/v of water/ethanol/0.050 M phosphate buffer solution pH 7.40 the one that allowed obtaining the most sensitive and stable amperometric signals. These results are in agreement with those reported by Karra et al. [47] who attributed the increase of the activity of GOx in the presence of phosphate buffer to the HPO_4^{2-} anions "trapped" within their platform.

Figure 4 displays amperometric recordings obtained at GCE modified with oxSWCNT-GOx (a) and SWCNT-Plys-GOx (b) for successive additions of 2.5×10^{-4} M glucose at a working potential of −0.100 V. At GCE modified with oxSWCNT-GOx, there is an unstable and noisy baseline. No response is obtained after the additions of glucose, indicating that, under these conditions, there is no formation of hydrogen peroxide due to a poor assembly of the enzyme at GCE/oxSWCNT as a consequence of the negative charges of the oxSWCNTs. On the contrary, at GCE/SWCNT-Plys-GOx (Fig. 4b) the baseline is stable and less noisy, and after successive additions of glucose, there is a well-defined and fast response, demonstrating an effective attachment of GOx mainly promoted by the interaction between the enzyme and the positive charges of the secondary amines of Plys.

The response to glucose obtained with GCE/SWCNT-Plys/GOx presents a linear range between 2.5×10^{-4} M and 2.5×10^{-3} M, a sensitivity of (65 ± 1) µA M^{-1} (r^2=0.9988), a detection limit of 8×10^{-5} M and a quantification limit of 2×10^{-4} M, (calculated as $3.3\times\sigma/S$ and $10\times\sigma/S$, respectively, where σ is the standard deviation of the blank signal and S is the sensitivity). The reproducibility for the sensitivities obtained from 21 electrodes prepared with 8 different dispersions was 1.7 %, demonstrating the high reproducibility of the overall process from the preparation of the dispersion and the incorporation of GOx to the response of the resulting biosensor. Regarding the reusability of the biosensor, after 8 successive calibrations with glucose, the sensitivity remained in a 99.4 % of the first one. It is important to remark that these measurements involved continuous operation for around 90 min, demonstrating good short-term stability. Regarding the long-term stability, the dispersion was stable for 10 days, without variation in the linear range (the sensitivities for the 1^{rst} and 10th day were (69 ± 2) and (73 ± 1) µA M^{-1}, respectively).

The biosensor was challenged with different samples, beverages like "Pepsi" and "Gatorade"; popular soft drinks constituted by several carbohydrates (glucose, fructose and sucrose), colorants and conservatives (such as citric acid); Alenys, an anti-histaminic drug that contains glucose as excipient; and honey which is a concentrated mixture of carbohydrates, proteins, enzymes and even hydrogen peroxide. In all cases the amperometric signals were obtained without any pretreatment. In order to adequate the injection volume. Alenys and honey samples, due to their viscosity, were diluted in phosphate buffer in a 1 : 10 ratio. Table 1 summarizes the glucose concentrations obtained with our biosensor and compares it with the values given in the literature. Doner [48] determined the glucose concentration in honey samples by selective adsorption chromatography through charcoal followed by hypoiodide oxidation while Goran et al. [49] obtained the glucose concentration in soft drinks by HPLC without

Table 1. Quantification of glucose in different samples using GCE/SWCNT-Plys/GOx. The measurements were performed at −0.100 V by triplicate.

Sample	Reported glucose concentration	Measured glucose concentration	% Error
Honey	(31±3) % w/w [48]	(34±2) % w/w	+9.0
Alenys	(4.8±0.1) % w/w	(5.3±0.1) % w/w	+10.0
Pepsi	(39±1) g/L [49]	(43±2) g/L	+9.0
Gatorade	(24±1) g/L [49]	(22.9±0.08) g/L	−5.0

pretreatment. A very good correlation was obtained with the values reported for each sample, with errors between 5.0 to 10.0%. Therefore, SWCNT-Plys-GOx was successfully used to measure the glucose concentration in different real samples using simple, fast and inexpensive methodologies without pretreatment of the samples, demonstrating the usefulness of the proposed biosensor for practical applications.

Similar or better analytical performance is obtained compared to the architectures based on the use of MWCNT, Prussian blue, chitosan and polyaniline [50]; chitosan and MWCNTs [51]; chitosan, gold nanoparticles, ionic liquid and MWCNTs [52]; or gold nanoparticles and TiO2 nanotubes [53]. Compared to the glucose biosensor prepared by self-assembling of GOx at GCE modified with MWCNTs noncovalently functionalized with Plys [28], the strategy proposed here offers a faster preparation of the biosensor and the most important aspect, the sensitive and selective detection of glucose at very low potentials not only in pure solutions but also in complex samples.

In summary, GCE/SWCNTs-Plys demonstrated to be a very active material for the development of electrochemical (bio)sensors and, even when there are several schemes that allow to obtain better analytical performance, our biosensor offers some additional advantages like i) the feasibility to quantify glucose at −0.100 V in a very sensitive and selective way; ii) the application of the biosensor for the quantification of glucose in real samples without any pretreatment, and iii) the quantification of glucose without adding metallic particles, polymers, redox mediators, or permselective membranes.

4 Conclusions

This work reports a new alternative to functionalize SWCNTs through the covalent attachment of Plys to obtain electrochemical (bio)sensors. Plys covalently attached to oxSWCNTs facilitates the dispersion of the nanostructures in aqueous solvents and the anchoring of GOx mainly by electrostatic forces with the positively charged Plys. The resulting GCE/SWCNT-Plys/GOx demonstrated to be highly competitive compared to other glucose biosensors and is quite advantageous if compared to its non-covalent counterpart.

Since the proposed functionalization method relies on the formation of amide bonds between carboxylic groups of oxSWCNTs and amine functions of Plys, this simple strategy can be used for other amino acids, peptides or aminated polymers, opening the doors to new biosensing developments using other biorecognition elements.

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