Sensors and Actuators B 225 (2016) 74–80

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Non-covalent functionalization of multi-walled carbon nanotubes with cytochrome c: Enhanced direct electron transfer and analytical applications

Marcos Eguílaz, Alejandro Gutiérrez, Gustavo Rivas *

INFIQC, Departamento de Físicoquimica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Córdoba 5000, Argentina

ARTICLE INFO

Article history:
Received 14 September 2015
Received in revised form 27 October 2015
Accepted 2 November 2015
Available online 10 November 2015

Keywords:
Multi-walled carbon nanotubes
Cytochrome c
CNT functionalization
Hydrogen peroxide
Electrochemical biosensor
Direct electron transfer.

ABSTRACT

This work reports the non-covalent functionalization (dispersion) of multi-walled carbon nanotubes (MWCNTs) with cytochrome c (Cyt c), the direct electron transfer (DET) after drop-coating deposition of MWCNTs-Cyt c dispersion on glassy carbon electrodes (GCE), and the analytical applications for the highly sensitive quantification of hydrogen peroxide. The dispersion and the resulting modified electrodes were studied by UV–visible spectroscopy, scanning electron microscopy, and electrochemical techniques. The drastic treatment for dispersing the MWCNTs (5.0 min sonication in water with ultrasonic tip) produces a partial denaturation that facilitates the interaction of Cyt c with the CNTs and makes possible an efficient electron transfer between the heme group and the electrode. A critical analysis of the influence of different experimental conditions on the efficiency of the dispersion and on the performance of GCE modified with MWCNTs-Cyt c dispersion is also reported. The analytical parameters obtained with GCE/MWCNTs-Cyt c for the amperometric quantification of hydrogen peroxide at −0.100 V were: sensitivity of (43 ± 1) mA M^{-1} cm^{-2}, linear range between 1.0×10^{-6} and 1.6×10^{-4} M, detection limit of 1.5×10^{-7} M, reproducibility of 3.1% and repeatability of 3.4%. The biosensor was successfully used for the quantification of hydrogen peroxide in mouthwash and spiked milk samples.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The unique physical and chemical properties of carbon nanotubes (CNTs) have made them an excellent component for the development of electrochemical (bio)sensors [1–3]. However, since CNTs present strong van der Waals and π-–π* interactions that induce bundling and prevent their dispersion in most organic and aqueous media [4], different strategies have to be developed to allow their application in the construction of electrochemical (bio)sensors. In this regard, the functionalization of CNTs by using high-frequency sonication in the presence of amphiphilic dispersing agents such as surfactants [5,6], polymers [7,8], proteins [9–11], and DNA [12,13] has demonstrated to be highly effective. The sonication process disaggregates the nanotubes from the bundles while the amphiphilic agent is adsorbed at the surface of CNTs in different extent depending on the nature, stabilizing them in solution either by electrostatic repulsion and/or steric hindrance, and/or other mechanisms [10]. Therefore, the knowledge of the factors that affect the exfoliation and the dispersing efficiency is a critical aspect when developing electrochemical (bio)sensors [14].

We are reporting for the first time a critical study of the non-covalent functionalization (dispersion) of multi-walled carbon nanotubes (MWCNTs) in the protein cytochrome c (Cyt c), the evaluation of the direct electron transfer (DET) of the Cyt c protein that supports the CNTs, and the development of an electrochemical biosensor for hydrogen peroxide (GCE/MWCNT-Cyt c). The dispersion of single-walled carbon nanotubes (SWCNT) with Cyt c has been proposed by Nagaraju et al. [15]; however, there was no critical analysis about the influence of the experimental conditions on the quality of the dispersion and on the electrochemical response of the gold electrode modified with the dispersion, and there is just few data about the analytical parameters of the resulting biosensor, with a very restricted linear range.

The DET of redox proteins is a topic that has received considerable attention for the development of third generation biosensors and as a model system to understand the mechanism of electron transfer in real biological systems [16–19]. Cyt c is a small redox hemeprotein of 104 aminoacids with peroxidase activity [20]. It is well known that the DET between Cyt c and conventional unmodified electrodes is generally slow due to the non-favourable contact between the prostetic group and the electrode, and the

* Corresponding author. Tel.: +54 351 4334169/80; fax: +54 351 4334188.
E-mail address: grivas@fcq.unc.edu.ar (G. Rivas).

http://dx.doi.org/10.1016/j.snb.2015.11.011
0925-4005/© 2015 Elsevier B.V. All rights reserved.

denaturation of the protein after immobilizing in a rigid environment [21]. Several modified electrodes, mainly based on nanomaterials such as carbon nanotubes, graphene, and nanoparticles, have been proposed to provide the Cyt c of a more favourable microenvironment that allows the DET [22–27].

In the following sections we discuss the effect of the amount of MWCNTs, the concentration of the protein, and the sonication time on the effectiveness of the dispersions by using UV–vis spectroscopy, scanning electron microscopy (SEM) and cyclic voltammetry (CV). The DET and biocatalytic activity of the Cyt c that supports the MWCNTs deposited at the surface of glassy carbon electrode (GCE) and the usefulness of GCE/MWCNT-Cyt c for the quantification of hydrogen peroxide are also discussed.

2. Experimental

2.1. Chemicals and solutions

Carbon nanotubes [MWCNTs (30 ± 15) nm diameter, (1–5) μm length, and purity higher than 95%], were supplied from Nanolab (USA). Cytochrome c (Cyt c) from horse heart was supplied by Sigma. Hydrogen peroxide (30% v/v aqueous solution), NaH_2PO_4, and Na_2HPO_4 were purchased from Baker. Other chemicals were of analytical grade and used without further purification. Commercial low fat milk (Argentine dairy company "La Serenisima") and mouthwash samples (Colgate Plax Whitening), were obtained from a local market.

A 0.050 M phosphate buffer solution pH 7.40 was employed as supporting electrolyte. A solution of 1.0 mg mL^{-1} Cyt c prepared in water was used as stock solution. Ultrapure water (ρ = 18.2 MΩ cm) from a Millipore-MilliQ system was used for preparing all aqueous solutions.

2.2. Apparatus

Sonication treatments were carried out with an ultrasonic processor VCX 130 W (Sonics and Materials, Inc.), of 20 kHz frequency with a titanium alloy microtip (3 mm diameter). A Digicen 21 ultracentrifuge (Orto Alresa) with a RT 151 rotor was used to centrifuge the samples after sonication. UV–vis absorption spectra were obtained with a Shimadzu UV-1700 Pharma spectrophotometer using a quartz cuvette of 1 mm path length.

Scanning electron microscopy (SEM) images were obtained with a field emission gun scanning electron microscope (FE-SEM, Zeiss, ΣIGMA model) equipped with secondary and back-scattered electron detectors. For this purpose, samples were prepared by drop-coating of MWCNTs dispersions onto GCE disks and solvent evaporation at room temperature.

Electrochemical experiments were carried out with Autolab (PGSTAT 128 N Eco-Chemie) and TEQ_04 potentiostats. Unmodified glassy carbon electrodes (GCE, CH Instruments, 3 mm diameter) and glassy carbon electrodes modified with MWCNTs-Cyt c dispersion were used as working electrodes. A platinum wire and Ag/AgCl, 3 M NaCl (BAS, Model RE-5B) were used as auxiliary and reference electrodes, respectively. All potentials are referred to this reference electrode. A magnetic stirrer under controlled speed provided the convective transport during the amperometric measurements.

2.3. Preparation of MWCNTs-Cyt c dispersion

MWCNTs were oxidized using a mixture of sulphuric and nitric acids (3:1 v/v) for 5 h under ultrasonic stirring. The oxidized CNTs were repeatedly washed with distiller water up to reach pH∼7. The dispersion was prepared by mixing 1.0 mg of oxidized MWCNTs with 1.0 mL of 0.060 mg mL^{-1} Cyt c solution (prepared in water) followed by sonication with a sonicator probe for 5.0 min. The amplitude was 50% and the sample was kept in an ice-bath during the procedure. After this treatment, the sample was centrifuged at 1500 rpm for 15 min, taking the supernatant for further work. For comparison, a dispersion of oxidized MWCNTs in water (MWCNTs-water) was prepared using similar conditions.

2.4. Preparation of glassy carbon electrodes modified with MWCNTs-Cyt c dispersion

GCE surfaces were polished with alumina slurries of 1.0, 0.3, and 0.05 μm for 1 min each, rinsed thoroughly with deionized water, sonicated for 30 s in water, and finally dried under a N_2 stream. GCE/MWCNTs-Cyt c was prepared by casting 20 μL of MWCNTs-Cyt c onto the glassy carbon surface, followed by the evaporation of the solvent at room temperature for 60 min. For comparison, the GCE was also modified with a dispersion of MWCNTs in water and once the solvent was evaporated, an aliquot of 20 μL of 0.060 mg mL^{-1} Cyt c solution (prepared in water) was deposited on the top of the electrode (GCE/MWCNTs/Cyt c). Both biosensors, GCE/MWCNTs-Cyt c and GCE/MWCNTs/Cyt c, were rinsed with deionized water before using.

2.5. Procedure

The electrochemical experiments were performed in a N_2-saturated 0.050 M phosphate buffer solution pH 7.40. Cyclic voltammetry (CV) experiments were carried out between 0.500 and −0.900 V at a scan rate of 0.050 V s^{-1}.

Amperometric experiments were performed in stirred solutions by applying −0.100 V as working potential and allowing the transient current to reach a steady-state value prior to the addition of the analyte and the subsequent current monitoring. All experiments were conducted at room temperature.

2.6. Determination of H_2O_2 in real samples

Mouthwash and low fat milk samples were used to evaluate the analytical performance of the proposed biosensor. Milk samples were spiked with known levels (10.0 and 30.0 mM) of hydrogen peroxide. The electrochemical measurements were performed by transferring aliquots of 10 μL of 1:100 stock solution of the mouthwash sample (diluted with the phosphate buffer) or 10 μL of the spiked milk samples to the electrochemical cell containing 5.0 mL of 0.050 M phosphate buffer solution pH 7.40. In all cases the determination of hydrogen peroxide was performed by amperometry at −0.100 V and the hydrogen peroxide content was determined by using the standard additions method.

3. Results and discussion

3.1. Characterization of MWCNTs-Cyt c dispersion

UV–vis spectroscopy is an effective tool to prove the efficient disaggregation of CNTs since the individual nanotubes strongly absorb around 265 nm, at variance with the aggregates that are hardly active in this frequency region [28]. This technique is also useful to study the interaction between the hemeprotein and CNTs since the wavelength of the maximum absorption and the intensity of the absorption bands, in particular the Soret one, are sensitive to the conformational state of the protein [25,29,30,32].

Fig. 1A shows the UV–vis spectra for: (a) 0.060 mg mL^{-1} native Cyt c in water; (b) 0.060 mg mL^{-1} Cyt c (in water) sonicated for 5.0 min with ultrasonic tip and centrifuged for 15 min at 1500 rpm; (c) the supernatant of the dispersion of 1.0 mg mL^{-1} MWCNTs in water after 15 min centrifugation at 1500 rpm; and (d) the supernatant of the dispersion of 1.0 mg mL^{-1} MWCNTs in 0.060 mg mL^{-1}

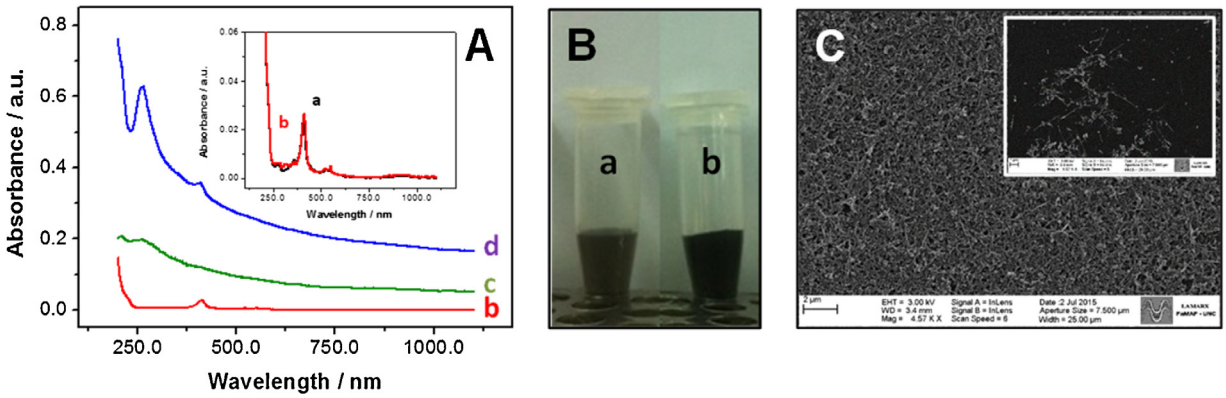

Fig. 1. (A) UV–vis absorption spectra for: (a) 0.06 mg mL^{-1} native Cyt c; (b) 0.06 mg mL^{-1} Cyt c sonicated for 5.0 min and centrifuged for 15 min at 1500 rpm; (c) supernatant of 1.0 mg mL^{-1} MWCNTs dispersed in water; (d) supernatant of 1.0 mg mL^{-1} MWCNTs dispersed in 0.06 mg mL^{-1} Cyt c. Sonication time 5.0 min, acoustic amplitude 50% and centrifugation at 1500 rpm for 15 min. (B) Pictures for dispersions of MWCNTs prepared in water (a) and 0.06 mg mL^{-1} Cyt c solution (b), obtained by ultrasonication for 5.0 min and centrifugation at 1500 rpm for 15 min. (C) SEM micrograph of glassy carbon disk modified with dispersion of MWCNTs-Cyt c. The inset corresponds to a SEM micrograph of glassy carbon disk modified with a dispersion of MWCNTs-water. Magnification: 4570×. Sonication time: 5.0 min, acoustic amplitude 50% and centrifugation at 1500 rpm for 15 min.

Cyt c followed by the centrifugation at 1500 rpm for 15 min. Native Cyt c (a) has two typical absorption bands at 409 nm (Soret band) and 530 nm (Q band), which are due to the chromophore of porphyrin ring [30,31]. The spectrum for sonicated Cyt c (b) presents a red-shift in the Soret band (wavelength of maximum absorption: 412 nm) as well as a split of the band Q in two small bands, both changes attributed to the denaturation of the protein [33]. The supernatant of the MWCNTs dispersed in water (c) presents a characteristic absorption band at 263 nm due to 1D van Hove singularities of individual nanotubes [34]. Finally, the spectrum of MWCNTs-Cyt c dispersion (d) presents both, the absorption band due to 1D van Hove singularities of individual nanotubes at 263 nm, which is three times higher than the one obtained for MWCNTs-water dispersion, and the characteristic Soret band of Cyt c at 410 nm, indicating the improvement in the disaggregation process due to the interaction between Cyt c and MWCNTs. The partial denaturation of Cyt c facilitates the dispersion of MWCNTs because the phenylalanine and tyrosine aminoacids of the protein interact more strongly with MWCNTs by π–π stacking effect and van der Waals attraction when the chain is more stretched [9,10].

Fig. 1B shows pictures of MWCNTs dispersions prepared in water (a) and Cyt c solution (b), obtained by ultrasonication for 5.0 min and centrifugation at 1500 rpm for 15 min. The comparison of these pictures demonstrates that higher amount of nanotubes can be exfoliated when MWCNTS are dispersed in Cyt c since Cyt c minimizes the strong van der Waals interaction between the nanotubes walls avoiding their aggregation and improving their compatibility with the solvent.

Fig. 1C displays SEM micrographs of glassy carbon disks modified with MWCNTs-Cyt c. The image reveals that MWCNTs-Cyt c dispersion covers the whole disk surface with an homogeneous distribution. For comparison, the inset presents SEM micrographs of glassy carbon disks modified with MWCNTs dispersed in water. At variance with GCE/MWCNTs-Cyt c, at GCE/MWCNT the CNTs are not homogeneously distributed on the electrode surface, demonstrating, in this way, the advantages of the presence of the protein as dispersing agent.

The analysis of the spectrophotometric and SEM results indicates that the presence of Cyt c not only facilitates the efficient disaggregation of CNTs but also makes possible the homogeneous and robust deposition of the nanostructures at glassy carbon surfaces.

The influence of the different modifications of GCE on the electroactivity of the resulting electrodes was evaluated from cyclic voltammetry experiments using hydroquinone (HQ) as redox probe. Fig. 2 depicts cyclic voltammograms for 1.0×10^{-3} M HQ (prepared in 0.050 M phosphate buffer solution pH 7.40) at GCE (a), GCE/MWCNTs (b), GCE/MWCNTs/Cyt c (c) and GCE/MWCNTs-Cyt c (d) at 0.050 V s^{-1}. A well-defined current peaks system with a peak-to-peak separation (ΔE_p) of 0.354 V is observed at the bare GCE (a). After modification with MWCNTs dispersed in water (b), the oxidation peak current increases 40%, and the ΔE_p decreases 141 mV (ΔE_p = 0.213 V), demonstrating that the presence of MWCNTs largely facilitates the electrooxidation of HQ. When GCE is modified by dropping the dispersion of MWCNTs in water followed by the adsorption of Cyt c (c), there is a large increase of ΔE_p (ΔE_p = 0.470 V) and a significant decrease in the associated peak currents due to the non-conductive nature of the protein that partially hinders the charge transfer between the redox probe and the electrode surface. On the contrary, when GCE is modified with MWCNTs-Cyt c (d) there is an increase in the oxidation peak current of 150% compared to GCE/MWCNTs/Cyt c and around 80% compared to GCE/MWCNTs, while the ΔE_p decreases 255 mV compared to GCE/MWCNTs/Cyt c (ΔE_p = 0.215 V) demonstrating that the non-covalent functionalization of the nanostructures and the consequent disaggregation of the nanotubes exalt the electrocatalytic properties of CNTs towards the oxidation of the redox marker, minimizing the blocking effect of the protein.

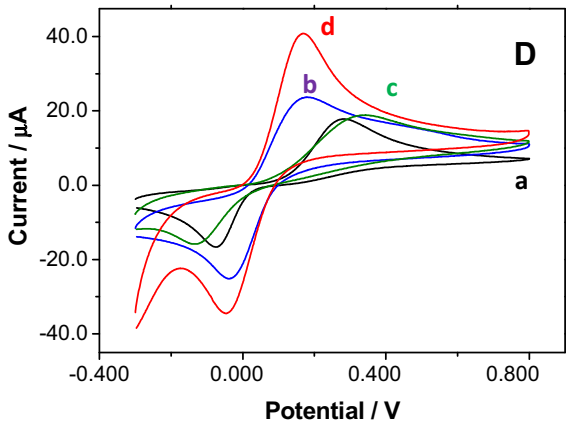

Fig. 2. Cyclic voltammograms obtained at GCE (a), GCE/MWCNTs (b), GCE/MWCNTs/Cyt c (c), and GCE/MWCNTs-Cyt c (d) in a solution of 1.0×10^{-3} M HQ prepared in 0.050 M phosphate buffer solution pH 7.40. Scan rate: 0.050 V s^{-1}.

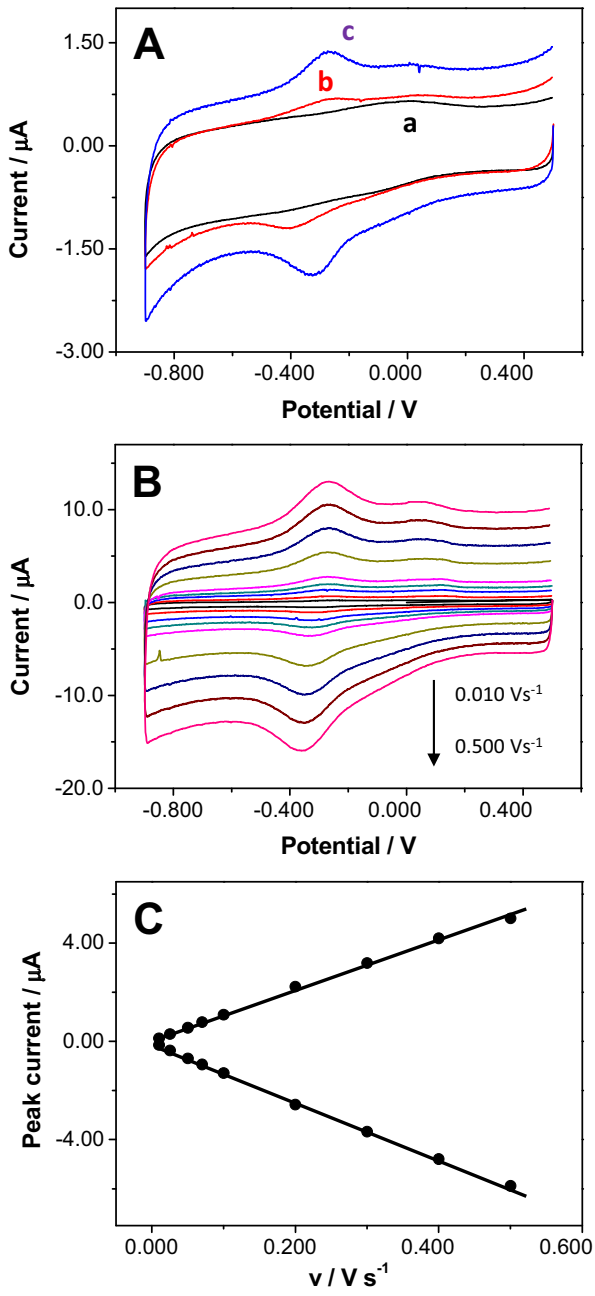

Fig. 3. (A) Cyclic voltammograms obtained at GCE/MWCNTs (a), GCE/MWCNTs/Cyt c (b), and GCE/MWCNTs-Cyt c (c) in a solution of N_2-satured 0.050 M phosphate buffer solution pH 7.40. Scan rate: 0.050 V s^{-1}. (B) Cyclic voltammograms of the GCE/MWCNTs-Cyt c at different scan rates: 0.010, 0.050, 0.070, 0.100, 0.200, 0.300, 0.400, and 0.500 V s^{-1}. (C) Dependence of peak currents on the scan rates.

3.2. Charge transfer of Cyt c at CNTs-modified GCE

Fig. 3A shows cyclic voltammograms obtained in a N_2-satured 0.050 M phosphate buffer solution pH 7.40 at 0.050 V s^{-1} at (a) GCE/MWCNTs, (b) GCE/MWCNTs/Cyt c, and (c) GCE/MWCNTs-Cyt c. At GCE/MWCNTs a poorly defined current peaks system appears around 0 V due to the redox processes associated with the quinone-like oxygen functional groups present at oxidized carbon nanotubes surfaces, generated during the acid treatment [35,36]. At GCE/MWCNTs/Cyt (c) (curve b) there is a pair of quasi-reversible current peaks, the cathodic one at −0.416 V (E_{pc}) and the anodic one at −0.260 V (E_{pa}); with a formal potential (E^0) of −0.338 V and ΔE_p of 0.156 V. Since this peaks system was not observed at GCE/MWCNTs, it is clear that it is due to the direct electron transfer of the prosthetic group of Cyt c, Fe(III). Considering that small changes in the accessibility of the heme group of an hemeprotein can modulate the reduction potential [37], the differences in the E^0 compared to the native protein (E^0 = 0.051 V) can be attributed to the conformational changes produced in the environment of the protein after the immobilization at GCE/MWCNTs [38–40].

The potentiodynamic profiles obtained at GCE modified with MWCNTs-Cyt c dispersion (c) shows clearly defined current peaks system, with E_{pc} = −0.320 V, E_{pa} = −0.268, ΔE_p = 0.052 V, E^0 = −0.294 V, and higher peak currents compared to GCE/MWCNTs/Cyt c. These changes in the voltammetric parameters can be ascribed to a more efficient charge transfer between the Fe(III) located within the Cyt c and the electrode surface due to the close proximity of the protein and CNTs in the dispersion of MWCNTs-Cyt c [23,41]. In this particular case, the non-covalent functionalization of MWCNTs with Cyt c significantly decreases the distance between the redox centre of Cyt c and CNTs and greatly increases the effective surface area of the CNTs for the immobilization of a larger amount of Cyt c in a more favourable orientation. Therefore, the MWCNTs-Cyt c dispersion can provide a convenient microenvironment for the direct electron transfer of Cyt c, largely improving the electrochemical performance of GCE/MWCNTs-Cyt c compared to GCE/MWCNTs/Cyt c.

The effect of the scan rate on the peak currents at GCE/MWCNTs-Cyt c was also evaluated (Fig. 3B). The linear relationship between the cathodic and anodic peaks currents (I_p) and scan rate (υ) in the range between 0.010 and 0.500 V s^{-1} confirms that the electron transfer is a surface-controlled quasi-reversible electrochemical process (Fig. 3C). The average surface concentration (Γ) of electroactive Cyt c present in GCE/MWCNTs-Cyt c was estimated according to the following equation [42]:

$$i_p = \frac{n^2F^2\upsilon\Gamma A}{4RT}$$

where υ is the scan rate, n is the number of the electrons involved in the redox process (n = 1 for Cyt c), F is the Faraday constant, A is the effective area of the electrode, R is the gas constant, and T is the temperature. The average surface concentration of Cyt c at GCE/MWCNTs-Cyt c was 1.9×10^{-10} mol cm^{-2}, which is higher than the theoretical monolayer coverage (1.4×10^{-12} mol cm^{-2}) [43] and higher than the one obtained by self-assembling Cyt c at gold modified with 4-aminothiophenol [15]. The surface coverage obtained at GCE/MWCNTs/Cyt c was 9.1×10^{-11} mol cm^{-2}, a value smaller than the one obtained at GCE/MWCNTs-Cyt c, confirming an enhanced protein loading after the non-covalent functionalization of MWCNTs with Cyt c. This improvement can be attributed to the sonication of the nanostructures in the presence of the protein that produces a chain of cavitation events that enables the separation of the tubes, the diffusion of the stretched protein between the nanotubes and further interaction with them. The electron transfer rate constant (k) was obtained by using the Laviron's model for a surface-controlled electrochemical system when the value of $n\Delta E_p \leq 200$ mV (n is the number of transferred electrons) [44]:

$$m = \frac{RT}{nF}\frac{k}{\upsilon}$$

where m is a parameter related to the ΔE_p. The value of the rate constant k was (3.6 ± 0.2) s^{-1} at GCE/MWCNTs-Cyt c, which is higher than the one obtained for GCE/MWCNTs/Cyt c [(0.88 ± 0.04) s^{-1}]. Moreover, the k value obtained is higher than those reported for Cyt c immobilized at carbon nanotube-DNA (0.89 s^{-1}) [45], graphene-gold nanoparticle (3.14 s^{-1}) [23], and graphene-ionic liquid (2.39 s^{-1}) [25] modified electrodes, and comparable to that of Cyt c immobilized on graphene-carbon nanotube composite (3.4 s^{-1}) [46]. The faster kinetics obtained here indicates that the

close proximity of the protein with MWCNTs at MWCNTs-Cyt c dispersion offers an excellent connection for the electron transfer between Cyt c and the electrode.

3.3. Bioelectrocatalytic properties of GCE/MWCNTs-Cyt c towards the reduction of H_2O_2

Hemeproteins have been widely used for the quantification of hydrogen peroxide due to their known electrocatalytic activity towards this important bioanalyte [47]. To evaluate the biocatalytic activity of GCE/MWCNTs-Cyt c towards the reduction of hydrogen peroxide, we performed amperometric experiments using not only the supernatant of MWCNTs-Cyt c dispersion, but also the whole dispersion. The sensitivity obtained from amperometric experiments performed at −0.100 V demonstrated that even when an excellent response is achieved with both, the GCE modified with the supernatant of the dispersion and the GCE modified with the whole dispersion, the GCE modified with the whole dispersion allowed to obtain higher sensitivity [(43 ± 1) mA M^{-1} cm^{-2} vs (16.9 ± 0.8) mA M^{-1} cm^{-2}]. Taking into account the importance of obtaining higher sensitivities and lower detection limits from the analytical point of view, we used the whole dispersion for further analytical applications.

Fig. 4A shows cyclic voltammograms obtained at GCE/MWCNTs-Cyt c in a 0.050 M phosphate buffer solution pH 7.40 without (dotted line) and with (solid line) 1.0×10^{-2} M H_2O_2. In the absence of H_2O_2, only the redox peaks system of the immobilized Cyt c is observed. After the addition of H_2O_2, the i–E profiles dramatically change, with a huge increment in the cathodic peak current and a large decrease in the anodic ones. The inset demonstrates that no similar cathodic peak is observed at GCE/MWCNTs (without the heme-protein) proving the role of Cyt c in the biocatalytic reduction of H_2O_2. These results clearly demonstrate that the protein retains the electrocatalytic properties towards the reduction of H_2O_2, even after being sonicated, converting GCE/MWCNTs-Cyt c in a promising platform for H_2O_2 sensing.

The optimization of MWCNTs-Cyt c was performed through the evaluation of the electrocatalytic properties of Cyt c from the amperometric response at −0.100 V in the presence of hydrogen peroxide. Fig. 4B shows the sensitivities for H_2O_2 obtained at GCE modified with MWCNTs-Cyt c dispersions prepared by sonication of different amounts of MWCNTs (0.5, 1.0, and 2.0 mg) in 1.0 mL of 0.5 mg mL^{-1} Cyt c (prepared in water) for 5.0 min. The sensitivity increases for electrodes modified with dispersions containing up to 1.0 mg of MWCNTs and decreases thereafter, suggesting that the amount of protein is not enough to disperse these amounts of MWCNTs. Therefore, 1.0 mg of MWCNTs was selected for further experiments.

Fig. 4C displays the H_2O_2 sensitivities obtained at GCE modified with MWCNTs-Cyt c dispersions obtained by sonication of 1.0 mg mL^{-1} MWCNTs with 0.5 mg mL^{-1} Cyt c (prepared in water) for different times between 1.0 and 20 min. The sensitivity increases with the ultracavitation time until reaching a maximum after 5.0 min. For electrodes modified with dispersions obtained after 20 min sonication, the sensitivity decreased in a factor of 18%. This decrease can be associated to a more important

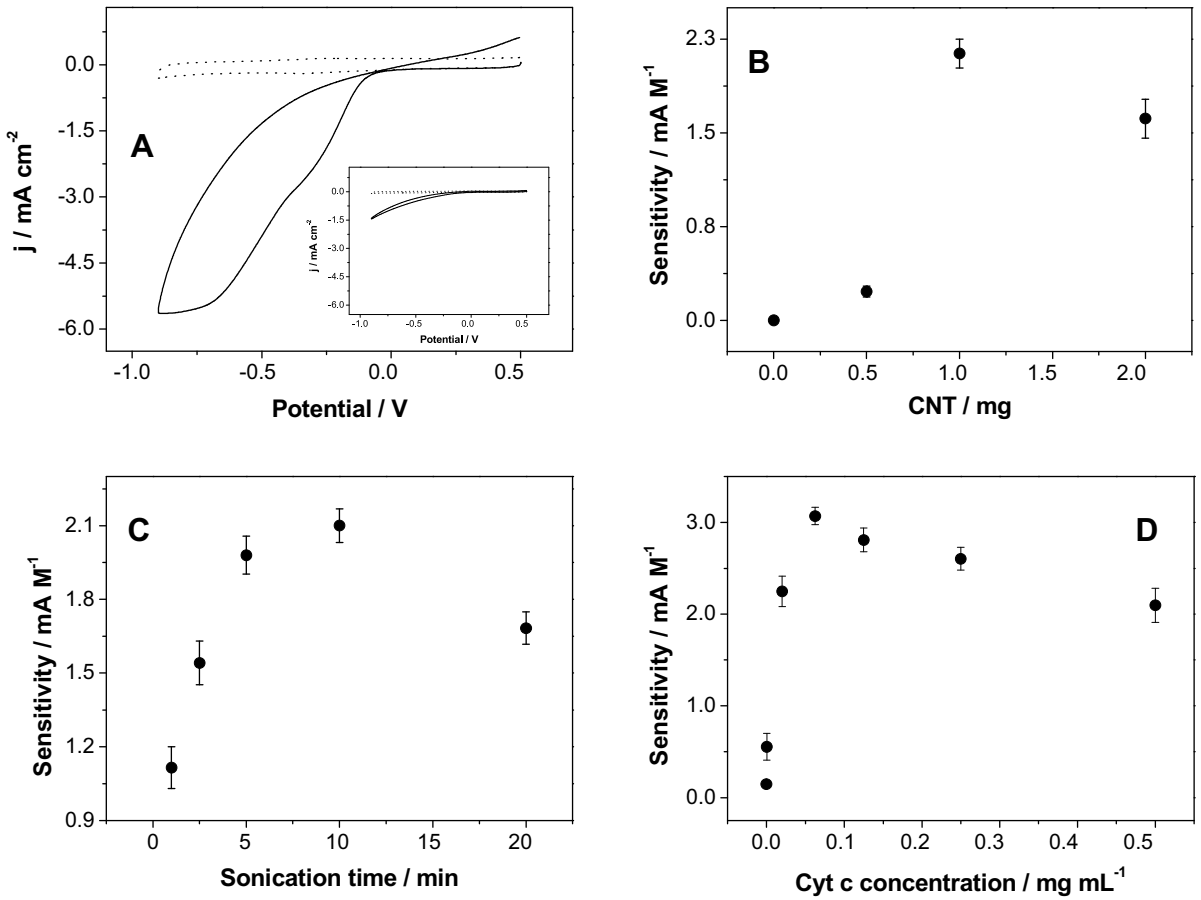

Fig. 4. (A) Cyclic voltammograms obtained at GCE/MWCNTs-Cyt c in a 0.050 M N_2-satured phosphate buffer solution pH 7.40 without (dotted line) and with (solid line) 1.0×10^{-2} M H_2O_2. Inset: cyclic voltammograms obtained at GCE/MWCNTs in the same conditions. Scan rate: 0.050 V s^{-1}. (B) Effect of the amount of MWCNTs on the sensitivity of GCE/MWCNTs-Cyt c towards H_2O_2. Sonication time: 5.0 min, Cyt c concentration: 0.5 mg mL^{-1}. (C) Effect of the sonication time on the sensitivity of GCE/MWCNTs-Cyt c towards H_2O_2. Amount of MWCNTs: 1.0 mg, Cyt c concentration: 0.5 mg mL^{-1}. (D) Effect of Cyt c concentration on the sensitivity of GCE/MWCNTs-Cyt c towards H_2O_2. Amount of MWCNTs: 1.0 mg. Sonication time: 5.0 min. Working potential: −0.100 V.

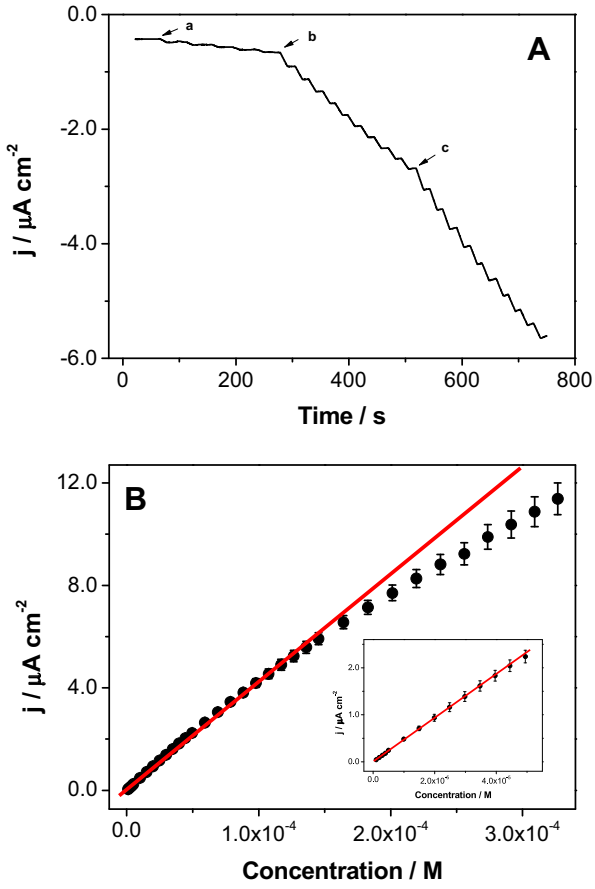

Fig. 5. (A) Amperometric recording for successive additions of H_2O_2 at GCE/MWCNTs-Cyt c: (a) 1.0×10^{-6} M, (b) 5.0×10^{-6} M, and (c) 1.0×10^{-5} M. (B) Calibration plot obtained from the amperometric recording shown in Fig. 5A. Working potential: −0.100 V.

denaturation of the protein when increasing the sonication time. It is important to remark that when MWCNTs and Cyt c were mixed using a vortex, the dispersion was not successful and when an aliquot was deposited onto the GCE, the deposit was unstable and the response was poor and non-reproducible (not shown). These results demonstrate that the sonication is necessary to allow the intimate interaction between MWCNTs and Cyt c, and that there is a critical time to obtain the highest sensitivity with the lowest denaturation effect. The best compromise was reached using a sonication time of 5.0 min.

The effect of the concentration of Cyt c used to disperse MWCNTs was also evaluated in the range between 0 and 0.50 mg mL^{-1} (Fig. 3D). The sensitivity to H_2O_2 increases when the dispersions were prepared with Cyt c up to 0.060 mg mL^{-1} and decreases for higher concentrations probably due to the insulating nature of the protein. According to the results previously shown, 0.060 mg mL^{-1} Cyt c was selected to establish the analytical characteristics of the developed biosensor.

Fig. 5A displays the amperometric recordings obtained with the GCE/MWCNTs-Cyt c biosensor at −0.100 V for successive additions of H_2O_2. A fast response was observed after the addition of H_2O_2 (steady-state reached in 2 s), demonstrating that the intimate contact of CNTs with the biorecognition element (Cyt c), and the efficient biocatalytic activity of the heme-protein that supports the CNTs makes possible to obtain an efficient amperometric response for hydrogen peroxide. Fig. 5B shows the calibration plot for hydrogen peroxide at GCE/MWCNTs-Cyt c obtained from the amperometric recordings ($n = 5$). The linear range goes from 1.0×10^{-6} to 1.6×10^{-4} M ($r = 0.997$), the sensitivity is (43 ± 1) mA M^{-1} cm^{-2}, which is 20-fold higher than the one obtained at GCE/MWCNTs without Cyt c (2.2 mA M^{-1} cm^{-2}), and the detection limit is 1.5×10^{-7} M (taken as $3.3\sigma/S$, where σ is the standard deviation of the blank signal and S the sensitivity). This detection limit is similar or lower than those obtained with other Cyt c-configurations based on carbon nanotubes [48–50]. It is important to remark that the amperometric quantification at SWCNT-Cyt c assembled at 4-aminothiophenol-gold modified electrode shows a linear range between 3.8 and 34.3 mM hydrogen peroxide, concentrations more than two orders of magnitude higher than the ones reported here [15].

The GCE/MWCNTs-Cyt c revealed a high reproducibility, with a relative standard deviation of 3.1% calculated from the sensitivities for hydrogen peroxide obtained with five biosensors prepared with five different MWCNTs-Cyt c dispersions. The reusability of the same electrode surface was excellent, with a relative standard deviation of 3.4% for five successive calibration plots using the same surface. The stability of MWCNTs-Cyt c dispersion stored at 4 °C was also evaluated from amperometric experiments performed at −0.100 V using GCE modified with this dispersion. After 14 days, the sensitivity remained in a 92% of the original value, indicating an excellent stability of the MWCNTs-Cyt c dispersion.

The GCE/MWCNTs-Cyt c biosensor was used to determine the hydrogen peroxide content in mouthwash and spiked low-fat milk samples. As described in Section 2.5, in order to minimize matrix effect, the determination was accomplished by using the standard addition method. Results obtained by triplicate yielded a mean value of (1.48 ± 0.03)% w/v for mouthwash sample, demonstrating an excellent agreement with the value reported by the laboratory (1.5% w/v). In the case of low-fat milk samples spiked with 10.0 mM and 30.0 mM H_2O_2, mean recoveries were 98% and 100% with relative standard deviations of 2.9% and 2.6%, respectively. The obtained results demonstrated the usefulness of the developed biosensor for the quantification of hydrogen peroxide at low potentials in real samples.

4. Conclusions

We reported the non-covalent functionalization of MWCNTs in Cyt c with a critical analysis of the influence of the experimental conditions on the exfoliation of the CNTs and on the performance of the resulting GCE modified with MWCNT-Cyt c dispersion. Spectroscopic experiments demonstrated that the drastic treatment for dispersing the MWCNTs (5.0 min sonication in water with an ultrasonic tip) produces a partial denaturation of the protein that facilitates the dispersion of MWCNTs and makes possible an efficient electron transfer between the heme group of the protein and the glassy carbon electrode after deposition of the dispersion MWCNTs-Cyt c. The resulting biosensor demonstrated to be a highly competitive strategy to quantify hydrogen peroxide, with excellent performance in different samples. It is important to remark that the resulting GCE/MWCNTs-Cyt c represents a new alternative to build supramolecular architectures, opening the doors to new and exciting possibilities for the development of biosensors using other biorecognition molecules.

References

[1] C. Gao, Z. Guo, J.-H. Liu, X.-J. Huang, Nanoscale 4 (2012) 1948.
[2] C.B. Jacobs, M.J. Peairs, B.J. Venton, Anal. Chim. Acta 662 (2010) 105.
[3] A. Walcarius, S.D. Minteer, J. Wang, Y. Lin, A. Mercoci, J. Mater. Chem. B 1 (2013) 4878.
[4] T. Premkumar, R. Mezzenga, K.E. Geckeler, Small 8 (2012) 1299.
[5] R. Rastogi, R. Kaushal, S.K. Tripathi, A.L. Sharma, I. Kaur, L.M. Bharadwaj, J. Colloid Interface Sci. 328 (2008) 421.
[6] E.E. Tkalya, M. Ghislandi, G. de With, C.E. Koning, Curr. Opin. Colloid Interface Sci. 17 (2012) 225.

[7] E.N. Primo, F.A. Gutierrez, G.L. Luque, P.R. Dalmasso, A. Gasnier, Y. Jalit, M. Moreno, M.V. Bracamonte, M. Eguílaz Rubio, M.L. Pedano, M.C. Rodriguez, N.F. Ferreyra, M.D. Rubianes, S. Bollo, G.A. Rivas, Anal. Chim. Acta 805 (2013) 19.
[8] M.M. Barsan, M.E. Ghica, C.M.A. Brett, Anal. Chim. Acta 881 (2015) 1.
[9] B.-G. Ma, Y.-Q. Li, J.-B. Liang, L.-M. Zhang, J.-Z. Yi, Biochem. Biophys. 1 (2013) 29.
[10] E. Edri, O. Regev, Langmuir 25 (2009) 10459.
[11] F. Gutierrez, M.D. Rubianes, G.A. Rivas, Sens. Actuators B 161 (2012) 191.
[12] E.N. Primo, P. Cañete-Rosales, S. Bollo, M.D. Rubianes, G.A. Rivas, Colloid Surf. B 108 (2013) 329.
[13] R. Wang, J. Sun, L. Gao, J. Zhang, J. Mater. Chem. 20 (2010) 6903.
[14] M. Eguílaz, N.F. Ferreyra, G.A. Rivas, Electroanalysis 26 (2014) 2434.
[15] D.H. Nagaraju, R.K. Pandey, V. Lakshminarayanan, J. Electroanal. Chem. 627 (2009) 63.
[16] K. Zhan, H. Liu, H. Zhang, Y. Chen, H. Ni, M. Wu, D. Sun, Y. Chen, J. Electroanal. Chem. 724 (2014) 80.
[17] Y. Wang, C. Bi, RSC Adv. 4 (2014) 31573.
[18] S.M. Silva, D.M. Pimentel, R.C.S. Luz, F.S. Damos, Int. J. Electrochem. Sci. 7 (2012) 1348.
[19] H.-C. Chen, Y.-M. Tu, C.-C. Hou, Y.-C. Lin, C.-H. Chen, K.-H. Yang, Anal. Chim. Acta 867 (2015) 83.
[20] F.R. Salemme, Ann. Rev. Biochem. 46 (1977) 299.
[21] M. Patila, I.V. Pavlidis, E.K. Diamanti, P. Katapodis, D. Gournis, H. Stamatis, Process Biochem. 48 (2013) 1010.
[22] J. Tanne, B. Dietzel, F.W. Scheller, F. Bier, Electroanalysis 26 (2014) 732.
[23] Y. Song, H. Liu, L. Wan, Y. Wang, H. Hou, L. Wang, Electroanalysis 25 (2013) 1400.
[24] M. Eguílaz, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, J. Electroanal. Chem. 644 (2010) 30.
[25] H. Chen, G. Zhao, J. Solid State Electrochem. 16 (2012) 3289.
[26] K.K. Reddy, K.V. Gobi, Electrochim. Acta 78 (2012) 109.
[27] Y. Wang, X. Bian, L. Liao, J. Zhu, K. Guo, J. Kong, B. Liu, Microchim. Acta 178 (2012) 277.
[28] F. Lu, S. Zhang, L. Zheng, J. Mol. Liq. 173 (2012) 42.
[29] H.-Z. Zhao, Q. Du, Z.-S. Li, Q.-Z. Yang, Sensors 12 (2012) 10450.
[30] R.R.R. Sardari, S.R. Zarchi, S. Hajihosseini, Z. Aghili, R. Rasoolzadeh, S. Imani, H. Borna, A.M. Zand, A. Nejadmoghadam, M. Ghalavand, Y. Panahi, Int. J. Electrochem. Sci. 8 (2013) 11578.
[31] J.F. Cerda, C.X. Guzman, H. Zhang, E.J. Amendola, J.D. Castorino, N. Millet, A.L. Fritz, D.N. Houchins, M.H. Roeder, Electrochem. Commun. 33 (2013) 76.
[32] S. Gupta, A. Irihamye, AIP Adv. 5 (2015) 037106.
[33] J.F. Cerda, C.X. Guzman, H. Zhang, E.J. Amendola, J.D. Castorino, N. Millet, A.L. Fritz, D.H. Houchins, M.H. Roeder, Electrochem. Commun. 33 (2013) 76.
[34] Y. Liu, L. Yu, S. Zhang, J. Yuan, L. Shi, L. Zheng, Colloid Surf. A 359 (2010) 66.
[35] Y.-L. Yang, B. Unnikrishnan, S.-M. Chen, Int. J. Electrochem. Sci. 6 (2011) 3743.
[36] S. Chakraborty, C.R. Raj, J. Electroanal. Chem. 609 (2007) 155.
[37] J.S. Vrettos, M.J. Reifler, O. Kievit, K.V. Lakshmi, J.C. de Paula, G.W. Brudvig, J. Biol. Inorg. Chem. 6 (2001) 708.
[38] X. Chen, R. Ferrigno, J. Yang, G.M. Whitesides, Langmuir 18 (2002) 7009.
[39] S.-F. Wang, T. Chen, Z.-L. Zhang, X.-C. Shen, Z.-X. Lu, D.-W. Pang, K.-Y. Wong, Langmuir 21 (2005) 9260.
[40] Y. Wang, K. Qian, K. Guo, J. Kong, J.-L. Marty, C. Yu, B. Liu, Microchim. Acta 175 (2011) 87.
[41] J. Xu, F.J. Shang, J.H.T. Luong, K.M. Razeeb, J.D. Glennon, Biosens. Bioelectron. 25 (2010) 1313.
[42] E. Laviron, J. Electroanal. Chem. 100 (1979) 263.
[43] R.E. Dickerson, T. Takano, D. Eisenberg, O.B. Kallai, L. Samson, A. Cooper, E. Margoliash, J. Biol. Chem. 246 (1971) 1511.
[44] E. Laviron, J. Electroanal. Chem. 101 (1979) 19.
[45] J.-W. Shie, U. Yogeswaran, S.-M. Chen, Talanta 74 (2008) 1659.
[46] B. Dinesh, V. Mani, R. Saraswathi, S.-M. Chen, RSC Adv. 4 (2014) 28229.
[47] Y.W. Lin, J. Wang, J. Inorg. Biochem. 129 (2013) 162.
[48] S.A. Kumar, S.-F. Wang, C.-T. Yeh, H.-C. Lu, J.-C. Yang, Y.-T. Chang, J. Solid State Electrochem. 14 (2010) 2129.
[49] K.-P. Lee, A.I. Gopalan, S. Komathi, Sens. Actuators B 141 (2009) 518.
[50] C. Xiang, Y. Zou, L.-X. Sun, F. Xu, Electrochem. Commun. 10 (2008) 38.

Biographies

Marcos Eguílaz Rubio received his degree in Chemical Sciences with a specialization in Environment from the Complutense University of Madrid (Madrid, España) in 2006 and his PhD in Analytical chemistry in 2012 from the same university. He performed post-doctoral research in the group of Electroanalysis and Electrochemical (bio)sensors of the Complutense University of Madrid between 2012 and 2013. At present, he is a postdoctoral fellow of CONICET in the Biosensors Group at the Physical Chemistry Department, Faculty of Chemical Sciences, National University of Cordoba, Argentina. His postdoctoral research is focused on the design and characterization of bio-functional analytical platforms based on carbon nanotubes and graphene functionalized with polymers.

Alejandro Gutiérrez Aguilar received his degree in Chemical Sciences from Guanajuato University (Guanajuato, México) in 2002 and his PhD in Analytical Chemistry in 2008 from the same university. He performed post-doctoral research in the group of Electrochemical sensors at the Universidad Autónoma Metropolitana (Iztapalapa, México) between 2009–2011 and 2012–2013. At present, he is a post-doctoral fellow of CONICET in the Biosensors group at the Pysical Chemistry Department, Faculty of Chemical Sciences, Cordoba National University, Argentina. His posdoctoral research is focused on the design of bioanalytical platforms for the quantification of bioanalytes of clinical interest based on nanostructures, polymers and biomolecules.

Gustavo A. Rivas obtained his PhD in Chemistry (1991) from Cordoba National University (Cordoba, Argentina). He did the postdoctoral training at University of Valence, Valence (Spain) in 1994 and 1995 and at New Mexico State University, Las Cruces (USA) between 1995 and 1996. At present, he is full professor at Cordoba National University and Superior Researcher at Argentine Research Council (CONICET). Dr. Rivas was the head of the Department of Physical Chemistry at the Faculty of Chemical Sciences between 2008 and 2010. He was President of the Argentinean Society of Analytical Chemists between 2005 and 2007. Professor Rivas is the recipient of the Ranwell Caputto Award from National Academy of Sciences of Argentina (2001), Rafael Labriola Award from Argentinean Society of Chemistry (2004), and Konex Award in Nanotechnology (2013). He has over 140 publications including peer-reviewed papers and book chapters. His research interests focus on the design and characterization of electrochemical (bio)sensors based on nanostructured materials, the development of new bioanalytical platforms for biosensing in batch and flow systems, the study of DNA damage, and the design of new strategies for nanomaterials and polymers modification.