



# Multi-walled carbon nanotubes functionalized with a new Schiff base containing phenylboronic acid residues: application to the development of a bienzymatic glucose biosensor using a response surface methodology approach

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## Abstract

An innovative supramolecular architecture is reported for bienzymatic glucose biosensing based on the use of a nanohybrid made of multi-walled carbon nanotubes (MWCNTs) non-covalently functionalized with a Schiff base modified with two phenylboronic acid residues (SB-dBA) as platform for the site-specific immobilization of the glycoproteins glucose oxidase (GOx) and horseradish peroxidase (HRP). The analytical signal was obtained from amperometric experiments at  $-0.050$  V in the presence of  $5.0 \times 10^{-4}$  M hydroquinone as redox mediator. The concentration of GOx and HRP and the interaction time between the enzymes and the nanohybrid MWCNT–SB-dBA deposited at glassy carbon electrodes (GCEs) were optimized through a central composite design (CCD)/response surface methodology (RSM). The optimal concentrations of GOx and HRP were  $3.0 \text{ mg mL}^{-1}$  and  $1.50 \text{ mg mL}^{-1}$ , respectively, while the optimum interaction time was 3.0 min. The bienzymatic biosensor presented a sensitivity of  $(24 \pm 2) \times 10^2 \mu\text{A dL mg}^{-1}$  ( $(44 \pm 4) \times 10^2 \mu\text{A M}^{-1}$ ), a linear range between  $0.06 \text{ mg dL}^{-1}$  and  $21.6 \text{ mg dL}^{-1}$  ( $3.1 \mu\text{M}$ – $1.2 \text{ mM}$ ) ( $R^2 = 0.9991$ ), and detection and quantification limits of  $0.02 \text{ mg dL}^{-1}$  ( $1.0 \mu\text{M}$ ) and  $0.06 \text{ mg dL}^{-1}$  ( $3.1 \mu\text{M}$ ), respectively. The reproducibility for five sensors prepared with the same MWCNT–SB-dBA nanohybrid was 6.3%, while the reproducibility for sensors prepared with five different nanohybrids and five electrodes each was 7.9%. The GCE/MWCNT–SB-dBA/GOx–HRP was successfully used for the quantification of glucose in artificial human urine and commercial human serum samples.

**Keywords** Multi-walled carbon nanotubes · Modified glassy carbon electrode · Non-covalent functionalization · Amperometry · Schiff base · Boronic acid · Glucose oxidase · Horseradish peroxidase · Glucose biosensor · Bienzymatic biosensor · Response surface methodology

## Introduction

Carbon-based nanomaterials have been largely used in biosensing applications due to their extraordinary properties. In particular, electrochemical biosensors modified with carbon nanotubes (CNTs) have demonstrated a largely improved performance [1–3]. However, it is important to remark that the key challenge to make possible the fully exploitation of the exceptional characteristics of carbon nanotubes is to minimize their aggregation. Consequently, different surface modifications have been implemented to modify the surface chemistry of these 1D carbon materials [4–6]. Among them, the non-covalent functionalization with biomolecules has

proved to be a very advantageous alternative since it not only allows the exfoliation of the nanostructures, but also gives them specific properties [7–10]. Recently, we have reported the non-covalent functionalization of multi-walled carbon nanotubes (MWCNTs) with a rationally designed Schiff base modified with phenylboronic acid residues (SB-dBA) to obtain nanohybrids with properties that mimic the specific interaction of lectins with glycocompounds [11].

In this work, we present a novel strategy for the preparation of a bienzymatic glucose biosensor through the use of glassy carbon electrodes (GCEs) modified with a nanohybrid of MWCNTs non-covalently functionalized with SB-dBA as platform for the specific anchoring of the glycoproteins glucose oxidase (GOx) and horseradish peroxidase (HRP). This new approach represents an interesting alternative for

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the highly sensitive and selective amperometric glucose biosensing using a cheap and easy-to-prepare platform without the need of expensive biomolecules like lectins or avidin or covalent attachment to anchor the biorecognition element.

The combination of peroxidases or pseudoperoxidases and GOx has demonstrated to be an excellent alternative for the development of bi- or pseudobi-enzymatic glucose biosensors. In the last years, several electrochemical bienzymatic biosensors have been proposed for glucose determination using GOx/HRP, GOx/peroxidases other than HRP or GOx/nanozymes. Gallay et al. [12] presented a bienzymatic glucose biosensor based on the use of a GCE modified with the nanohybrid obtained by non-covalent functionalization of MWCNTs with avidin and GOx, and final anchoring of HRP. Rama et al. [13] used mass-produced stainless-steel pins modified with carbon ink to develop simple and portable amperometric glucose biosensors using GOx/HRP and ferrocyanide as electron-transfer mediator. Ortiz et al. [14] reported an amperometric glucose biosensor using MWCNTs non-covalently functionalized with concanavalin A as platform to allow the immobilization of GOx and HRP. Márquez et al. [15] described the use of an electrodeposited calcium alginate hydrogel as a biocompatible matrix for HRP and GOx immobilization using 3,3',5,5'-tetramethylbenzidine as mediator. Amor-Gutierrez et al. [16] presented a simple and low-cost multiplexed paper-based electrochemical device for the quantification of glucose in the presence of GOx and HRP using ferrocyanide as electron-transfer mediator. Liu et al. [17] reported the sensitive glucose biosensing through the simultaneous entrapment of GOx and HRP during the HRP-catalyzed polymerization of noradrenaline in the presence of hydrogen peroxide. Wang et al. [18] proposed the use of reduced graphene oxide as platform for the immobilization of GOx, HRP, and the redox mediator poly(toluidine blue). The modification of GCE with a nanohybrid of MWCNTs functionalized with cytochrome c and GOx, using hydroquinone as redox mediator, was reported by Eguílaz et al. [19]. Izadyar et al. [20] proposed the use of GOx and a recombinant Mn peroxidase from corn for the successful bienzymatic biosensing of glucose. Gao et al. [21] presented a platform made of Au@Fe<sub>3</sub>O<sub>4</sub> nanoparticles modified with GOx and chloroperoxidase by layer-by-layer assembly. Gallay et al. [22] reported a pseudobienzymatic glucose biosensor based on the use of GCEs modified with a nanohybrid of avidin-modified MWCNTs, biotinylated GOx, and in situ electrogenerated Ru nanoparticles to mimic peroxidase.

In the following sections, we discuss the optimization of the experimental conditions to prepare the bienzymatic amperometric biosensor using a response surface methodology (RSM) coupled with a CCRD (central composite rotatable design) matrix, the analytical performance, and the

practical application of the resulting biosensor in samples of clinical relevance.

## Experimental

### Reagents

Glucose oxidase (GOx) (type X-S, *Aspergillus niger*, 50,000 units/g solid, catalog number G7141), horseradish peroxidase (HRP) (type I,  $\geq 50$  units/mg solid, catalog number P8125), human immunoglobulin G (IgG), human serum albumin (HSA), ascorbic acid (AA), uric acid (UA), hydroquinone (HQ), and multi-walled carbon nanotubes powder (MWCNTs, diameter  $(30 \pm 15)$  nm, length 1–5  $\mu\text{m}$ ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were reagent grade and used without further purification. The supporting electrolyte was 0.050 M phosphate buffer solution pH 7.40. All the aqueous solutions were prepared with ultrapure water ( $\rho = 18 \text{ M}\Omega \text{ cm}$ ) from a Millipore-MilliQ system (Molsheim, France).

### Apparatus and procedure

Electrochemical experiments were performed with a TEQ\_4 potentiostat. Electrochemical impedance spectroscopy (EIS) measurements were carried out with a PGSTAT30 potentiostat (Metrohm, Herisau, Switzerland). The working electrode was a GCE modified with the MWCNT-SB-dBA nanohybrid, GOx, and HRP (GCE/MWCNT-SB-dBA/GOx-HRP). The auxiliary and reference electrodes were a platinum wire and a Ag/AgCl (3 M NaCl), respectively. All potentials are referred to the latter.

A TB04TA ultrasonic cleaner (Testlab, Bernal, Argentina) of 40-kHz frequency and 160 W of ultrasonic power was used for performing the sonication treatments.

### Optimization of the experimental conditions using CCD-RSM design

A CCRD combined with RSM was used for the simultaneous optimization of statistically significant variables (factors) on the analytical performance of the bienzymatic amperometric glucose biosensor. In this work, a set of 17 experiments, including three replicates at the central point, were designed using CCRD with three factors (GOx concentration, HRP concentration, and interaction time) at three levels using DesignExpert version 12.0 (Stat-Ease Inc., Minneapolis, USA). The amperometric response obtained in the presence of  $5.0 \times 10^{-4}$  M hydroquinone and  $3.6 \text{ mg dL}^{-1}$  glucose was chosen as the dependent variable (response).

## Preparation of glassy carbon electrode modified with MWCNT–SB–dBA nanohybrid and the glycoenzymes (GCE/MWCNT–SB–dBA/GOx–HRP)

MWCNT–SB–dBA nanohybrid was prepared according to the conditions reported by Tamborelli et al. [11]. Briefly, 2.0 mg of MWCNTs was mixed with 0.50 mg SB–dBA dissolved in 1.0 mL of *N,N*-dimethylformamide (DMF) followed by sonication with an ultrasonic bath for 30.0 min (Fig. 1).

Before modification, GCEs (3.0 mm diameter) were polished with slurries of different alumina size (1.0, 0.3, and 0.05  $\mu\text{m}$ ), followed by an exhaustive rinsing with MilliQ water, sonication for 5 s in water, and modification by deposition of 10  $\mu\text{L}$  of MWCNT–SB–dBA nanohybrid. Once the solvent was evaporated at room temperature, the bienzymatic glucose biosensor was obtained by dropping 10  $\mu\text{L}$  of a solution containing 3.00  $\text{mg mL}^{-1}$  GOx + 1.50  $\text{mg mL}^{-1}$  HRP (prepared in 0.050 M phosphate buffer pH 7.40) at the resulting GCE/MWCNT–SB–dBA, allowing to interact for 3.0 min (Fig. 1). After washing with 0.050 M phosphate buffer solution pH 7.40, the bienzymatic biosensor was ready to use.

### Procedure

All electrochemical experiments were performed at room temperature in a 0.050 M phosphate buffer solution pH 7.40. Amperometric experiments were performed under mechanical stirring at  $-0.050$  V in the presence of  $5.0 \times 10^{-4}$  M hydroquinone as redox mediator.

EIS parameters were the following: amplitude: 0.010 V, frequency range:  $1.0 \times 10^{-2}$  to  $1.0 \times 10^5$  Hz, and working potential: 0.050 V. Hydroquinone/benzoquinone ( $1.0 \times 10^{-3}$  M, prepared in the phosphate buffer solution) was used as a redox probe. The EIS spectra were analyzed and fitted using the Z-view program.

### Analytical applications for glucose quantification in samples of clinical interest

We evaluated the usefulness of the proposed bienzymatic biosensor for glucose quantification in commercial human serum samples (Standatrol S-E, Wiener lab., Rosario, Santa Fe, Argentina) and artificial human urine samples prepared according to the procedure described previously by Sarigul et al. [25].

A deproteinization step was necessary to eliminate the bilirubin interference for the amperometric determination of glucose in these serum samples [23]. The reconstituted commercial human serum samples were diluted four times with a cold mix of methanol:ethanol (1:1) and vortex-mixed for 1.0 min. Then, the samples were kept on ice bath for 20.0 min

and centrifuged at 8000 rpm for 20.0 min [24]. After that, the supernatant was transferred into a vial and stored at 4 °C until further analysis.

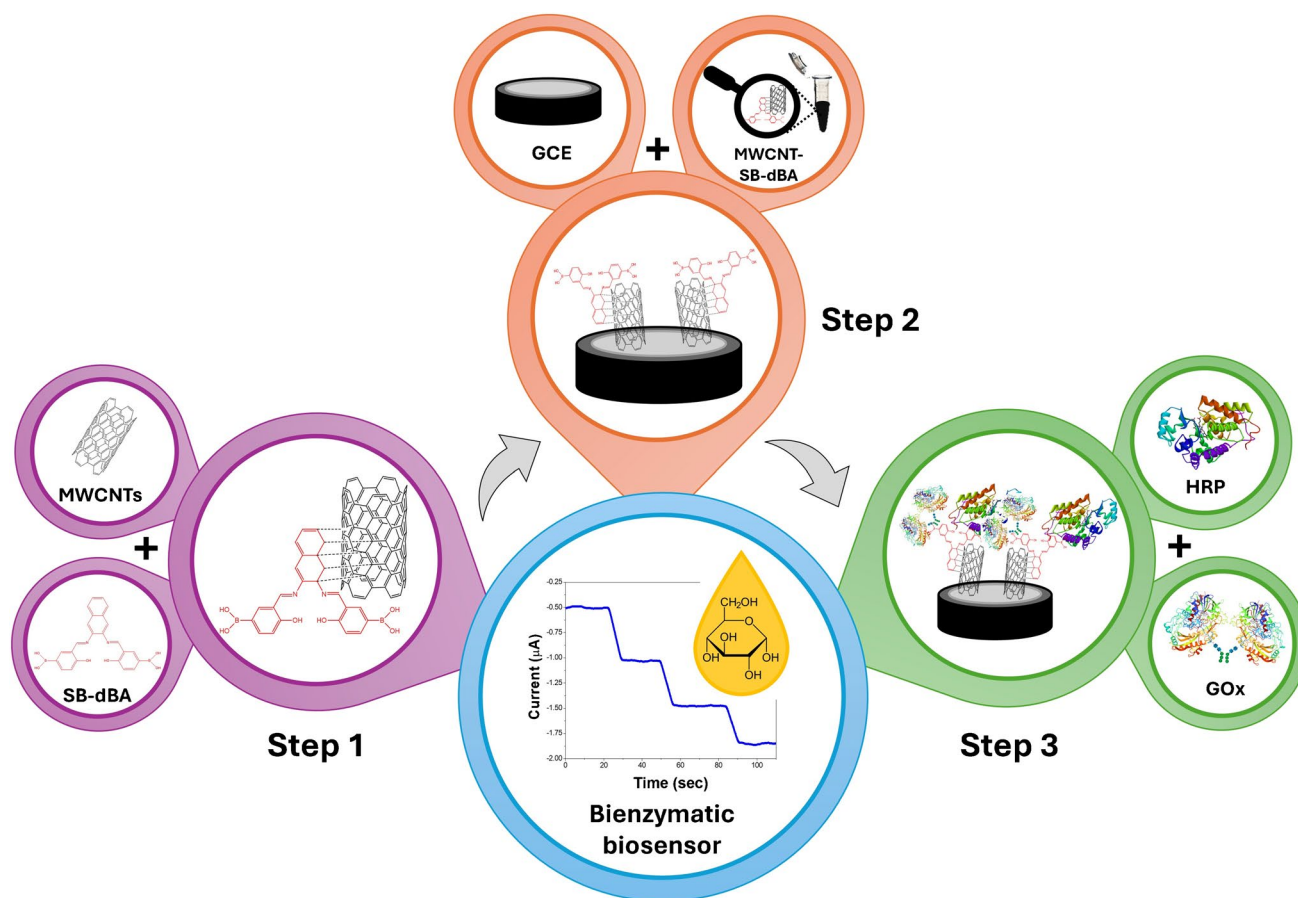
Enriched samples of artificial human urine were obtained by adding glucose concentrations equivalent to the glucose levels detected with the reagent strips used in the clinical laboratory for testing glucose in urine samples.

## Results and discussion

### Specificity of the interaction between GOx and HRP with GCE/MWCNT–SB–dBA

SB–dBA have demonstrated the advantages to present a dual role, both as an exfoliation agent for MWCNTs and as a specific site for anchoring glyco biomolecules [11]. In this work, the two glycoproteins GOx and HRP were anchored at the SB–dBA that supports the MWCNTs to obtain a bienzymatic glucose biosensor.

Figure 2A displays the amperometric response obtained at GCE/MWCNT–SB–dBA and GCE/MWCNT (without the ligand) modified by interaction with 3.00  $\text{mg mL}^{-1}$  GOx and 1.50  $\text{mg mL}^{-1}$  HRP for 3.0 min. There is a well-defined response for successive additions of glucose at the GCE modified with MWCNT–SB–dBA nanohybrid and the enzymes (red line), while no response was obtained at GCE modified with MWCNTs (blue line) since GOx and HRP cannot be immobilized at the electrode due to the absence of the phenylboronic acid residues of the Schiff base. These results demonstrate the importance of SB–dBA as anchoring point for the specific immobilization of the two glycoenzymes used to develop the bienzymatic biosensor. Figure 2B displays a bars plot for the charge transfer resistances ( $R_{CT}$ ) obtained from the Nyquist diagrams shown as inset for GCE/MWCNT–SB–dBA and GCE/MWCNT before and after the interaction with 3.00  $\text{mg mL}^{-1}$  GOx and 1.50  $\text{mg mL}^{-1}$  HRP for 3.0 min using  $1.0 \times 10^{-3}$  M hydroquinone/benzoquinone as redox probe. The corresponding equivalent circuit used for fitting the spectra (Randles circuit) is also indicated in the inset, where  $R_s$  is the resistance of the solution,  $R_{CT}$  corresponds to the charge transfer resistance,  $C_{dl}$  is the double-layer capacitance, and  $W$  the Warburg impedance. The  $R_{CT}$  increases from  $(0.9 \pm 0.1) \times 10^2 \Omega$  at GCE/MWCNT–SB–dBA (black bar, a) to  $(1.7 \pm 0.1) \times 10^2 \Omega$  at GCE/MWCNT–SB–dBA/GOx–HRP (red bar, b) due to the blocking of the surface as a consequence of the non-conductive nature of the proteins, indicating that the glycoenzymes are immobilized at GCE/MWCNT–SB–dBA. In the case of GCE/MWCNT, almost no change in  $R_{CT}$  was obtained after the interaction with 3.00  $\text{mg mL}^{-1}$  GOx and 1.50  $\text{mg mL}^{-1}$  HRP for 3.0 min ( $(0.83 \pm 0.03) \times 10^2$  and  $(0.93 \pm 0.08) \times 10^2 \Omega$  for GCE/MWCNT (blue bar, c) and GCE/MWCNT/GOx–HRP (green bar, d), respectively), confirming the high specificity of



**Fig. 1** Schematic representation of the preparation of MWCNT–SB-dBA nanohybrid, further modification of the GCE surface (GCE/MWCNT–SB-dBA), and interaction with HRP and GOx to obtain the amperometric bienzymatic glucose biosensor (GCE/MWCNT–SB-dBA/HRP-GOx)

the glycoproteins interaction at GCE/MWCNT–SB-dBA.  $R_{CT}$  for GCE (pink bar, e) is also shown for comparative purposes.

### Optimization of GCE/MWCNT–SB-dBA/GOx-HRP preparation using central composite design/response surface methodology

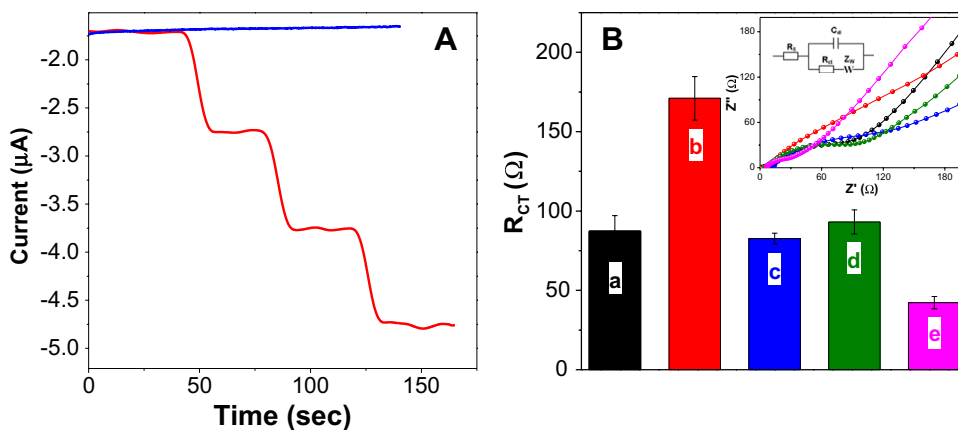
GOx and HRP concentrations and the interaction time of the enzymes with GCE/MWCNT–SB-dBA play a critical role on the performance of the GCE/MWCNT–SB-dBA/GOx-HRP biosensor. The statistical optimization of these independent variables was performed using a central composite rotatable design (CCRD) combined with response surface methodology (RSM) to maximize the amperometric response of 3.6 mg dL<sup>-1</sup> glucose at  $-0.050$  V in the presence of  $5.0 \times 10^{-4}$  M hydroquinone as redox mediator. Table 1 shows the experimental design values of the three factors used in the matrix of the experiments, as well as the responses obtained (reduction currents of the redox mediator after the addition of glucose).

The relationship between the experimental response and the optimization variables can be given by the following second-order polynomial equations in terms of coded factors (Eq. 1) or actual factors (Eq. 2):

$$\begin{aligned} \text{Coded factors : } I = & 0.7113 - 0.0884A + 0.1086B \\ & - 0.0797C - 0.0313A \cdot B + 0.0563A \cdot C \\ & + 0.0062B \cdot C - 0.0606A^2 - 0.0318B^2 - 0.0029C^2 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Actual factors : } I = & -0.093903 + 0.387277\text{HRP} + 0.512291\text{GOx} \\ & - 0.034537\text{Time} - 0.055556\text{HRP} \cdot \text{GOx} \\ & + 0.010000\text{HRP} \cdot \text{Time} + 0.001111\text{GOx} \cdot \text{Time} \\ & - 0.107804\text{HRP}^2 - 0.056484\text{GOx}^2 - 0.000052\text{Time}^2 \end{aligned} \quad (2)$$

where  $I$  represents the reduction current of the redox mediator, while  $A$ ,  $B$ , and  $C$  correspond to the HRP concentration, the GOx concentration, and the interaction time, respectively. Analysis of variance (ANOVA) was used to evaluate the quality of this quadratic regression model, and the results



**Fig. 2** **A** Amperometric response obtained for successive additions of  $3.6 \text{ mg dL}^{-1}$  glucose at GCE/MWCNT-SB-dBA/GOx-HRP (red line) and GCE/MWCNT/GOx-HRP (blue line). GOx concentration:  $3.00 \text{ mg mL}^{-1}$ . HRP concentration:  $1.50 \text{ mg mL}^{-1}$ . MWCNT-SB-dBA-glycoenzymes interaction time: 3.0 min. Redox mediator:  $5.0 \times 10^{-4} \text{ M}$  hydroquinone. Working potential:  $-0.050 \text{ V}$ . **B** Bars plot for the charge transfer resistance ( $R_{CT}$ ) obtained at GCE/MWCNT-SB-dBA (black bar, a), GCE/MWCNT-SB-dBA/GOx-HRP (red bar, b), GCE/MWCNT (blue bar, c), GCE/MWCNT/GOx-HRP (green bar, d), and GCE (pink bar, e). GOx concentration:  $3.00 \text{ mg mL}^{-1}$ ; HRP concentration:  $1.50 \text{ mg mL}^{-1}$ ; MWCNT-SB-dBA-glycoenzymes interaction time: 3.0 min. Redox probe:  $1.0 \times 10^{-3} \text{ M}$  hydroquinone/benzoquinone. Amplitude:  $0.010 \text{ V}$ . Frequency range:  $1.0 \times 10^{-2}$  to  $1.0 \times 10^5 \text{ Hz}$ . Working potential:  $0.050 \text{ V}$ . Inset: Nyquist plots obtained at the corresponding electrodes and the equivalent circuit used to fit the experimental results. Supporting electrolyte:  $0.050 \text{ M}$  phosphate buffer solution pH 7.40

are shown in Table 2. The  $F$  value (48.17) and the remarkably low  $p$  value ( $<0.0001$ ) indicate that the proposed model is statistically significant. In addition, Fig. 3 shows that there is a very good fitting between the actual results and the currents predicted by the model.

According to the coded model (Eq. 1), HRP concentration (A) and the interaction time (C) have a negative effect since an increase in of any of them produces a decrease in the biosensor response. On the contrary, an increment in the concentration of GOx (B) produces a higher response, indicating that this variable has a positive effect. Figure 4 displays 3D surface plots for the reduction current of the redox mediator after glucose addition based on the quadratic regression model for different concentrations of HRP and GOx at three time levels (3.0, 10.5, and 18.0 min). When the GOx concentration increases from  $1.50$  to  $3.00 \text{ mg mL}^{-1}$  and HRP concentration decreases from  $3.00$  to  $1.50 \text{ mg mL}^{-1}$ , the current shows a drastic enhancement, demonstrating the importance of the ratio between GOx and HRP concentrations on the amperometric response of the bienzymatic glucose biosensor. In fact, a considerably higher GOx concentration (compared to HRP) is required to obtain the most efficient cascade response, indicating that the determining step is the production of hydrogen peroxide as a byproduct of the GOx-catalyzed glucose oxidation. Regarding the interaction time, for longer times (from 3.0 to 18.0 min), the current decreases, probably due to an increase of the amount of non-conductive proteins on the electrode surface, with the consequent negative influence on the electron transfer process, and/or a competition between the glycoenzymes

**Table 1** CCRD matrix of the experimental design and experimental responses

| Run | Factor 1<br>HRP (mg mL <sup>-1</sup> ) | Factor 2<br>GOx (mg mL <sup>-1</sup> ) | Factor 3<br>Time (min) | Response<br>Current ( $\mu\text{A}$ ) |
|-----|--|--|------------------------|---------------------------------------|
| 1   | 1.50                                   | 3.00                                   | 3.0                    | 1.00                                  |
| 2   | 1.50                                   | 1.50                                   | 3.0                    | 0.70                                  |
| 3   | 1.50                                   | 1.50                                   | 18.0                   | 0.45                                  |
| 4   | 2.25                                   | 2.25                                   | 10.5                   | 0.70                                  |
| 5   | 2.25                                   | 2.25                                   | 10.5                   | 0.75                                  |
| 6   | 1.26                                   | 2.25                                   | 10.5                   | 0.70                                  |
| 7   | 3.24                                   | 2.25                                   | 10.5                   | 0.50                                  |
| 8   | 2.25                                   | 2.25                                   | 20.4                   | 0.60                                  |
| 9   | 3.00                                   | 3.00                                   | 3.0                    | 0.60                                  |
| 10  | 2.25                                   | 2.25                                   | 10.5                   | 0.70                                  |
| 11  | 2.25                                   | 2.25                                   | 0.6                    | 0.80                                  |
| 12  | 1.50                                   | 3.00                                   | 18.0                   | 0.70                                  |
| 13  | 3.00                                   | 3.00                                   | 18.0                   | 0.60                                  |
| 14  | 3.00                                   | 1.50                                   | 3.0                    | 0.50                                  |
| 15  | 3.00                                   | 1.50                                   | 18.0                   | 0.40                                  |
| 16  | 2.25                                   | 1.26                                   | 10.5                   | 0.50                                  |
| 17  | 2.25                                   | 3.24                                   | 10.5                   | 0.80                                  |

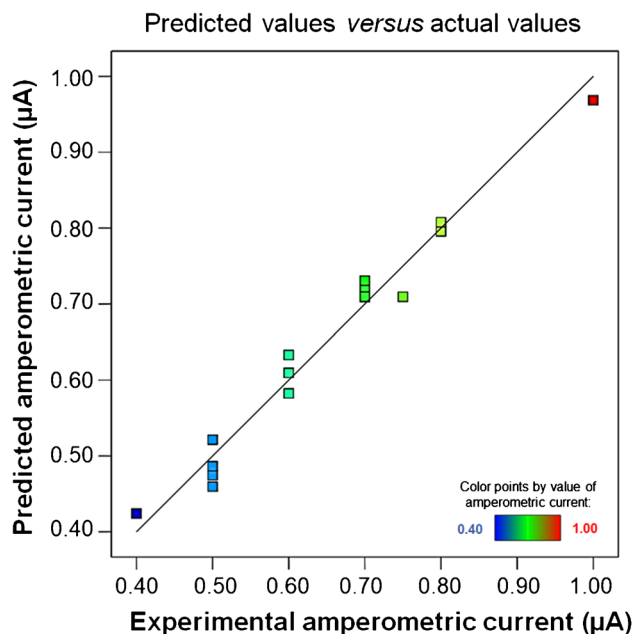
for the available sites at the platform due to the different sizes and density of glycosidic residues. From CCRD/RSM, the optimal level of the variables predicted to obtained the maximum amperometric current with GCE/MWCNT-SB-dBA/GOx-HRP was GOx concentration =  $3.00 \text{ mg mL}^{-1}$ , HRP concentration =  $1.50 \text{ mg mL}^{-1}$ , and interaction

time = 3.0 min. The results presented here demonstrate that RSM is a valuable tool to obtain the most efficient biosensor response through the adequate optimization of the amount of enzymes and time to build the bienzymatic biosensor.

Amperometric experiments performed with GCE/MWCNT–SB–dBA/HRP (Figure S1,a) did not show any response after successive additions of  $3.6 \text{ mg dL}^{-1}$  glucose, as expected, due to the absence of GOx, the enzyme responsible to generate the hydrogen peroxide. Similar experiments performed with GCE/MWCNT–SB–dBA/GOx presented a well-defined response after the additions of glucose due to the reduction of the oxidized redox mediator (Figure S1,b). The presence of GOx and HRP at the surface of GCE/MWCNT–SB–dBA produced a response that is the double of the one obtained in the presence of GOx, demonstrating the importance of the cascade enzymatic reaction to amplify the response (Figure S1,c). The inset shows bar plot for the currents obtained from the amperometric recordings after three additions of  $3.6 \text{ mg dL}^{-1}$  glucose.

### Analytical performance of the biosensor

Figure 5A depicts the amperometric response for successive additions of glucose obtained at GCE/MWCNT–SB–dBA/GOx–HRP at  $-0.050 \text{ V}$  in the presence of  $5.0 \times 10^{-4} \text{ M}$  hydroquinone. A well-defined signal, due to the reduction of the quinone produced during HRP regeneration, was obtained even for  $0.06 \text{ mg dL}^{-1}$  glucose. Figure 5B displays the corresponding calibration plot obtained from the amperogram shown in Fig. 5A. There is a linear relationship between  $0.06 \text{ mg dL}^{-1}$  and  $21.6 \text{ mg dL}^{-1}$  ( $3.1 \mu\text{M}$ – $1.2 \text{ mM}$ ) ( $R^2 = 0.9991$ ), with a sensitivity of  $(24 \pm 2) \times 10^2 \mu\text{A dL mg}^{-1}$  ( $(44 \pm 4) \times 10^2 \mu\text{A M}^{-1}$ ), a detection limit of  $0.02 \text{ mg}$



**Fig. 3** Predicted values of the amperometric response from RSM design versus the experimental currents obtained from amperometric experiments after the addition of  $3.6 \text{ mg dL}^{-1}$  glucose at  $-0.050 \text{ V}$  in the presence of  $5.0 \times 10^{-4} \text{ M}$  hydroquinone as redox mediator

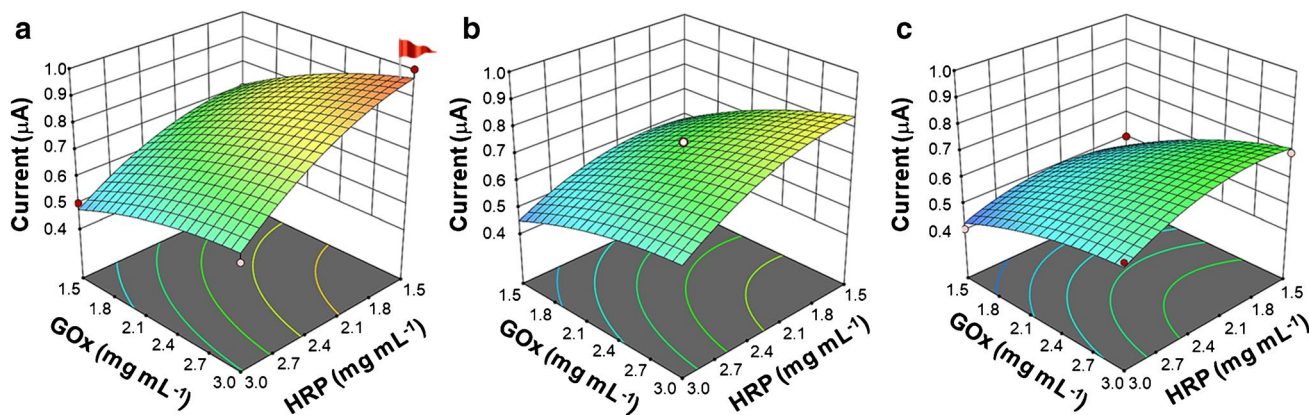
$\text{dL}^{-1}$  ( $1.0 \mu\text{M}$ ) (obtained as  $3 \times$  standard deviation (SD) of the blank signal/sensitivity), and a quantification limit of  $0.06 \text{ mg dL}^{-1}$  ( $3.10 \mu\text{M}$ ) (obtained as  $10 \times$  standard deviation (SD) of the blank signal/sensitivity).

Table 3 summarizes the analytical characteristics of the most relevant electrochemical bienzymatic glucose biosensors reported in the last years. GCE/MWCNT–SB–dBA/GOx–HRP presented a detection limit lower than most of

**Table 2** ANOVA for the CCRD quadratic model

| Source           | Sum of squares | Degree of freedom | Mean square | F value | p value |                 |
|------------------|----------------|-------------------|-------------|---------|---------|-----------------|
| <b>Model</b>     | 0.3615         | 9                 | 0.0402      | 48.17   | <0.0001 | Significant     |
| A–HRP            | 0.0895         | 1                 | 0.0895      | 107.39  | <0.0001 | Significant     |
| B–GOx            | 0.1352         | 1                 | 0.1352      | 162.10  | <0.0001 | Significant     |
| C–Time           | 0.0727         | 1                 | 0.0727      | 87.24   | <0.0001 | Significant     |
| AB               | 0.0078         | 1                 | 0.0078      | 9.37    | 0.0183  | Significant     |
| AC               | 0.0253         | 1                 | 0.0253      | 30.36   | 0.0009  | Significant     |
| BC               | 0.0003         | 1                 | 0.0003      | 0.3748  | 0.5598  |                 |
| A <sup>2</sup>   | 0.0230         | 1                 | 0.0230      | 27.55   | 0.0012  | Significant     |
| B <sup>2</sup>   | 0.0063         | 1                 | 0.0063      | 7.56    | 0.0285  | Significant     |
| C <sup>2</sup>   | 0.0001         | 1                 | 0.0001      | 0.0632  | 0.8087  |                 |
| <b>Residual</b>  | 0.0058         | 7                 | 0.0008      |         |         |                 |
| Lack of fit      | 0.0042         | 5                 | 0.0008      | 1.00    | 0.5685  | Not significant |
| Pure error       | 0.0017         | 2                 | 0.0008      |         |         |                 |
| <b>Cor total</b> | 0.3674         | 16                |             |         |         |                 |

$R^2 = 0.9841$  Adjusted  $R^2 = 0.9637$  Predicted  $R^2 = 0.8494$  Adeq precision = 24.9806

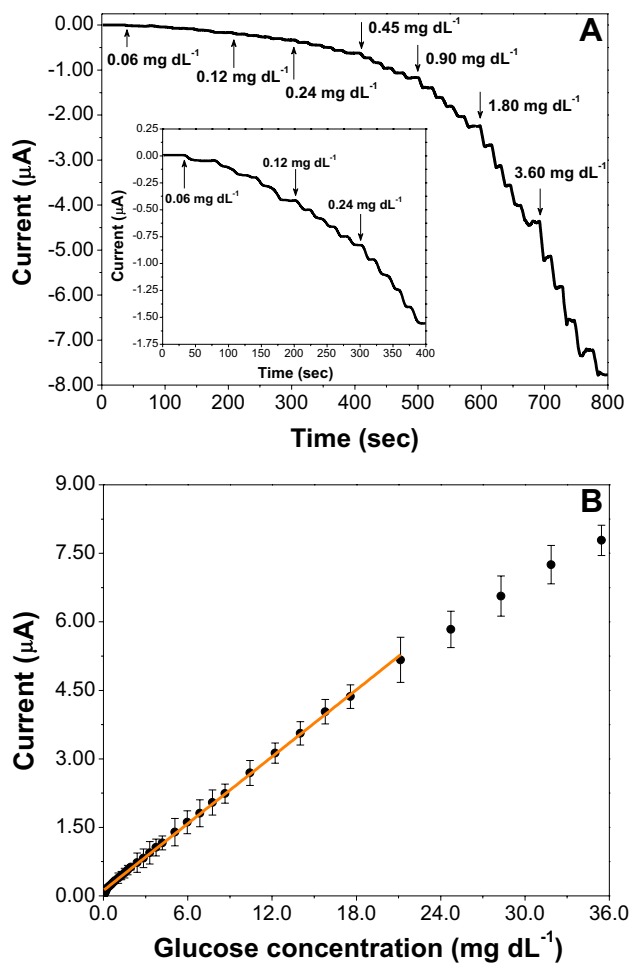


**Fig. 4** 3D response surface plots showing the effect of GOx and HRP concentration on the experimental current at three different interaction times: **a** 3.0 min, **b** 10.5 min, and **c** 18.0 min. The dots represent experimental data points

these biosensors [13, 15, 16, 18–20, 22], similar to the one proposed by Gallay et al. [12], and higher than those reported by Ortiz et al. [14] and Liu et al. [17]. However, it is important to mention that [14] requires a more expensive platform based on the use of concanavalin A and [17] needs a polymerization step for the preparation of each biosensor.

The reproducibility for five biosensors prepared with the same MWCNT–SB–dBA nanohybrid (reproducibility intra-dispersion) was 6.3%, while the reproducibility for five different nanohybrids (reproducibility inter-dispersion using five electrodes for each dispersion) was 7.9%. Sensitivities obtained from amperometric experiments at  $-0.050$  V remained in a  $(90 \pm 1)\%$  of the one obtained the first day after 3 days for biosensors stored at  $4$  °C. The selectivity of the biosensor was evaluated in the presence of possible interferences usually present in human blood serum. Figure S2 displays the amperometric response obtained after the addition of glucose and possible interferences present in human serum at physiological level. With the exception of bilirubin, no interference was observed in the presence of the other compounds.

The biosensor was challenged with artificial human urine and human blood serum. In the case of urine, since glucose was not present in the sample, it was spiked with glucose at concentrations similar to those detected through the different colors of the commonly used laboratory reagent strips for the detection of glucose in urine: 75, 225, 450, 900, and  $1800$  mg dL $^{-1}$ . Figure 6 depicts bar plots for the recovery percentages obtained for the different glucose concentrations added to the urine sample as well as the associated error. An excellent correlation was obtained, with percentages of 94.0, 90.0, 104.3, 105.7, and 101.8, respectively, for the glucose concentrations previously indicated and errors lower than 10%. Taking into account the bilirubin interference shown in Figure S2, the reconstituted human serum samples were pretreated as indicated in the Experimental section. The glucose concentration obtained after three determinations



**Fig. 5** **A** Amperometric response of GCE/MWCNT–SB–dBA/HRP–GOx to successive additions of glucose. Inset: amperometric recordings obtained for additions of 0.06, 0.12, and 0.24 mg dL $^{-1}$  glucose. **B** Calibration plot obtained from the amperometric recordings shown in (A). GOx concentration:  $3.00$  mg mL $^{-1}$ . HRP concentration:  $1.50$  mg mL $^{-1}$ . Interaction time: 3.0 min. Redox mediator:  $5.0 \times 10^{-4}$  M hydroquinone. Working potential:  $-0.050$  V. Supporting electrolyte:  $0.050$  M phosphate buffer solution pH 7.40

**Table 3** Comparison of techniques, platforms, and analytical performance of electrochemical bienzymatic biosensors for glucose reported in the last years

| Technique         | Platform  | LR ( $\mu\text{M}$ ) | LOD ( $\mu\text{M}$ ) | Real samples           | Ref.      |
|-------------------|---|----------------------|-----------------------|------------------------|-----------|
| Amperometry       | GCE/MWCNT-GOx-Av/bHRP                           | 25–750               | 1.2                   | Nasal spray, milk      | 12        |
| Chronoamperometry | SSPC/GOx/HRP                                    | 50–1000              | 30                    | Honey, orange juice    | 13        |
| Amperometry       | GCE/MWCNT-ConA/GOx-HRP                          | 2–410                | 0.31                  | Human serum            | 14        |
| Chronoamperometry | SPGE/ <i>e</i> -alginate/GOx-HRP                | 2000–12,000          | 126                   | Blood                  | 15        |
| Chronoamperometry | PE/GOx/HRP                                      | 500–15,000           | 400                   | Orange juice, beverage | 16        |
| Amperometry       | Pt/GOx-HRP-PNA                                  | 0.50–420             | 0.08                  | Human serum            | 17        |
| Amperometry       | GCE/RGO/pTB-GOx-HRP                             | 80–3000              | 50                    | Plasma                 | 18        |
| Amperometry       | GCE/(MWCNT-Cyt <i>c</i> /GOx) <sub>2</sub> /Naf | 100–1000             | 8.0                   | Beverages              | 19        |
| Amperometry       | Au/GOx-PPMP-BSA-GA/Naf                          | 20–14,000            | 3.1                   | -                      | 20        |
| Amperometry       | GCE/MWCNT-Av/RuNPs/bGOx                         | 20–1230              | 3.3                   | Beverages              | 22        |
| Amperometry       | GCE/MWCNT-SB-dBA/GOx-HRP                        | 3.1–1200             | 1.0                   | Human serum, urine     | This work |

LR linear range, LOD detection limit, GCE glassy carbon electrode, MWCNT multi-walled carbon nanotubes, GOx glucose oxidase, Av avidin, bHRP biotinylated horseradish peroxidase, SSPC stainless-steel pin modified with carbon ink, HRP horseradish peroxidase, ConA concanavalin A, SPGE screen printed gold electrode, *e*-alginate electrodeposited calcium alginate hydrogel, PE paper-based electrode, Pt platinum electrode, PNA poly(noradrenaline), RGO reduced graphene oxide, pTB poly(toluidine blue), Cyt *c* cytochrome *c*, Naf Nafion, Au gold electrode, PPMP plant-produced manganese peroxidase, BSA bovine serum albumin, GA glutaraldehyde, RuNPs ruthenium nanoparticles, bGOx biotinylated glucose oxidase, SB-dBA Schiff base containing phenylboronic acid residues

was  $(88 \pm 5)$  mg dL<sup>-1</sup>, demonstrating an excellent agreement with the value reported by Wiener laboratory (84 mg dL<sup>-1</sup>).

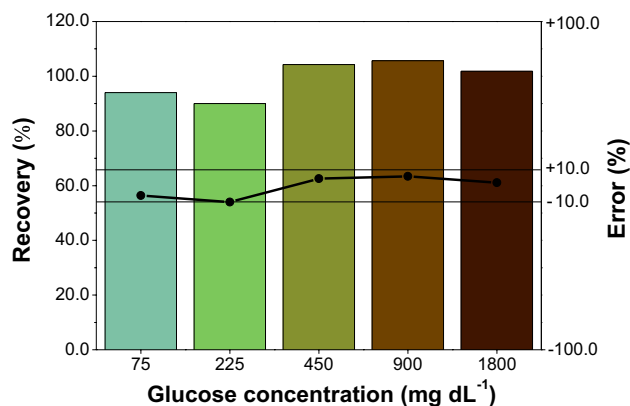
## Conclusions

We proposed an innovative strategy to build an amperometric bienzymatic glucose biosensor based on the use of non-covalently functionalized MWCNTs with a new Schiff

base modified with phenylboronic acid residues as a platform for the anchoring of GOx and HRP and response surface methodology to optimize the experimental conditions for the electrochemical quantification of glucose. The lectin-mimic properties of the rationally designed Schiff base offer a simple and cheap way to immobilize GOx and HRP without using expensive biomolecules like avidin or lectins. The efficient cascade transport of electrons through the enzymes and the fast charge transfer of the redox mediator at the MWCNT-modified GCE made possible the development of a highly competitive bienzymatic biosensor with successful application for the quantification of glucose in biological fluids. The effectiveness of the proposed biosensor demonstrates once more the advantages of a careful selection of the carbon nanotube functionalization agent to tune their properties and obtain a custom-made biosensing platform.

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**Fig. 6** Bar plot for the recovery percentages and associate errors obtained from the amperometric response of GCE/MWCNT-SB-dBA/GOx-HRP in artificial urine samples enriched with the glucose concentrations detected with the urinalysis reagent strips used in the clinical laboratory. Redox mediator:  $5.0 \times 10^{-4}$  M hydroquinone. Working potential:  $-0.050$  V. Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40. Number of biosensors used for each concentration = 3



**Author contribution** A.T.: Methodology, Formal analysis, Investigation, Visualization. V.V.: Conceptualization, Validation, Writing – review & editing, Supervision, Funding acquisition. B.V.: Methodology, Formal analysis. M.L.M.: Methodology, Formal analysis, Investigation, Visualization. S.B.: Writing – review & editing. D.V.-Y.: Writing – review & editing. P.H.-I.: Conceptualization, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. G.R.: Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. P.D.: Conceptualization, Validation, Writing – review & editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Data availability** The data will be available upon request to the corresponding author.

## Declarations

**Ethical approval** This research did not involve animal samples. Human serum and urine samples used were commercial and artificial, respectively.

**Conflict of interest** The authors declare no competing interests.

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