Electrochimica Acta 387 (2021) 138530

Contents lists available at ScienceDirect

Electrochimica Acta

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

Multi-wall carbon nanotubes electrochemically modified with phosphorus and nitrogen functionalities as a basis for bioelectrodes with improved performance

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ARTICLE INFO

Article history:
Received 6 January 2021
Revised 20 April 2021
Accepted 29 April 2021
Available online 6 May 2021

Keywords:
Electrochemical modification
PQQ-GDH
Electron-transfer
Biocatalyst
Sensing

ABSTRACT

In this study, multi-wall carbon nanotubes (MWCNTs) were electrochemically modified with nitrogen and phosphorus species and employed as platform to immobilize pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) for the fabrication of bioelectrodes for glucose detection. Depending on the upper potential limit used during the electrochemical modification of MWCNTs, the nature and amount of the nitrogen and phosphorus species incorporated in the carbon material surface can be selectively controlled. These species act as anchoring groups for the immobilization of the PQQ-GDH. The value of the upper potential limit used in the electrochemical modification influences the electron-transfer rate between the electrode and the enzyme. The performance of the bioelectrodes for glucose oxidation and detection is improved by the electrochemical modification conditions, leading to an increased sensitivity towards glucose oxidation from 39.2 to 53.6 mA g_{MWCNT}^{-1} mM^{-1} in a linear range between 0.1 to 1.2 mM. This electrochemical modification is considered as an alternative for the preparation of highly sensitive glucose bioelectrodes.

1. Introduction

In many applications involving the development of biosensing or energy conversion devices, the electron-transfer (ET) between an immobilized enzymatic element and the electrode substrate is considered the triggering factor for an optimal operation of the envisaged bioelectrochemical device. However, the prosthetic group of the enzyme which is often deeply buried within the protein structure makes direct electron transfer (DET) between the biocatalyst and the electrode difficult [1,2]. Therefore, continued efforts have focused on facilitating and promoting the ET from the redox center within the enzyme and the electrode. Different immobilization methods have been extensively used to preserve the activity of the enzyme, improving the orientation of the enzyme or even "wiring" the active center of the enzyme with the electrode transducer, providing a path for a DET mechanism [3–6]. Unfortunately, denaturation and conformational changes in the enzymatic element occur frequently during the immobilization process and can compromise the stability and catalytic behavior of the biomolecule [6].

Carbon nanotubes (CNTs) have been employed as a platform for the development of biosensors as well as bioelectrodes used for electrochemical energy generation due to their remarkable chemical stability, biocompatibility, and unique catalytic and electronic properties [7,8]. Moreover, despite the comparable dimensions of CNTs and the typical size of enzymes within a few nanometers, some studies have suggested that the structural features of CNTs can contribute to reduce the distance between the active-center of the enzyme and the electrode surface [8,9]. More importantly, one important aspect that promotes the versatility of CNTs lies in their tunable-surface chemistry, which can be done through different procedures including non-covalent or covalent functionaliza-

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https://doi.org/10.1016/j.electacta.2021.138530

tion methods [10,11]. In this sense, the modification of CNTs with surface functionalities provides a versatile route for the synthesis of active materials with enhanced properties for specific applications.

First approaches of functionalization were focused on the oxidation of CNTs employing acid solutions to incorporate oxygen functionalities, such as carboxylic acid moieties, which can anchor the terminal amino-groups at the enzyme structure with the CNT-modified surface, promoting the immobilization of the bioelement [12]. Nowadays, other reactions (for example, amidation, hydrogenation, electrografting, etc.), based on the generation of highly reactive radical species that can react with the carbon atoms in the CNT structure, have become a promising strategy for the assembly of building blocks in the synthesis of bioelectrodes with an improved performance [13–16].

The functionalization of CNT surfaces with different types of active species (such as oxygen and nitrogen) by chemical and electrochemical modification procedures have demonstrated being effective for anchoring surface functionalities enabling the formation of covalent bonds with amino acids in the enzymes [13,17,18]. For example, CNT surfaces modified with maleimide groups by electrografting of primary amines were used to fabricate surface-modified electrodes, which showed high catalytic currents and a long-term storage stability [5]. Furthermore, Gutiérrez-Sanchez et al. have demonstrated that direct interactions of aldehyde functions and sugar residues at the glycosylation shell of laccase *via* imino bond of N-functionalized CNTs improve the biocatalytic activity of the obtained biocathodes, resulting in a three times higher response than electrodes prepared using adsorbed enzyme or other type of covalent bonds [19]. Similarly, recent works employing electrochemically functionalized MWCNTs with pyrenediones species have shown the role of the functional species in providing electron pathways for a mediated electron transfer with the redox enzyme [20]. Although previous reports have demonstrated the use of phosphonic groups for an effective electrochemical communication with proteins such as hemoglobin over the electrode surface [21,22], the modification of CNTs with P species as functional groups for immobilization of enzymes is scarce. In a recent work, we have shown that electrochemical entrapment within a CNT matrix incorporating N and P species provides an effective means for electrical communication with immobilized enzymes [23].

In this work, multi-wall carbon nanotubes (MWCNTs) were electrochemically functionalized with N and P species for the development of bioelectrodes as platforms for biosensors and biofuel cell applications. The functional carbon materials promote an enhanced electron-transfer between an oxidoreductase, namely pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH), employed as model enzyme and the electrode. Thus, a biofunctional metal-free MWCNTs electrode is developed in which the amount of phosphorus and nitrogen species introduced on the carbon nanotube surface can be controlled. Depending on the upper potential limit used for electrochemical modification, the operational stability, electron-transfer kinetics, catalytic activity and sensitivity towards glucose oxidation can be significantly improved, providing an interesting platform to modulate the electrocatalytic performance of the bioelectrodes.

2. Experimental section

2.1. Reagents and equipment

MWCNTs with 95% purity (8 nm diameter, 10–30 µm length) were purchased from Cheap Tubes (Cambridgeport, USA). N,N-dimethylformamide (DMF), extra pure, provided by Scharlau, was used as solvent to disperse the CNTs. The specific surface area, obtained from N_2 adsorption isotherms at −196 °C in an automatic adsorption system (Autosorb-6, Quantachrome) and using the Brunauer, Emmett and Teller (BET) method, was 208 $m^2\ g^{-1}$.

The apo enzyme, soluble glucose dehydrogenase (s-GDH), was provided by Roche Diagnostics (Germany). Sulfuric acid (98%) analytical reagent to prepare the electrolyte, was obtained from VWR Chemicals. 4-Aminophenyl phosphonic acid (4-APPA, +98%) was purchased from Tokyo Chemical Industry (TCI-Belgium). Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate tri hydrate ($K_2HPO_4 \bullet 3H_2O$), obtained from VWR Chemicals, were used to prepare phosphate buffer solution (0.1 M PBS, pH 7.2). 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES, ≥99.5%-titration), D-(+)-glucose (ACS reagent) and pyrroloquinoline quinone (PQQ, ≥95%-HPLC) were purchased from Sigma-Aldrich. All solutions were prepared using ultrapure water (18 MΩ cm). The gases Ar (99.999%) and H_2 (99.999%) were provided by Air Liquide.

Electrochemical measurements were performed either using a BIOLOGIC SP-300 or an Autolab PGSTAT302N potentiostat and a standard three-electrode cell with the glassy carbon (GC) electrodes modified with the MWCNTs as the working electrode (WE), a graphite rod as counter electrode (CE) and a Ag/AgCl (3 M KCl), introduced in the same electrolyte and connected with the working solution through a Luggin capillary as reference electrode (RE).

2.2. Electrochemical modification of MWCNTs with 4-aminophenyl phosphonic acid

Dispersions of 1 mg mL^{-1} of MWCNTs in DMF were prepared. Prior to the deposition of MWCNTs, the GC surface (3 mm diameter) was sanded with emery paper and polished using 1 and 0.05 µm alumina slurries, then rinsed with ultrapure water. Afterwards, a 5 µL aliquot of the MWCNTs dispersion was dropped onto the GC surface and dried under an infrared lamp to remove the solvent. This procedure was repeated twice. No additional binder has been incorporated during this procedure.

The electrochemical modification of MWCNTs was performed by cyclic voltammetry in deoxygenated 0.5 M H_2SO_4 + 1 mM aqueous 4-APPA solution. During the measurement inert gas (Ar) flow was maintained. Modification of the MWCNTs was achieved by 10 CV cycles at 10 mV s^{-1} reaching different upper potential limits. After electrochemical treatment, the carbon electrodes were washed with excess of ultrapure water, removing the remaining electrolyte.

2.3. Physicochemical characterization

X-Ray photoelectron spectroscopy (XPS) was performed in a VG-Microtech Mutilab 3000 spectrometer using Al Kα radiation (1253.6 eV). The P2p spectra have been analyzed considering the spin-orbit splitting into $P2p_{3/2}$ and $P2p_{1/2}$ with a 2:1 peak area ratio and 0.87 eV splitting [24].

Scanning electron micrographs were taken using an ORIUS SC600 model Field Emission Scanning Electron Microscopy (FE-SEM) and a ZEISS microscope, Merlin VP Compact model. The samples were coated with a thin layer of carbon to avoid decomposition of the enzyme and the different oligomers incorporated by the electrochemical modification of 4-APPA.

2.4. Modification of 4-APPA-functionalized MWCNTs with PQQ-GDH

Glassy carbon electrodes modified with MWCNTs functionalized with 4-APPA were modified with PQQ-GDH as a bioactive species. For this, s-GDH was reconstituted with PQQ. 36 mg of s-GDH were dissolved in 10 mM HEPES buffer solution (pH=7.0), containing 150 mM $CaCl_2 \bullet 2H_2O$ and 520 µM freshly prepared PQQ solution, attaining a concentration of 36 mg mL^{-1} of PQQ-GDH. In this procedure, the PQQ cofactor is introduced in the apo-enzyme struc-

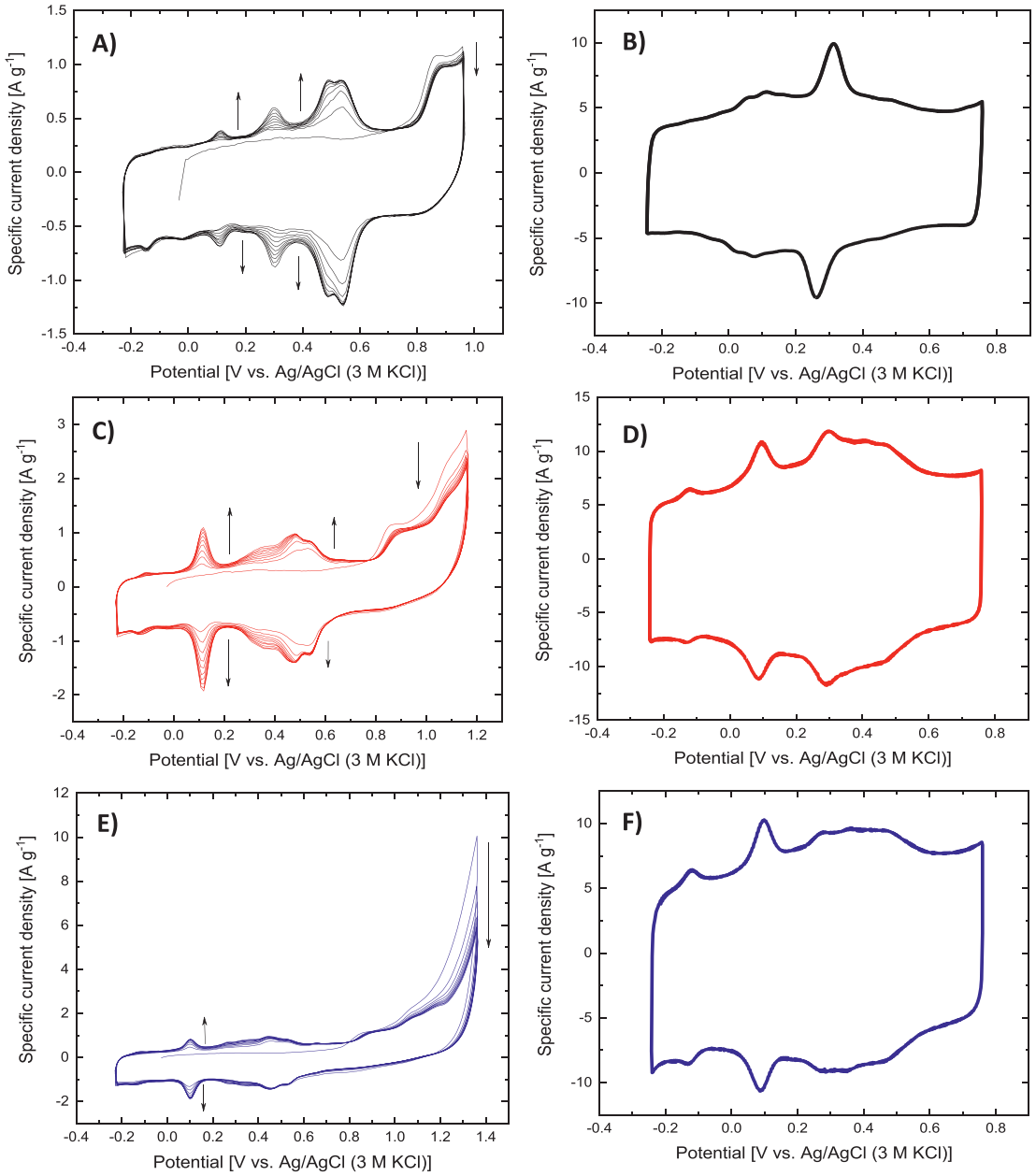

Fig. 1. Cyclic voltammograms of MWCNTs in 0.5 M H_2SO_4 + 1 mM 4-APPA at 10 mV s^{-1}, 10 cycles under Ar atmosphere at different upper potential limits: A) 0.97 V, C) 1.17 V and E) 1.37 V. Cyclic voltammograms for MWCNT-APPA-X electrodes in 0.5 M H_2SO_4, B) MWCNT-APPA-0.97, D) MWCNT-APPA-1.17 and F) MWCNT-APPA-1.37 at 50 mV s^{-1}, under Ar atmosphere.

ture as active center, and the complex is stabilized by the presence of three Ca^{2+} ions [25]. At the same time, one of the Ca^{2+} ions are required for the activation of the cofactor and oxidation of the substrate (glucose). The enzyme solution was treated at 1600 rpm for 30 min in a vortex and stored at 4 °C until used.

Bioelectrodes were fabricated dropping a 5 µL aliquot of the enzyme solution onto the MWCNTs functionalized with 4-APPA. The aliquot was dried at 4 °C for 1 hour, promoting the incubation process of the enzyme on the electrode surface. Subsequent washing with 0.1 M PBS (pH=7.2) removed loosely bound enzymes before drying the electrode under an Ar flow. The surface concentration of PQQ-GDH was calculated from the redox processes in the voltammogram using Eq (1).

$$\Gamma = \frac{Q}{nFA} \quad (1)$$

Where Γ is the surface concentration of PQQ-GDH (mol m^{-2}), n is the number of electrons in the redox reaction, F is the Faraday constant (C mol^{-1}), A is the electrode surface area (m^2) and Q is the charge (C) obtained from the integration of the anodic peak associated with the redox process of the enzyme.

For the estimation of charge and peak current values (i_a, anodic peak current and i_c, cathodic peak current) the background specific current has been estimated and subtracted. For this, the electrochemical response associated with the contribution of functionalized electrode surfaces in the absence of immobilized enzyme was considered.

The apparent electron transfer constant (k_s^{app}) for the redox center of the PQQ-GDH with the electrochemically 4-APPA modified MWCNTs at different upper potential limits, was derived using Laviroń́s model [26] based on the classical Butler–Volmer theory [27].

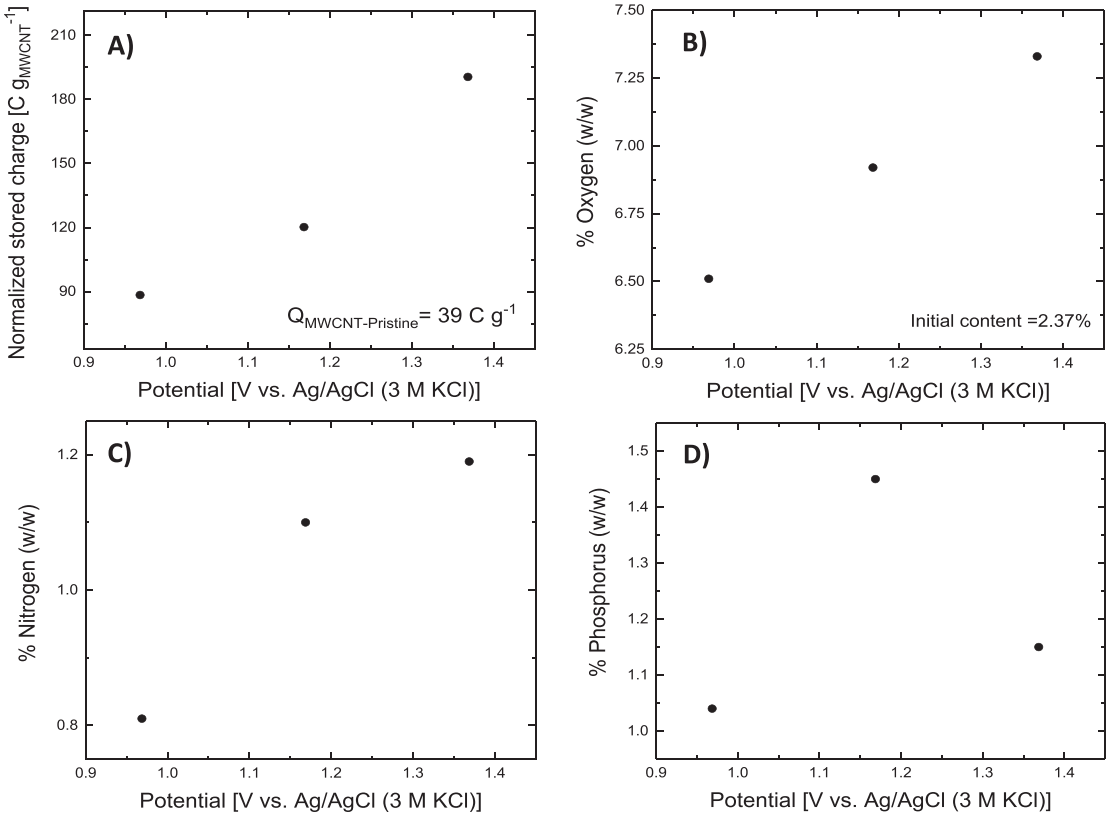

Fig. 2. A) Normalized charge vs. upper potential limit used for 4-APPA oxidation, B) Amount of oxygen vs. upper potential limit, C) Amount of nitrogen vs. upper potential limit and D) Amount of phosphorus vs. upper potential limit. Note: Charge was determined in the potential range from −0.25 to 0.75 V vs. Ag/AgCl (3 M KCl) and at scan rate of 50 mV s^{-1}, under Ar atmosphere.

2.5. Electrochemical characterization and biocatalytic activity towards glucose oxidation

The electrochemical behavior of MWCNTs modified with 4-APPA and PQQ-GDH (MWCNT-PQQ-GDH) was evaluated in 0.1 M PBS solution (pH=7.2), in absence and presence of glucose using cyclic voltammetry. The catalytic behavior towards glucose oxidation was studied by chronoamperometry using the same electrochemical cell configuration. Considering that the oxidation process of glucose by PQQ-GDH is oxygen independent, the electrolyte was equilibrated with air. The current output of the different electrodes was allowed to stabilize at 0.35 V in 0.1 M PBS (pH 7.2) for 1 h before the addition of glucose aliquots of a stock solution (0.1 M) obtaining concentrations between 5 μM and 6 mM. During chronoamperometry, stirring was maintained to improve the mass transport of glucose towards the electrode surface. A cyclic voltammogram was recorded at the beginning and at the end of the experiment to control that the integrity of the electrode. All measurements were carried out in triplicate with three electrodes prepared using the same conditions. The limit of detection (LOD) was determined by progressively measuring more diluted concentrations of the analyte. The LOD was the lowest concentration at which the current signal could be clearly distinguished from the blank. Moreover, the limit of quantification (LOQ) was calculated as 3.3 times the LOD (LOQ = 3.3 LOD).

In this work, the electrodes prepared are named as MWCNT-APPA-X for those prepared without enzyme, with X the upper potential limit that was employed for the electrochemical modification, and MWCNT-APPA-X-GDH for the electrodes with immobilized PQQ-GDH.

Electrochemical stability was determined by subsequent chronoamperometry experiments. In the first experiment, MWCNT-APPA-X-GDH electrodes were polarized from the open circuit potential to 0.35 V and were kept at this potential for 10 min. Then an aliquot of 0.1 M glucose was added to achieve a concentration of 20 mM. The electrodes were stored at 4 °C and tested each day under the same conditions for 8 subsequent days. The second stability test consisted of maintaining the electrode at an applied potential of 0.35 V for 24 h without addition of glucose under continued stirring.

3. Results and discussion

3.1. Electrochemical modification of MWCTNs with 4-APPA

Cyclic voltammograms recorded during electrochemical oxidation of 4-APPA on MWCNTs using different upper potential limits are shown in Fig. 1. Irreversible oxidation peaks can be observed at potentials higher than 0.8 V that correspond to the oxidation of 4-APPA (Figs. 1-A, C and E). During oxidation of 4-APPA, the radical species generated can promote both the formation of adsorbed oligomer chains as well as species covalently bonded to the surface of MWCNTs [28,29]. The presence of those functional species on the electrode surface generates different highly reversible redox processes appearing in the voltammogram recorded after the modification procedure and in the absence of 4-APPA in the electrolyte solution (Fig. 1-B, D and F), as result of the adequate interaction between adsorbed and covalently attached functionalities at the MWCNTs support [29].

Continuous cycling of the electrode in the presence of 4-APPA generates an increase in the charge of the different reversible redox processes observed between −0.25 V and 0.75 V. Fig. 2-A shows the average of the integrated charge within the indicated potential range, determined for the forward and backward scans

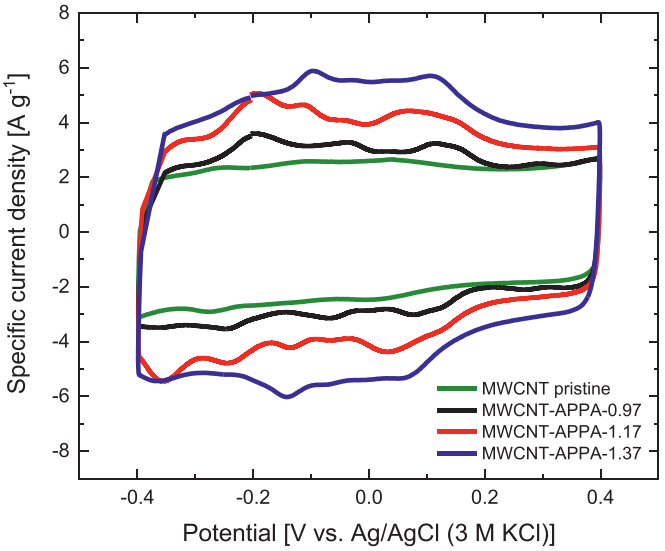

Fig. 3. Cyclic voltammograms for the MWCNT and MWCNT-APPA-X electrodes in 0.1 M PBS (pH = 7.2) at 50 mV s^{-1} under Ar atmosphere.

from voltammograms normalized by the amount of deposited MWCNTs. It can be observed that the integrated normalized charge increases with the upper potential limit used for the oxidation of 4-APPA in comparison with the values observed for pristine MWCNTs, reaching values twice as higher as the initially observed for MWCNTs only with polarization up to 0.97 V, and more than four times for the highest upper potential limit studied. Fig. 2-B, C and D summarize the amount of oxygen, nitrogen and phosphorus, respectively, as determined by XPS for MWCNT-APPA electrodes at different upper potential limits. The quantification performed by XPS elucidates the degree of modification and incorporation of heteroatoms on the MWCNT surface. The corresponding XPS spectra are presented in Fig. S1.

The increased amount of oxygen and nitrogen on the MWCNT-APPA-X surfaces (Fig. 2B and C) shows a direct dependence with the upper potential limit used for the oxidation of 4-APPA. The oxidative conditions achieved at higher applied potentials promote the incorporation of oxygen functionalities in the MWCNTs, the adsorption of oligomer chains on the surface, and the covalent binding of APPA species [28]. Interestingly, the phosphorus content shows a maximum value of incorporation at 1.17 V, suggesting that higher applied potentials favor phosphonic acid oxidation and desorption due to hydrolysis reactions. An upper potential limit of functionalization at higher potentials promotes the formation of oxidized species with higher binding energy (such as, amine and oxidized N groups) as well as phosphonic species (see Fig. S1), suggesting that the degree of incorporation of functionalization species can be controlled by the selection of the upper potential limit [28,29].

3.2. Electrochemical characterization of MWCNTs modified with 4-APPA and PQQ-GDH

Fig. 3 shows the electrochemical response of MWCNT-APPA-X electrodes in 0.1 M PBS (pH 7.2). Pristine MWCNTs present a quasi-rectangular shape under these conditions due to double-layer charging. However, the MWCNT-APPA-X samples show additionally different redox processes, as those observed under acidic conditions but with lower current. Thus, indicating that the electroactive performance is also maintained under neutral pH conditions.

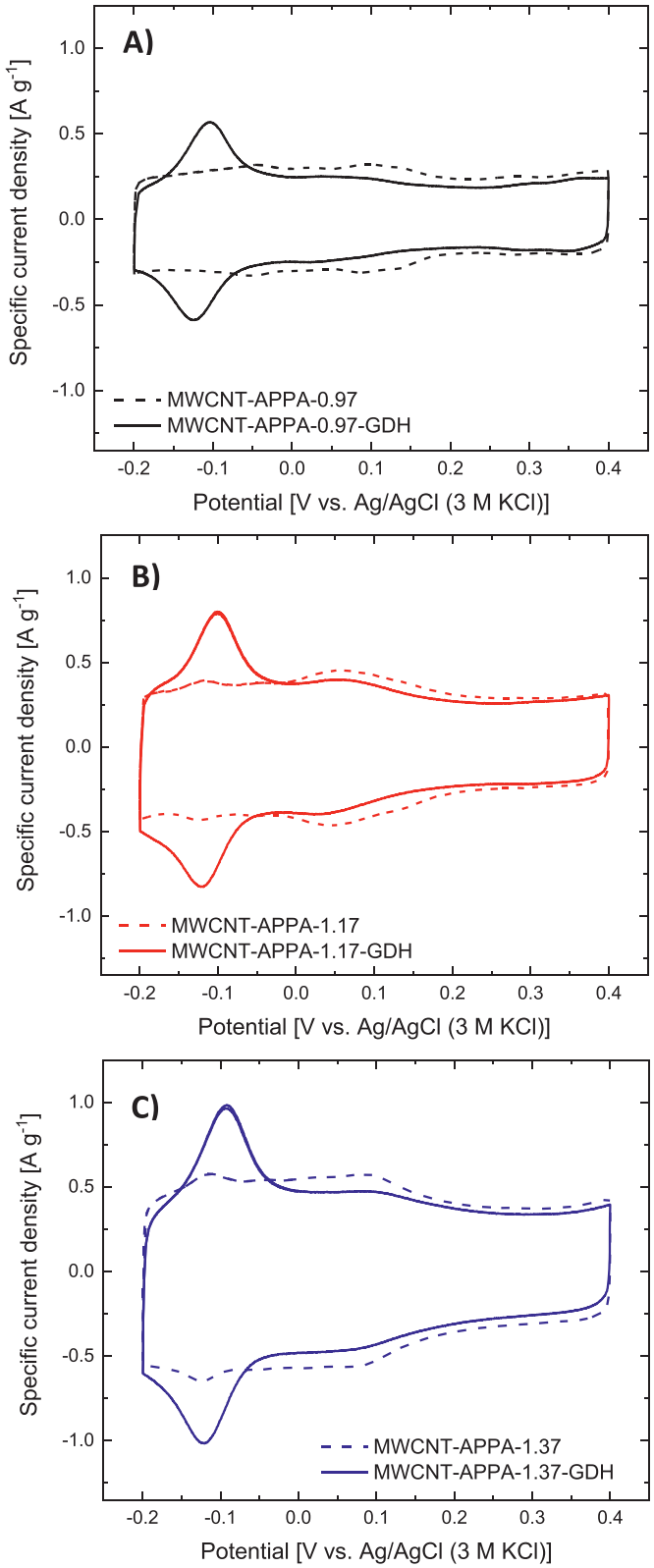

Fig. 4. Representative cyclic voltammograms for MWCNT-APPA and MWCNT-APPA-X-GDH at different upper potential limits of modification: A) 0.97 V, B) 1.17 V and C) 1.37 V in 0.1 M PBS (pH = 7.2), at 5 mV s^{-1} under Ar atmosphere.

Fig. 4 shows the cyclic voltammograms obtained for the modified electrodes after immobilization of PQQ-GDH. It can be observed a small decrease of the double-layer charge as compared

Table 1
Electrochemical parameters associated with the PQQ-GDH electron-transfer process on MWCNT-APPA-X-GDH electrodes.

Electrodes	Γ^{**} [mol cm^{-2}]	ΔE^{**} [mV]	k_s^{app} [s^{-1}]	$i_a{}^{*}$ [A g^{-1}]	$i_c{}^{*}$ [A g^{-1}]	$\frac{i_a}{i_c}$
MWCNT-APPA-0.97-GDH	$(1.4 \pm 0.3) \times 10^{-11}$	28.8 ± 5.1	69.2	0.37 ± 0.07	0.37 ± 0.07	1.00 ± 0.05
MWCNT-APPA-1.17-GDH	$(2.1 \pm 0.2) \times 10^{-11}$	30.1 ± 10.2	71.5	0.47 ± 0.06	0.44 ± 0.05	1.06 ± 0.02
MWCNT-APPA-1.37-GDH	$(1.9 \pm 0.4) \times 10^{-11}$	25.8 ± 4.6	81.7	0.38 ± 0.07	0.35 ± 0.05	1.07 ± 0.07

All values have been determined by the average of three different electrodes synthetized and characterized in the same conditions for each upper potential limit.
* Anodic and cathodic current density and charge of the PQQ-GDH were determined subtracting the contribution of the MWCNT-APPA-X without enzyme (see Experimental section).
** Values determined at 5 mV s^{-1}.

with the electrodes in the absence of enzyme. Additionally, a clear electrochemical redox couple is observed for all modified MWCNT-APPA-X-GDH electrodes at a formal potential (E^0) of about −0.12 V, which can be assigned to the two-electron redox process of PQQ in the active center of the holo-enzyme PQQ-GDH [30]. This result is in agreement with a similar electrochemical behavior observed for immobilized PQQ-GDH using different electrodes [31,32].

The peak potential separation associated with the PQQ center in the holo-enzyme (~29 mV) has values corresponding to a theoretical reversible two-electron transfer process [33], and is in agreement with the values of an i_a/i_c ratio ≈ 1. Taking into account the pH conditions, the redox process of the cofactor within the enzyme takes place by formation of an intermediate quinone species and single protonation of PQQ [34].

Table 1 shows the values of surface coverage of PQQ-GDH for the different electrodes. These values change in correlation with the phosphorus content (Fig. 2-D), reaching the maximum value for MWCNTs modified with APPA at an upper potential limit of 1.17 V, which is the one with the highest phosphorus content. This can be explained due to the electrostatic interactions between PQQ-GDH, which presents a net positive charge at the pH conditions employed during the immobilization process [35], and the different deprotonated terminal groups in the phosphorus moieties [36] incorporated on the MWCNTs surface.

The electrochemical study at different scan rates shows a linear-dependence of the oxidation and reduction currents, associated with the two electron-transfer process for the redox center of the enzyme immobilized on the modified MWCNTs (see Figs. S2 and S3). This corresponds to a surface confined redox process [37–39], confirming the binding of the enzyme by the functional groups on the MWCNTs surface. Interestingly, the slope of the linear-fitting of the peak current dependence with the scan rate (v_{scan}), could be correlated with the concentration of the active species on the electrode surface (coverage of PQQ-GDH) [40,41], which shows a maximum value for the MWCNT-APPA-1.17 electrodes, in agreement with the results shown in Table 1, which confirms that a higher concentration of enzyme is reached with this electrode.

The apparent rate constant, k_s^{app}, (Table 1) was calculated from the trumpet-plots (see Fig. S4), providing values between 69 and 82 s^{-1}. An increase of the k_s^{app} with the upper potential limit used during electrode modification suggests that the interaction between the MWCNT-APPA electrodes and PQQ-GDH is improved, providing a fast electron-transfer between the electrode and the redox center of the enzyme.

The morphological characterization obtained by FE-SEM of the MWCNT-APPA-X and MWCNT-APPA-X-GDH electrodes is presented in Fig. 5.

Typical bundles can be observed in the deposits of MWCNTs (see Fig. S5) and the modified MWCNTs with 4-APPA (see Fig. 5-A, C and E). However, the oxidation of MWCNTs in presence of 4-APPA causes the formation of a thin film and small deposits on the MWCNTs' surface, related to the oligomer chains formed on the surface of the modified electrode. Once PQQ-GDH is immobilized on the electrochemically modified MWCNTs (see Fig. 5-B, D and F), the formation of a continuous enzyme coating can be observed. Depending on the applied potential for the modification with APPA, the morphology of the coating changes considerably. The electrodes modified using an upper potential limit of 0.97 V present a coating of PQQ-GDH which resembles the roughness and bundles-like structure of MWCNTs, suggesting a low thickness of the enzymatic coating. An increase of the upper potential limit promotes the immobilization of a dense and thick enzyme coating with high porosity and a sponge-like texture, as observed for a potential limit of 1.17 V and evolving into a more defined globular morphology for a potential limit of 1.37 V.

3.3. Electrocatalytic response of MWCNT-APPA-X-GDH electrodes towards glucose oxidation

Fig. 6-A, C and E show cyclic voltammograms in the presence and absence of glucose for MWCNT-APPA-X-GDH electrodes. For the three electrodes, the appearance of an oxidation current upon addition of glucose can be observed. The current starts from a potential of about −120 mV and increases with respect to the voltammogram in absence of glucose at more positive applied potentials. The non-functionalized MWCNTs do not show this oxidation current in presence of glucose (Fig. S6). Fig. 6-B, D and F show chronoamperograms obtained with the same electrodes at an applied potential of 0.35 V vs. Ag/AgCl (3 M KCl) and the response when glucose is added at a concentration of 20 mM. After addition of glucose, the anodic current increases as consequence of the catalytic activity of the enzyme and the successful electron transfer between the immobilized enzyme and the modified electrode surface. The bioelectrode modified at an upper potential limit of 1.37 V (MWCNT-APPA-1.37-GDH) shows the highest values of current change (Δi), corresponding to 1.47 A $g_{MWCNT}{}^{-1}$ by cyclic voltammetry and 3.06 μA by chronoamperometry. In contrast, with the other potential limits applied for the modification of MWCNTs, the currents obtained for glucose oxidation were 0.49 A $g_{MWCNT}{}^{-1}$ and 0.89 μA for both electrodes. This behavior can be correlated with the apparent rate constant estimated before (Table 1). It can be observed that this value is higher for the MWCNT-APPA-1.37-GDH electrode. In this sense, despite the lower amount of immobilized enzyme in the MWCNT-APPA-1.37-GDH electrode, the enzyme seems to be immobilized under more favorable conditions; thus, enabling an improved electron transfer between the enzyme and the electrode.

Regarding the electrocatalytic behavior and activity of the enzyme immobilized on the differently modified MWCNTs-based electrodes, Figs. 7-A, C and E show the chronoamperograms carried out at 0.35 V vs. Ag/AgCl (3 M KCl) under stirring conditions for glucose oxidation in the concentration range between 5.0 μM and 6.0 mM in PBS (pH = 7.2). All electrodes present a rapid response to the changes in glucose concentration. The limit of detection (LOD) was determined to be 5 μM for the electrodes modified at 1.17 V and 1.37 V. In contrast, the bioelectrodes synthesized at 0.97 V exhibit a LOD of 10 μM.

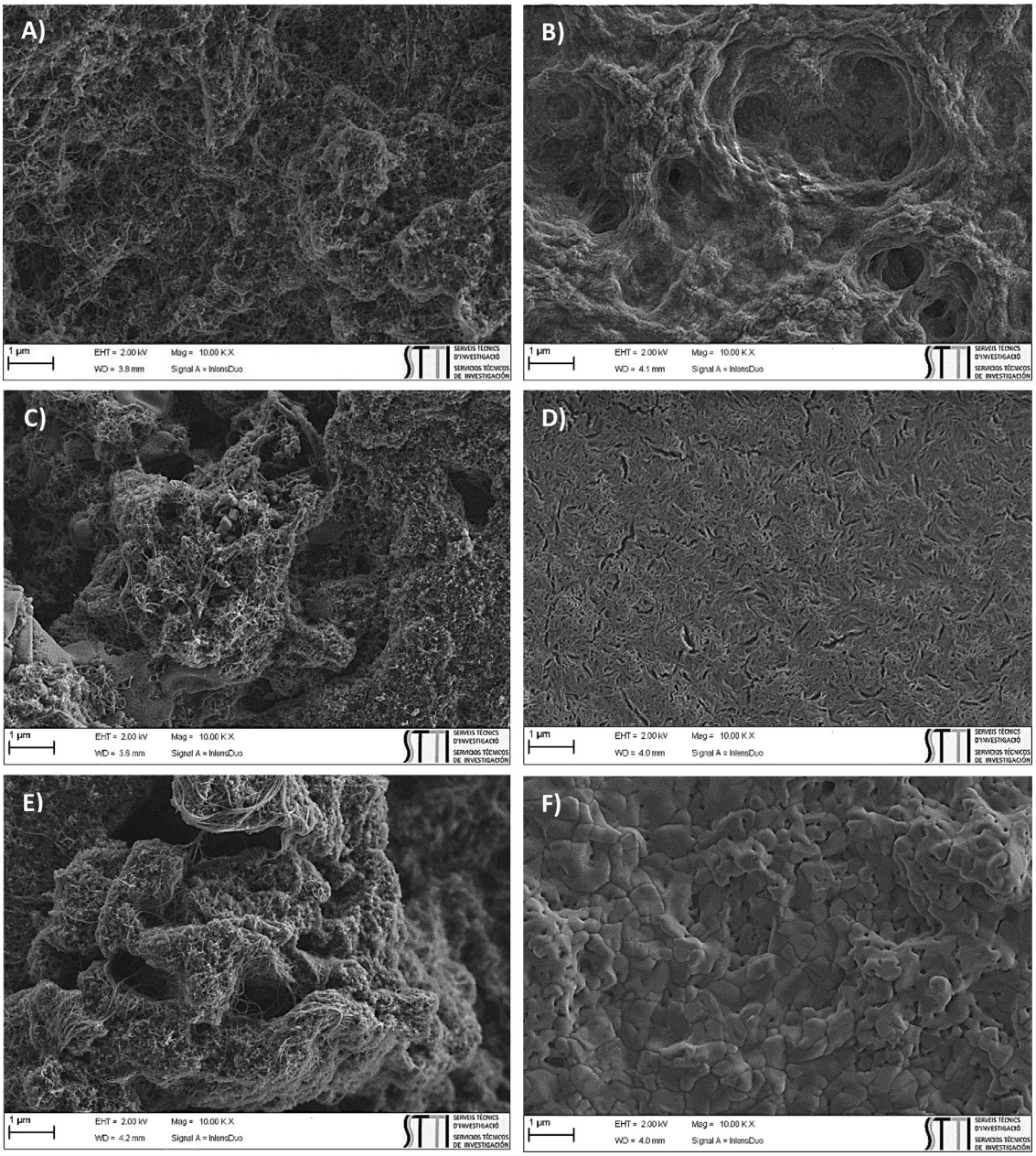

Fig. 5. FE-SEM micrographs of A) MWCNT-APPA-0.97, C) MWCNT-APPA-1.17 and E) MWCNT-APPA-1.37. B) MWCNT-APPA-0.97-GDH, D) MWCNT-APPA-1.17-GDH and F) MWCNT-APPA-1.37-GDH. All micrographs were attained with a magnification of 10.000 X.

Fig. 7-B, D and F show the calibration curves for the MWCNT-APPA-X-GDH electrodes that present a Michaelis-Menten behavior, in which a linear range at low glucose concentrations is observed. Afterwards, a plateau zone appears corresponding to the saturation of the enzyme. In the case of MWCNT-APPA-1.17-GDH and MWCNT-APPA-1.37-GDH electrodes, a change in the slope at low glucose concentrations is observed, suggesting either changes in the surface chemistry or swelling effects [42,43], leading to modifications in the interaction with the enzyme. In case of the electrode synthesized at 0.97 V, this behavior is less noticeable due to the lower degree of functionalization. Thus, the linear range was determined to be between 0.1 mM and 1.2 mM for all electrodes.

Interestingly, the sensitivity of the electrodes presents an important enhancement with the increase in the upper potential limit used during the electrochemical modification of MWCNTs, obtaining at 1.37 V a value 37% higher than for the electrode modified at 0.97 V (Table 2). This is most likely due to the faster kinetics in the electron-transfer process between the enzyme and the electrode surface, in agreement with the increase in k_s^{app} (Table 1). Moreover, taking into account that the active center of the enzyme is a redox active species with an external-electron transfer mechanism, the functionalities incorporated during the electrochemical modification, could facilitate the transfer of electrons, as a mediating process, because the redox processes observed in the functionalized MWCNT-APPA appear at higher potentials than the redox process associated to the redox active center of the enzyme [44].

The apparent Michaelis-Menten constant (K_m^{app}) (Table 2) was determined by Lineweaver-Burk fitting (Fig. S7). The obtained values are in agreement with reported values for PQQ-GDH in solution, which are between 0.5 mM and 22 mM [45], and also with previous works in which this parameter presents values between

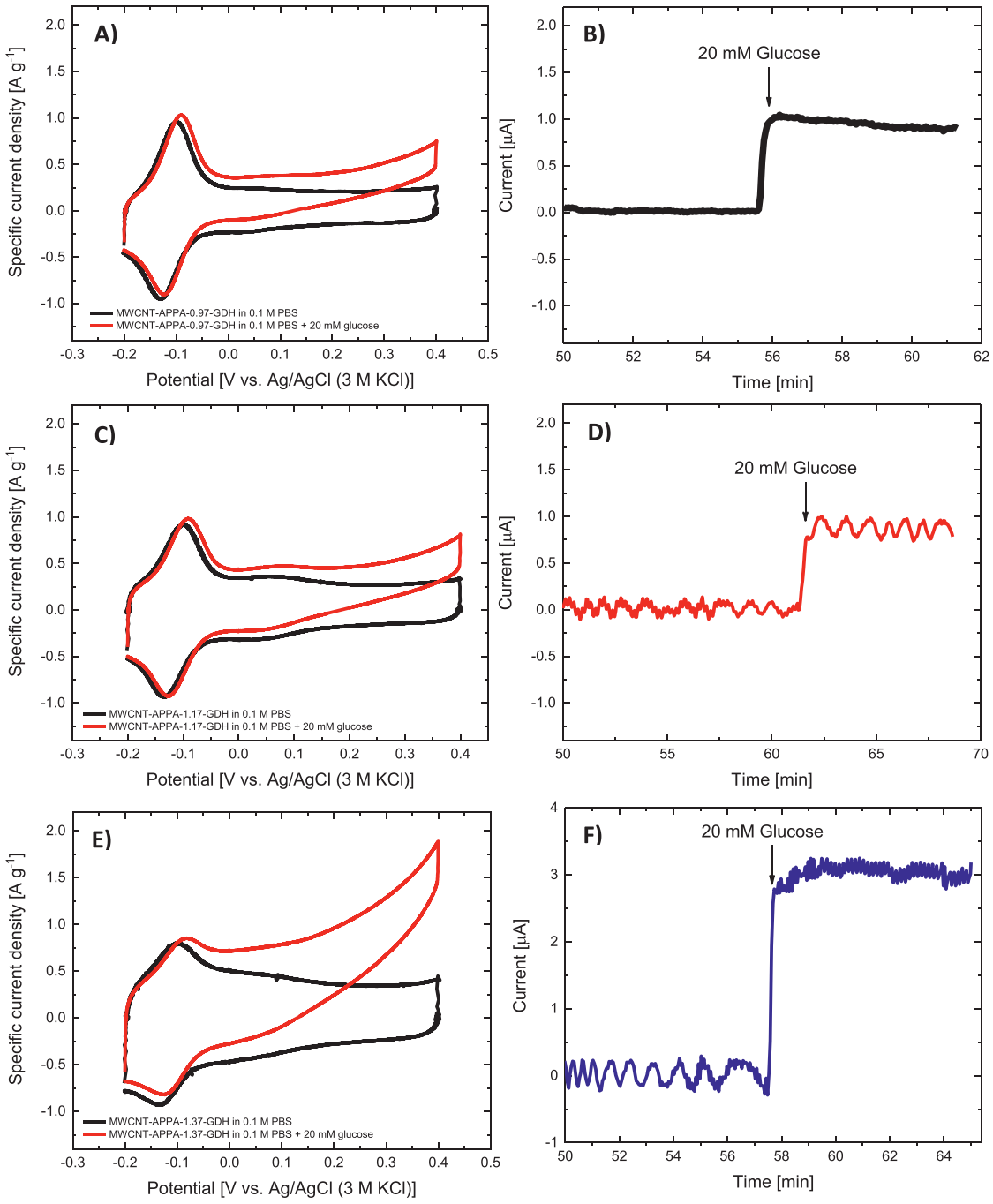

Fig. 6. Cyclic voltammograms for MWCNT-APPA-X-GDH electrodes in 0.1 M PBS (pH = 7.2) in absence (black line) and presence (red line) of 20 mM glucose at 5 mV s^{-1} under Ar atmosphere: A) 0.97 V, C) 1.17 V and E) 1.37 V. Amperometric response at 0.35 V for MWCNT-APPA-X-GDH electrodes modified at B) 0.97, D) 1.17 and F) 1.37 V, after the addition of 0.1 M glucose in 0.1 M PBS (pH = 7.2) to achieve 20 mM glucose in solution under room atmosphere and stirring conditions.

Table 2
Analytical figures of merit for the quantification of glucose for MWCNT-APPA-X-GDH electrodes.

Parameter*	Bioelectrode MWCNT-APPA-0.97-GDH	MWCNT-APPA-1.17-GDH	MWCNT-APPA-1.37-GDH
Sensitivity [(mA g_{MWCNT}^{-1}) mM^{-1}]	39.2 ± 2.8	42.2 ± 4.5	53.6 ± 4.7
R^2	0.995	0.990	0.992
Linear range (mM)	0.1–1.2	0.1–1.2	0.1–1.2
LOD (mM)	0.01	0.005	0.005
LOQ (mM)	0.033	0.0165	0.0165
K_m^{app} (mM)	2.5	2.7	1.6

* All parameters were determined employing three different electrodes fabricated using the same conditions.

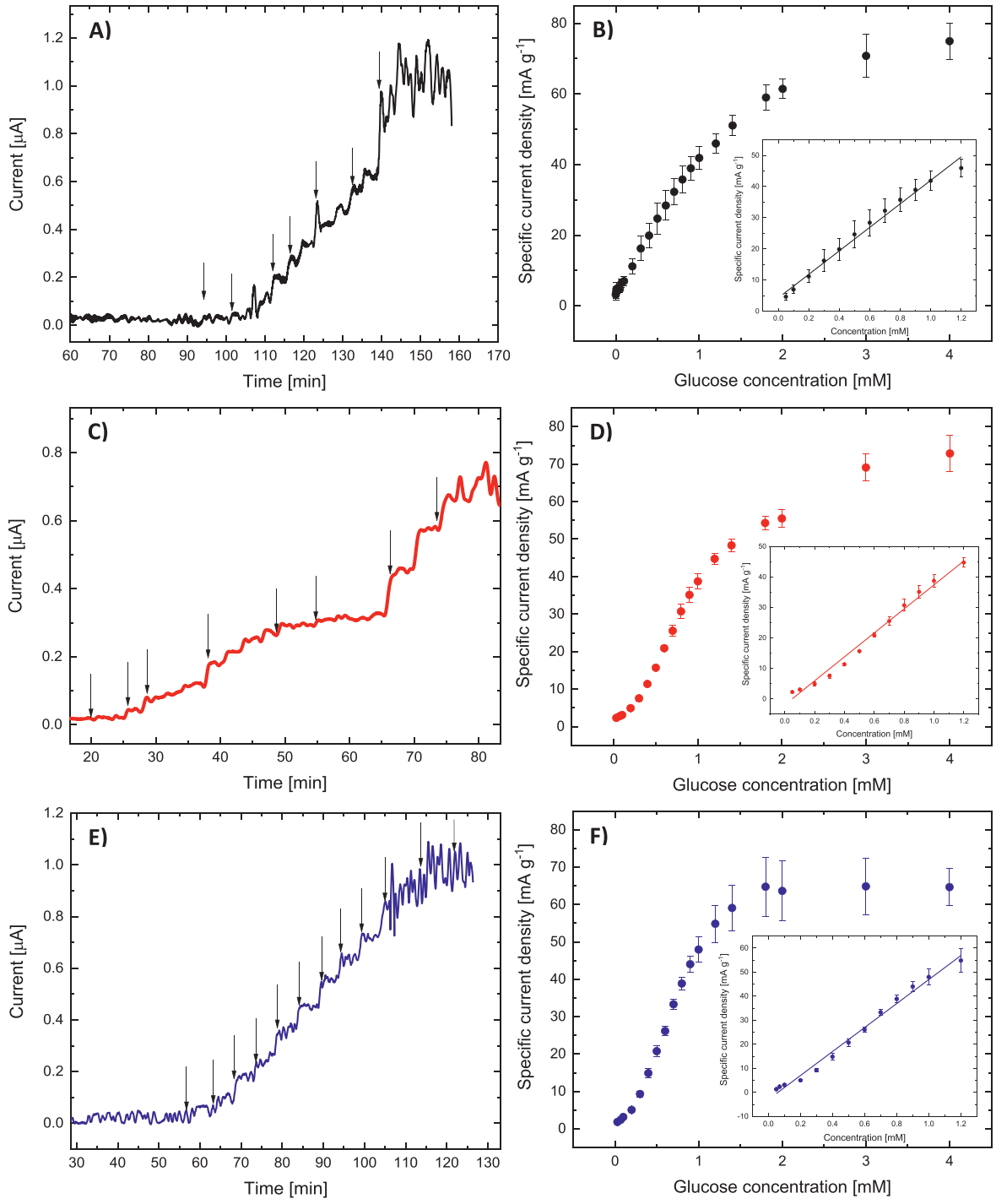

Fig. 7. Chronoamperometry profiles at 0.35 V vs. Ag/AgCl (3 M KCl) in 0.1 M PBS (pH=7.2) for successive additions of glucose (marked with arrows), from 5 µM to 6 mM, A) MWCNT-APPA-0.97-GDH, C) MWCNT-APPA-1.17-GDH and E) MWCNT-APPA-1.37-GDH. Calibration curves for B) MWCNT-APPA-0.97-GDH, D) MWCNT-APPA-1.17-GDH and F) MWCNT-APPA-1.37-GDH. Insets: Calibration curves in the concentration range between 0.1 mM and 1.2 mM. Error bars are estimated as a triple of the standard deviation ($n = 3$).

0.2 mM to 3 mM for immobilized enzyme [45,46]. The lower K_m^{app} value is obtained with the MWCNT-APPA-1.37-GDH electrode, indicating a higher apparent affinity of the enzyme to its substrate glucose.

A comparison of the results obtained in this work with other relevant bioelectrode platforms previously reported in literature and employing PQQ-GDH is summarized in Table S1. The proposed bioelectrode architecture based on MWCNTs functionalized with N and P groups shows a comparable performance, while making use of a simple, controlled, and reproducible electrode modification procedure.

4. Conclusions

The electrochemical modification of MWCNTs with 4-APPA has demonstrated the incorporation of different phosphorus and nitrogen surface species which promote the immobilization and electrical communication with PQQ-GDH. The bioelectrodes have been

used in the oxidation of glucose at neutral pH conditions. Depending on the upper potential limit used during the electrochemical modification of MWCNTs, the morphological features of the enzymatic coating and the interaction with the enzymatic element can be tuned for improving the electron-transfer kinetics, obtaining values for apparent electron transfer rate constants ranging from 69 to 82 s^{-1}. This adequate electron transfer could be associated with a better orientation of the redox-active center of the enzyme to the electrode. Moreover, the different biocatalytic electrodes show different amounts of immobilized enzyme, catalytic oxidation current, and sensitivity towards glucose oxidation.

The MWCNT-APPA-1.37-GDH bioelectrode shows the highest values of glucose oxidation current (1.47 A g^{-1}, obtained by cyclic voltammetry and 3.06 μA obtained by chronoamperometry). The sensitivity obtained for this electrode is (53.63 ± 4.72) mA $g_{MWCNT}{}^{-1}$ mM^{-1}. Therefore, the electrochemical modification of MWCNTs with phosphorus and nitrogen species, from oxidation of 4-APPA, can be considered as a promising alternative to control and improve the electrochemical performance of enzyme-modified bioelectrodes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit authorship contribution statement

Andrés Felipe Quintero-Jaime: Conceptualization, Methodology, Investigation, Writing – original draft. **Felipe Conzuelo:** Conceptualization, Methodology, Investigation, Writing – original draft. **Wolfgang Schuhmann:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Diego Cazorla-Amorós:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition. **Emilia Morallón:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing, Funding acquisition.

Acknowledgments

The authors would like to thank Ministerio de Ciencia e Innovación (PID2019–105923RB-I00) for the financial support. A.F.Q.J. gratefully acknowledges Generalitat Valenciana for the financial support through Santiago Grisolia grant (GRISOLIA/2016/084), to the University of Alicante for the support in the mobility program through the Escuela de Doctorado (EDUA).

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