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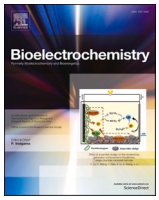

Polynorepinephrine and polydopamine-bacterial laccase coatings for phenolic amperometric biosensors

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ABSTRACT

The successful fabrication of biosensors is greatly limited by the immobilization of their bioreceptor, thus we propose a facile and reproducible two-step method to modify graphite electrodes with a bacterial laccase, relying on a fast and controllable potentiostatic process to coat graphite surfaces with biomolecule-compatible thin films of polynorepinephrine (ePNE) and polydopamine (ePDA). Both polymers, synthesized with a similar thickness, were functionalized with bacterial laccase, displaying distinct electrochemical transducing behaviours at pH 5.0 and 7.0. ePNE layer enables adequate electron transfer of anionic and cationic species in acidic and neutral media, whereas transduction across ePDA strongly depends on pH and redox probe charge. ePNE stands out by improving the amperometric responses of the biosensing interface towards a phenolic acid (gallic acid) and a flavonoid (catechin), in respect to ePDA. The optimal graphite/ePNE/laccase interface outperforms biosensing interfaces based on fungal laccases at neutral pH, displaying detection sensitivities of 104 and 14.4 μA cm^{-2} mM^{-1} for gallic acid and catechin, respectively. The fine synthetic control of the ePNE bio-inspired transduction layer and the use of an alkaliphilic bacterial laccase enabled the construction of an amperometric biosensing interface with extended pH range of polyphenols detection present in food products and agro-industrial waste.

1. Introduction

Laccases are multi-copper oxidases (EC 1.10.3.2) that catalyse the oxidation of a variety of organic compounds, such as ortho- and *para*-diphenols and aryl diamines, through electron transfer between the substrate, the different copper atoms of the enzyme active sites, and the oxygen [1]. The four copper atoms of laccase play a role in the catalytic process [2]: the mononuclear site, formed by type 1 copper (T1), is directly involved in the oxidation of the substrate, whereas the trinuclear site, formed by type 2 copper (T2) and two type 3 copper atoms (T3 and T3), is responsible for the reduction of molecular oxygen. When oxidized, the substrate transfers one electron to T1 copper that is then transferred through the intramolecular His-Cys-His bridge to the trinuclear cluster where molecular oxygen is reduced to water. It is recognized that electron transfer from the substrate to the T1 copper is the rate-limiting step in the catalytic process of laccases, where the difference between the potential values for the substrate and the T1 copper redox conversions is the main driving force for the reaction, allied to the substrate binding affinity. The T1 copper redox potential, usually 0.4–0.8 V *vs.* Normal Hydrogen Electrode (NHE), depends on several factors, but especially on the composition of its coordination sphere, and according to its value, laccases from different sources may be classified as high, medium, or low redox potential [2].

Laccases can be extracted from different natural sources, mostly from fungi and high plants, but also from bacteria and their selectivity towards a range of substrates explains their extensive use in the

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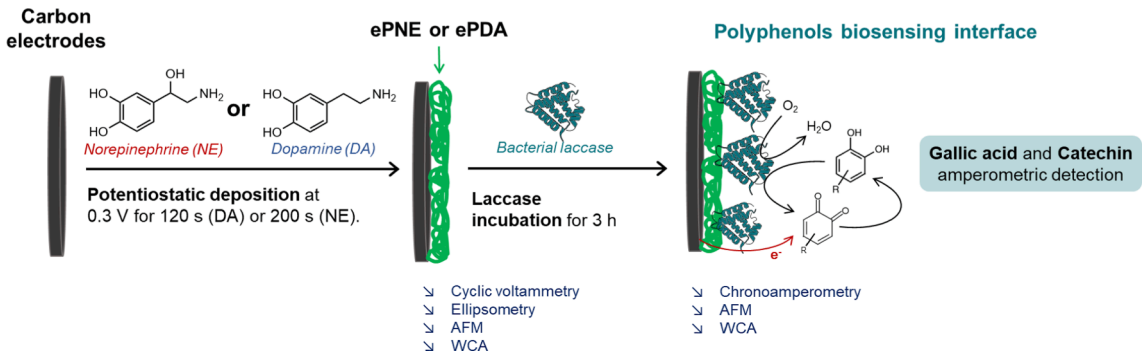

Fig. 1. Schematic illustration of the electrode modification with polycatecholamines followed by laccase incubation and catalytic performance evaluation. AFM and WCA stands for Atomic force microscopy and Water contact angle, respectively.

development of biosensing interfaces [1], cathode materials to enhance oxygen reduction reaction in biofuel cells [3], in delignification and decolorization processes of wood [4], and in many bioremediation processes, involving detoxification of effluents from textile, paper and petrochemical industries [5]. Regarding the construction of biosensors, the wide range of compounds that can be oxidized by laccases may become a problem, if one wants to distinguish amongst several types of phenols, namely those with powerful health benefits that should be valorised, from those considered toxic for the environment in low concentrations.

Compared to fungal laccases (enzymes with high and medium redox potentials), bacterial laccases are far less explored, mostly due to their typically lower redox potentials [6]. However, they have revealed encouraging catalytic performances in industrial processes, such as textile wastewater treatments [7] and lignin valorisation [8,9], since they are active in a wider pH range, stable at higher temperatures, and less susceptible to inhibitory agents compared to the eukaryotic laccases, extracted from fungi and plants [10]. The same characteristics are also important to consider in the development of biosensors, since it is worth noting that the redox potential of a laccase is not the only factor affecting the substrate oxidation rate. There are some examples supporting the increased stability, selectivity and superior performance at higher pH values of low redox potential laccase from *Bacillus subtilis* [11], compared to fungal laccases, towards the oxidation of aromatic amines [12], and detection of hydroquinone [13].

In what concerns the bacterial laccases, the advantages and disadvantages of employing these enzymes in the electrochemical biosensors field are still overlooked. Thus, it is a scientific topic that should be pursued. Besides the search for robust laccases, there are also other critical challenges that still preclude the biosensing technological progress, namely, effective enzyme immobilization and improvements in signal transduction. A possible candidate to address these challenges is the bio-inspired approach based on polydopamine (PDA) dip coating, that became a reference material in surface modification due to its simple chemical preparation on virtually any substrate [14]. The versatile catechol moities found in PDA can interact with other functional groups either by non-covalent interactions, such as π-π, π-cation, and hydrogen bonds, and also by establishing covalent bonds with amines and thiols (through Michael-type addition or Schiff base formation). Both types of interactions can explain the successful attachment of biomolecules to polydopamine-coated electrodes, although the covalent bonds are more frequently mentioned due to their stronger character [15]. In our recent work we have demonstrated the suitability of electrochemically synthesized PDA coatings as bioconjugation platforms for affinity and enzymatic sensors [16–18]. Electrochemical synthesis also provides faster deposition rates, mitigates material loss due to its surface specificity, and offers fine control over the coatings physicochemical properties – crucial characteristics for biosensors development. PDA is by far the most acquainted catechol-derived coating; however, there are other promising catecholamines, possessing additional chemical groups, such as norepinephrine (NE) [19–21], whose electrosynthesis and physicochemical properties have been recently reported [22]. It was proved that potentiodynamically synthesized ePNE with a similar thickness of ePDA, exhibited better electroactivity, lower hindrance for the electron transfer of soluble ionic species, keeping a high adhesiveness. These properties are advantageous for the development of electrochemical transduction interfaces, but as far as we are concerned, there are very few studies addressing polynorepinephrine performance as an amperometric biosensing interface up to now, for glucose [23,24] and Mycobacterium tuberculosis detection [25].

Hereby, as illustrated in the scheme of Fig. 1, we carried out a comparative study of the performance of two electrosynthesized polymers, ePNE and ePDA as transducing matrices in amperometric biosensors for the detection of phenolic compounds. First, thin ePNE and ePDA films were grown potentiostatically on carbon electrodes and then modified with a technical bacterial laccase, with demonstrated catalytic activity, in solution, for the oxidation of several phenolic compounds [8]. In this configuration, the phenolic substrates are oxidized by the immobilized enzyme into phenoxy radicals, which are then reduced at the modified electrode surface, originating a cathodic current proportional to the analyte concentration. Amongst the numerous phenolic compounds, we have selected catechin and gallic acid as target analytes, since they represent important classes of phenols, namely flavonoids and phenolic acids, respectively, and are both abundant in agro-industrial wastes [26]. The conventional methods for quantification of phenolic content in the environmental samples are based on spectrophotometry (*e.g.*, Folin-Ciocalteu method [27] that is very affected by non-phenolic interferents) or chromatography, both time consuming, expensive, and requiring specialized technicians to perform the analyses. Therefore, the search for specific enzymatic biosensors can greatly contribute for a fast and efficient screening of industrial waste.

2. Experimental section

2.1. Chemicals

DL-norepinephrine hydrochloride (NE), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (+)-catechin hydrate, gallic acid and hexaammineruthenium(III) chloride were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); dopamine hydrochloride (DA) was purchased from Alfa-Aesar (Haverhill, Massachusetts, USA). Potassium hexacyanoferrate (III) was acquired from Merck (Darmstadt, Germany). Citrate-phosphate buffers (CPB) were prepared from citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$, Sigma-Aldrich) and di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) from Panreac-AppliChem (Chicago, Illinois, USA) in the following concentrations: CPB pH 7.0 (17 mM $C_6H_8O_7{\cdot}H_2O$ and 66 mM $Na_2HPO_4{\cdot}12H_2O$) and CPB pH 5.0 (27 mM $C_6H_8O_7{\cdot}H_2O$ and 47 mM

$Na_2HPO_4{\cdot}12H_2O$, both adjusted with sodium hydroxide, Sigma-Aldrich). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Buchs, Switzerland). All reagents were all analytical grade and used without further purification. Ultrapure water was obtained from a purification system, MILLI-Q A10 (18 MΩ·cm, 25 °C) from Merck-Millipore (Burlington, Massachusetts, USA). Industrial alkaliphilic bacterial laccase product (MetZyme® L371) was kindly provided by MetGen (Oy, Kaarina, Finland).

2.2. Laccase preparation

Laccase solutions were prepared by diluting 1:10 or 1:50 the enzyme preparation in CPB pH 7.0 or pH 5.0, followed by centrifugation at 13,400 rpm for 10 min on a MiniSpin® centrifuge (Eppendorf, Hamburg, Germany). The supernatant was used to evaluate the enzyme activity in solution and to modify the polycatecholamine electrodes. The protein content of the supernatant of 1:50 dilution (0.28 ± 0.01 $mg{\cdot}mL^{-1}$) was estimated by the Bradford assay using bovine serum albumin for the calibration curve [28].

2.3. Laccase activity

Enzymatic activity of the laccase preparation was assessed by UV–Vis spectroscopy (UV2600 spectrophotometer, Shimadzu, Kyoto, Japan) following the oxidation of ABTS at 420 nm ($\varepsilon = 3.6 \times 10^4\ M^{-1}{\cdot}cm^{-1}$) [29]. The assay was carried out at 22 ± 2 °C, by mixing 250 μL of 3 mM ABTS with 50 μL of diluted laccase 0.28 $mg{\cdot}mL^{-1}$, in 1 mL total volume of CPB pH 5.0. The absorbance was recorded for 60 s and a value of 40 $U{\cdot}mL^{-1}$ could be estimated (one unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol ABTS per minute).

For homogeneous catalysis studies, 0.5 mM catechin and gallic acid in CPB pH 5.0 and 7.0 were incubated with laccase, using different concentrations, as described below. The formation of the oxidation products was followed between 220 and 700 nm (Thermo Scientific spectrophotometer, Genesys 180 model, Rodano, Milan, Italy), and spectra were recorded at the requested times.

2.4. Carbon electrodes

A glassy carbon (GC) disk (exposed area of 0.44 cm^2) was employed as working electrode for the electropolymerization studies in a single compartment Teflon cell. The electrode surface was polished on synthetic fiber cloths (Buehler Microcloth, Esslingen, Germany), using alumina suspension (Buehler Alpha Micropolish, Esslingen, Germany) of decreasing grain sizes (5.0, 1.0 and 0.3 μm), with a washing procedure by sonication for 3 min between polishing steps and 15 min at the end, to remove alumina residues from the electrode surface. Afterwards, the polished electrode was cleaned in an UV chamber (ProCleaner™ Plus, Bioforce Nanosciences, Utah, USA), for 40 min, to remove any remaining organic residues [30]. For AFM analysis, freshly cleaved highly oriented pyrolytic graphite (HOPG) electrodes from Agar Scientific (Stansted Mountfitchet, United Kingdom) were used. Graphite plate (Ref. 10132) from Alfa-Aesar was cut into rectangular slides and tested as substrate for disposable amperometric biosensing interfaces. The electrode was polished using a waterproof SiC (silicon carbide) sandpaper (p800), followed by 15 min water sonication. The geometric area of the working electrode was limited by covering the remaining surface with Teflon tape. The active geometric area of each electrode was precisely measured by using a calliper (*ca.* 2 cm^2 with an error of 0.01 cm^2). All carbon electrodes were dried using a nitrogen flux.

2.5. Electrode modification and electrochemical assays

Potentiostatic polymerizations were carried out using 5 mM dopamine and norepinephrine solutions in CPB pH 7.0, on glassy carbon, HOPG, or graphite surfaces, at 0.3 V, for 120 s (ePDA) and 200 s (ePNE). CPB has been chosen as supporting electrolyte based on previous electropolymerization studies from the group [16,17,21]. This buffer solution was successfully used to grow polydopamine and other polycatecholamines, in contrast, for instance, to other common electrolytes (*e.g.* phosphate buffer saline or lithium perchlorate) with superior ionic strength, which may affect the initial adsorption of oligomers to the surface, precluding the quantitative growth of the polymer. The neutral pH was used to avoid any chemical polymerization of catecholamines by residual oxygen. Furthermore, solutions were previously deaerated with N_2 for 20 min. After modification, the electrodes were extensively washed with ultrapure water and characterized in deaerated monomer-free solutions, CPB pH 7.0. Following this step, the electrodes modified with polycatecholamines were immersed in diluted laccase preparation (0.28 $mg{\cdot}mL^{-1}$) for 3 h. Afterwards, the electrodes were placed in buffer solution (pH 7.0) for 30 min, to remove any weakly bounded laccase from the polymeric film. The assessment of the catalytic activity of laccase modified electrodes towards gallic acid and catechin was performed by chronoamperometry, in CPB buffered solutions at pH 5.0 and 7.0. The catechin assay was performed with the addition of 40 μL of DMSO per mL of CPB buffer. The assays were conducted by stepping the potential from open-circuit potential to 0.0 V and –0.1 V, for the solutions at pH 5.0 and 7.0, respectively, and pulses of 200 s and 100 s were applied upon successive additions of gallic acid or catechin, respectively. The electrolyte solution was aerated during the measurements to ensure a constant oxygen concentration. All electrochemical assays were performed inside a Faraday cage, using a platinum counter electrode and a saturated calomel reference electrode RE-401 (Radiometer Analytical S.A., Lyon, France), and were carried out using a PARSTAT 2263 produced by AMETEK (Princeton Applied Research, Pennsylvania, USA). Electrochemical impedance spectroscopy (EIS) was performed in a solution of 1 mM $K_3Fe(CN)_6$ in CPB pH 5.0 and 7.0, and acquired at 0.2 V (half-wave potential of the redox probe at GC), using a frequency range from 1 kHz to 10 mHz and a perturbation amplitude of 10 mV. Equivalent circuit fitting was performed with IviumSoft software (v 4.985).

2.6. Ellipsometry

The optical parameters (refractive index and extinction coefficient) and the thickness of the polynorepinephrine and polydopamine layers were obtained by ex-situ ellipsometry, using a single wavelength (He-Ne laser, 632.8 nm) ellipsometer SE400 (SENTECH Instruments GmbH, Berlin, Germany) in a PSA configuration, at 70° incidence angle. The computation of the data was undertaken considering a physical model composed by a semi-infinite substrate and a homogeneous layer of polymer film.

2.7. Atomic force microscopy

The morphology of modified HOPG surfaces was characterized on a Multimode 8 HR, Nanoscope V (Bruker, Massachusetts, USA), using Peak Force Tapping mode and ScanAsyst control. The images were obtained in ambient conditions (≈21 °C) at a scan rate of ≈1.0 Hz, using a ScanAsyst-air probes (Bruker, Massachusetts, USA) with a spring constant of 0.44 $N{\cdot}m^{-1}$. Thickness measurements were carried out by scratching the ePDA and ePNE modified HOPG using an edge of a thick paper.

2.8. Water contact angle measurements

The modified electrodes wettability was evaluated through water contact angle analysis using a Krüss DSA30 (Hamburg, Germany) goniometer. A volume of 1 μL of water was placed on the surface of the electrode and the resulting contact angle was measured using Drop Analysis software by Krüss.

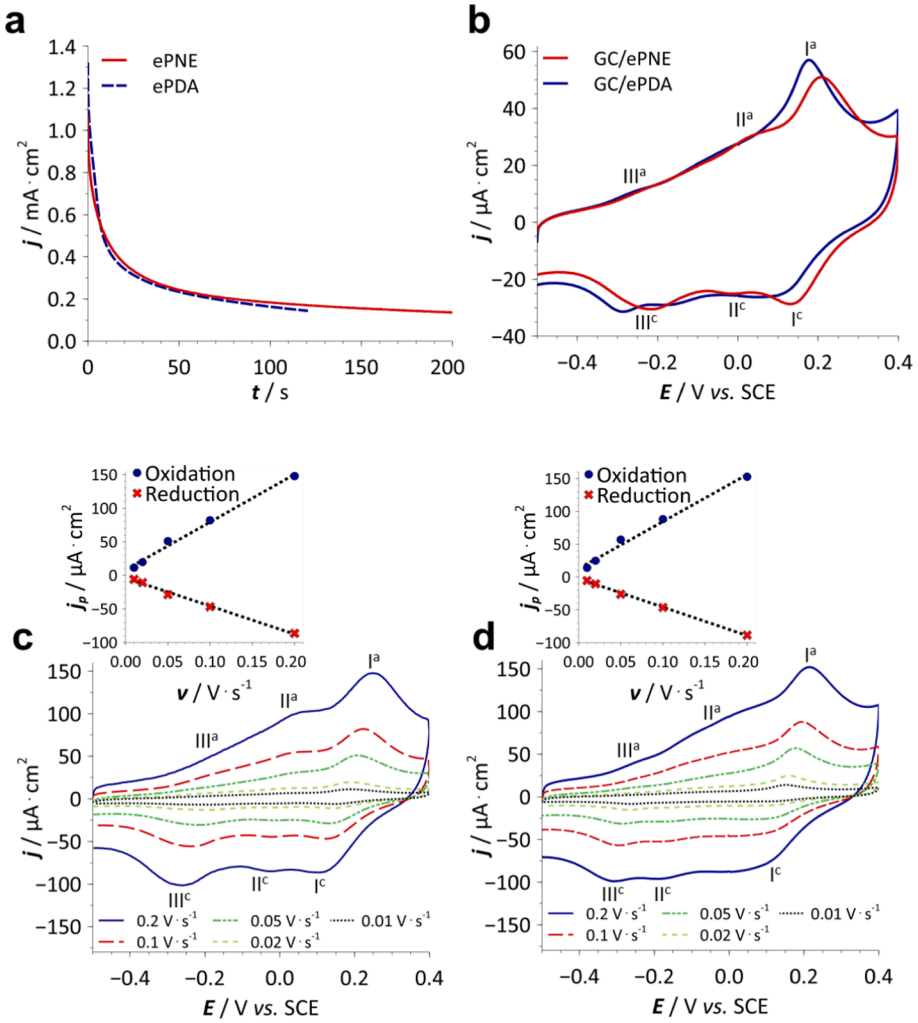

Fig. 2. (a) Potentiostatic synthesis (0.3 V) of ePNE (200 s) and ePDA (120 s) films from 5 mM DA and NE in CPB pH 7.0 solutions, respectively. (b) Cyclic voltammograms of ePNE and ePDA in CPB pH 7.0, recorded at 50 mV·s^{-1}. (c) and (d) Cyclic voltammograms at different potential scan rates for ePNE and ePDA modified electrodes in CPB pH 7.0, respectively. The insets show the representations of the peak current density (process I) *versus* potential scan rate of ePNE (oxidation: Y=8.4 + 710.06 X, R^2 = 0.9925; reduction: Y = − 4.0–418.15 X, R^2 = 0.9943), and ePDA films (oxidation: Y=13.0 + 715.88 X, R^2 = 0.9890; Y = − 2.5–433.4 X, R^2 = 0.9984).

3. Results and discussion

3.1. Potentiostatic synthesis and electrochemical characterization of polycatecholamine films

The chronoamperograms corresponding to the potentiostatic growth of ePNE and ePDA films are presented in Fig. 2a. The applied potential was selected based on the potentiodynamic synthesis of ePDA and ePNE, corresponding to half of the maximum current value assigned to monomer oxidation [17,22]. A low potential value applied in the synthesis is crucial to obtain a non-overoxidized polymer with superior electrochemical activity [31], allowing a moderate rate of reactive species creation, leading to slow polymerization kinetics, and therefore contributing for the regularity of the formed polymer. Previous microgravimetric studies indicated a faster potentiodynamic polymerization rate for ePDA than for ePNE [22], during the initial 10 potential cycles. For this reason, the applied potential pulse for ePNE formation was longer than that of ePDA in order to achieve films with comparable thickness, as discussed below. The current of the transients shown in Fig. 2a, decrease over time, revealing that both polymers have low conductivity when compared to conventional conducting polymers (*e.g.* PEDOT, polyaniline), where the current rises after a nucleation step, due to a 3D growth of a conducting phase under diffusion control [32,33]. The catecholamines polymerization, rather than any diffusional regulation of their growth, is limited by the increasing resistance of the layer as it thickens, which inevitably leads to a continuous decrease of the rate of charge transfer, only determined by the ohmic constraints. Thus, although ePNE and ePDA display similar current profiles, the decrease in current observed for ePNE is less accentuated, suggesting the presence of a less insulating polymer layer. The cyclic voltammograms obtained for both polymers in CPB pH 7.0, reveal the typical two-electron two-proton redox processes of polycatecholamines, as thoroughly discussed in the literature [16,22,34]. The main process (I) in Fig. 2b, occurring at higher potentials, corresponds to o-quinone/o-diphenolic moieties conversion, the intermediate process (II) can be assigned to the presence of indole-type species and the more negative process (III) is assigned to the indolinequinone/dihydroxyindoline groups. Indolinic species result from a chemical step involving an intramolecular cyclization of catecholamines, upon oxidation to o-quinones, due to nucleophilic attack of the nitrogen atoms to the catechol rings, whereas the indolic species, arise from an isomerization process of the indolinic species. Despite their similarities (Fig. 2c and 2d), the redox properties of ePNE are better-defined than those of ePDA, and the presence of indolic moieties is clearly denoted [34]. The insets of Fig. 2c and d show the linear relationship between the oxidation and reduction peak currents of the process I against the potential sweep rate for ePNE and ePDA,

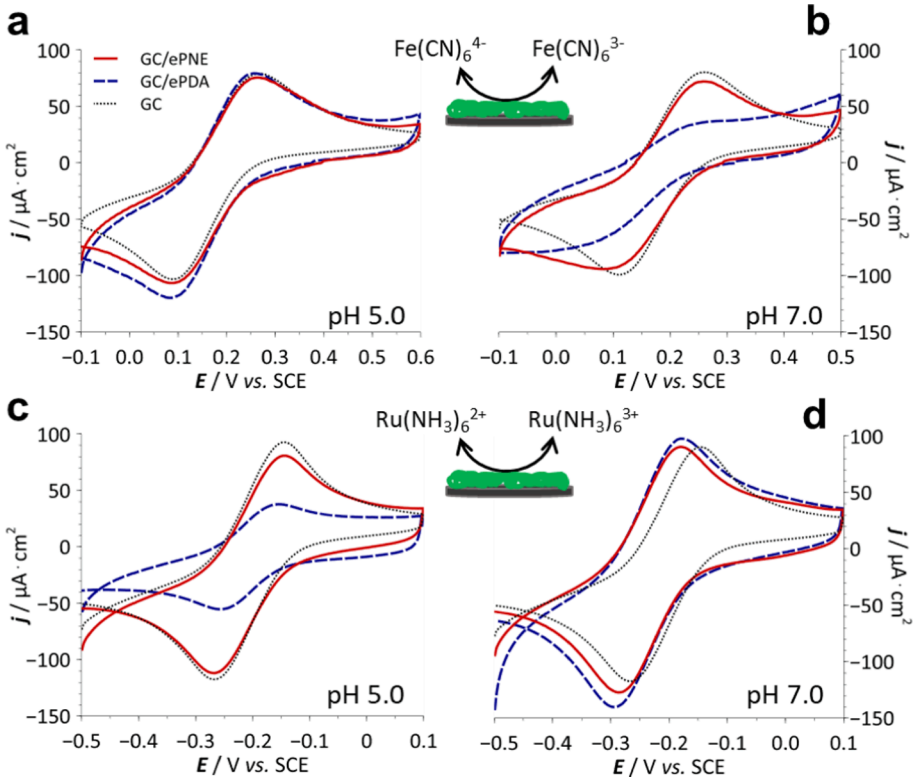

Fig. 3. Cyclic voltammograms, recorded at 50 mV·s^{-1}, of bare GC and GC modified with ePDA, ePNE, grown potentiostatically at 0.3 V for 120 and 200 s, respectively. 1 mM $K_3[Fe(CN)_6]$ in CPB pH 5.0 (a) and pH 7.0 (b); 1 mM $[Ru(NH_3)_6]Cl_3$ in CPB pH 5.0 (c) and pH 7.0 (d).

respectively, revealing that electrochemical conversions of the immobilized redox active groups for both polymers are not determined by mass-transfer phenomena. The surface coverage of this groups can be estimated following relationship: $j_p = (nF)^2(4RT)^{-1}\Gamma\nu$ (n, number of transferred electrons; F, Faraday constant; R, gas constant; T, absolute temperature; ν, sweep rate) [35]. Considering the slope of the oxidation processes I^a presented in Fig. 2c and Fig. 2d, and assuming a two-electron transfer, the surface coverage of the polymers redox active groups is estimated to be 1.90×10^{-10} mol cm^{-2} for the case of ePDA, and 1.87×10^{-10} mol cm^{-2} for the case of ePNE, confirming identical coverages of the electrode by both polymers.

When preparing an amperometric biosensor interface, it is important to evaluate the ability of the polymer modified electrode for the electrochemical conversion of positively and negatively charged soluble ionic species, at the working pH of the enzyme. Fig. 3 illustrates the redox behaviour of 1 mM $[Fe(CN)_6]^{4-/3-}$ or $[Ru(NH_3)_6]^{2+/3+}$ species, at CPB pH 5.0 and 7.0, on ePNE and ePDA films. The electrochemical response of a bare GC electrode is also included for comparison. In general, the redox potentials of the anionic (pH 5: $E_{1/2} = 0.178$ V, $\Delta E_p = 0.177$ V and pH 7: $E_{1/2} = 0.185$ V, $\Delta E_p = 0.150$ V) and cationic probes ($E_{1/2} = -0.206$ V, $\Delta E_p = 0.122$ V) on bare GC are nearly unchanged upon modification with ePDA or ePNE (Fig. 3a–c), with the exception of $[Fe(CN)_6]^{4-/3-}$ conversion on ePDA, at pH 7, which presents a more irreversible profile with a larger peak separation ($\Delta E_p = 0.270$ V). In the

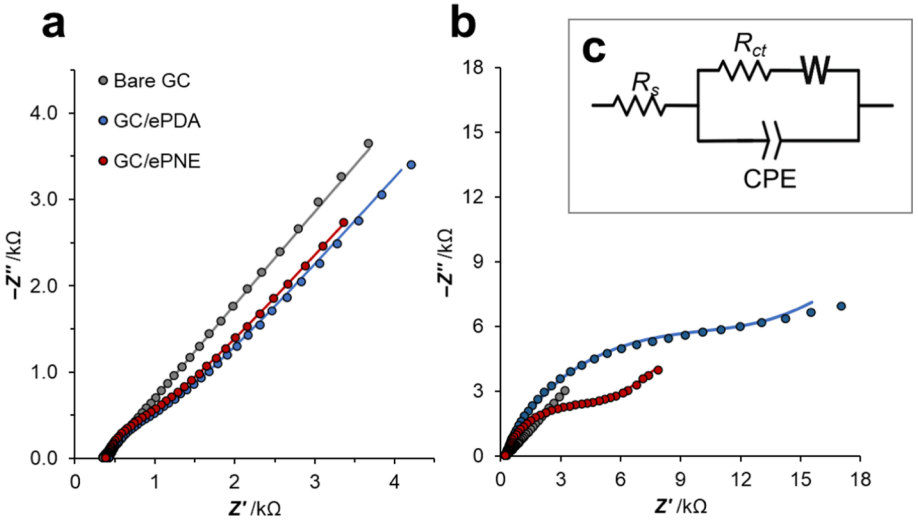

Fig. 4. Nyquist plots of GC electrode before and after modification with ePDA and ePNE, acquired in 1 mM $K_3[Fe(CN)_6]$ CPB solution at pH 5.0 (a) and pH 7.0 (b). Equivalent circuit used to fit the EIS data (c).

Table 1
Charge transfer resistance (R_{ct}) values estimated for the EIS data shown in Fig. 4, and corresponding absolute fitting errors.

Electrode	R_{ct} /kΩ	
	pH 5	pH 7
GC	0.23 ± 0.02	0.15 ± 0.01
GC/ePDA	0.98 ± 0.03	13 ± 1
GC/ePNE	0.75 ± 0.01	4.97 ± 0.03

specific case of $[Ru(NH_3)_6]^{2+/3+}$ redox conversion, at pH 7, on both polymers (Fig. 3d), there is a negative potential shift (*ca.* 30 mV), indicating a more facile electron transfer reaction. It is also observed that ePNE does not hinder the oxidation or reduction currents of either the anionic or the cationic species, in any of the solutions studied. In contrast, on ePDA modified GC electrode, there is a decrease in the anodic and cathodic peak currents at pH 5.0, for the $[Ru(NH_3)_6]^{2+/3+}$ redox conversion, and at pH 7.0 for the negatively charged $[Fe(CN)_6]^{4-/3-}$ redox probe. This behaviour suggests that ePDA polymer charge, which changes according to the solution pH, affects its transduction properties. In fact, both ePDA and ePNE are expected to change their net charge, at distinct pH values, being positive at pH below 4–5 and negatively charged above this value [22], which has been attributed to the presence of semiquinones (pK_a of 4.7 [36]) and thus, despite of having very similar thickness, as discussed below, ePNE interface allows a better electrochemical conversion than ePDA, revealing either a more

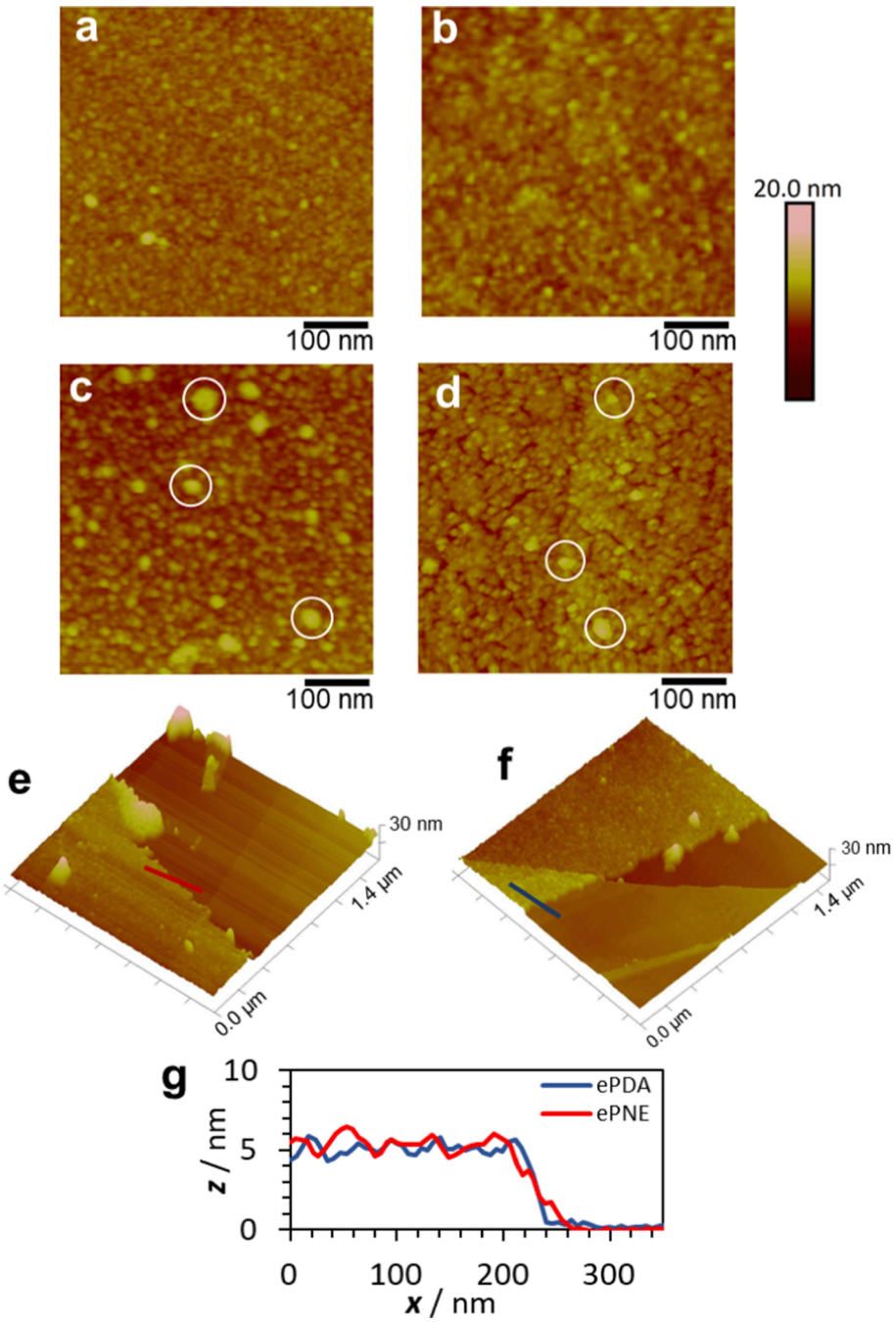

Fig. 5. AFM topographic images of ePNE (a) and ePDA (b) films, grown potentiostatically on HOPG electrodes, from 5 mM NE and DA in CPB pH 7.0, at 0.3 V for 200 and 120 s, respectively, and after their incubation in 0.28 mg·mL^{-1} laccase solution for 3 h: (c) ePNE/Lac and (d) ePDA/Lac. The 3D images of ePNE (e) and ePDA (f) coatings also exhibit clean HOPG areas after intentional scratching; the corresponding cross section analysis are shown in panel (g).

porous layer or a less insulating polymer.

To further validate the best transduction performance of ePNE, compared to ePDA, electrochemical impedance spectroscopy (EIS) assays were carried out using the same anionic probe. Fig. 4a and b show the Nyquist plots at pH 5.0 and 7.0, respectively, corresponding to unmodified and modified GC electrode with ePDA and ePNE. To fit the EIS spectra the very simple modified Randles circuit of Fig. 4c was used [22,37]. It includes an electrolyte resistance element (R_s) in series with a parallel circuit comprising a charge transfer element (R_{ct}), in series with a Warburg impedance element (W), related to diffusion, and a constant phase element (CPE), accounting for a non-ideal double-layer capacitance. Table 1 compiles the corresponding fitted R_{ct} values for bare and modified GC electrodes. It is clear that EIS spectra significantly changes from pH 5.0 to 7.0 for both polymers. At pH 5.0 (Fig. 4a), the impedimetric responses of the modified interfaces are similar to that of bare GC electrode, presenting very small incomplete semicircles indicating low charge transfer resistances (>1 kΩ, Table 1) within the high-frequency range, and typical semi-infinite diffusion regions for middle-range frequencies. The results agree with a facile electron transfer on ePNE and ePDA, recorded by cyclic voltammetry at pH 5.0 (Fig. 3a). At pH 7.0 (Fig. 4b), ePNE impedimetric response presents a smaller semicircle than that of ePDA (*ca.* 5 *versus* 13 kΩ, Table 1), demonstrating a lower charge transfer resistance for the conversion of the $[Fe(CN)_6]^{4-/3-}$ probe on ePNE, further supporting the cyclic voltammetric assays (Fig. 3b).

3.2. Morphology, thickness and wettability of modified electrode

AFM topographic images were taken on modified HOPG electrodes with ePNE and ePDA thin films before (Fig. 5a and b, respectively) and after (Fig. 5c and d, respectively) laccase incubation. HOPG was employed in the topographic analysis of the polycatecholamines, since it has an atomically flat surface, allowing to perfectly distinguish the deposited thin polymeric films from the underlying carbon electrode, in contrast with previous polydopamine topographic studies on GC, where the roughness of the coatings was highly associated to the polished GC surfaces [16]. The morphologies of the ePDA and ePNE are consistent with those observed before for these polycatecholamines, revealing very uniform films with a regular granular morphology [16,17]. Root mean-square roughness values, Rq, of 0.80 nm and 0.88 nm are obtained for ePNE and ePDA, respectively.

For the determination of the polymeric film thickness, ePNE and ePDA grown on HOPG, again to minimize the error from the underlying substrate roughness, were intentionally scratched to produce an area that encompasses polymer and the underlying HOPG substrate, thus suitable for cross section analysis (Fig. 5e and f, respectively). The images of bare HOPG surface show the typical atomically flat terraces, with a height variation of 0.33 nm or multiples of that, corresponding to a single or multilayers of carbon atoms [38]. Through the profiles taken, it can be concluded that the thickness of both films is very similar, 5.3 ± 0.5 and 5.1 ± 0.4 nm, for ePNE and ePDA, respectively, as intended for this comparative study. The results confirm the requirement of using a superior growth charge for ePNE (43 $mC{\cdot}cm^{-2}$) than that measured for ePDA (32 $mC{\cdot}cm^{-2}$) (Fig. 2a). In fact, it is known that during the electropolymerization of catecholamines there is a chemical formation of indoline-type species, which do not contribute extensively for polymer formation [16]. The coatings should be mainly formed by polycatecholamines in open form. According to our previous data [22], the ePNE electrosynthesis originates a larger number of cyclized intermediates in solution compared to those formed during ePDA deposition. Thus, longer electrodeposition times are required to produce ePNE films with the same thickness as ePDA coatings.

The complex refractive index, $N=n-ki$, comprising the optical parameters n (refractive index) and k (extinction coefficient) as compiled in Table 2, could be determined for both films, taking into consideration the thickness obtained by AFM. The values fully agree with those previously reported for polydopamine film prepared on GC electrode [1.84 (±0.09) – 0.363 (±0.006)i], under similar conditions [17]. The calculated refractive index of polycatecholamines is high, when compared to well-known conducting polymers, such as polypyrrole [39], or PEDOT [40], confirming the presence of optically dense films. Nevertheless, ePNE presents a slightly lower refractive index than ePDA, which should reveal a less dense film, supporting the better electrochemical response of ionic species on this film. On the other hand, the values of the extinction coefficient, that is related to the electronic π-π* transitions, is higher for ePNE than for ePDA, suggesting that the former may have a larger amount of double bonds in polymer backbone, whether they are conjugated or not [41]. In fact, when comparing the cyclic voltammograms of Fig. 2c and d, the redox processes assigned to the indolic form (II), are better defined for ePNE than for the ePDA coating.

Table 2
Optical parameters retrieved from ellipsometric measurements: complex refractive index (N) and thickness (L); Water contact angle (θ_{WCA}) data.

Film	$N=n-ki$	L /nm	θ_{WCA} /°	
			without laccase	with laccase
ePNE	1.80(±0.01) − 0.52 (±0.02)i	5.3 ± 0.5	25 ± 2	65 ± 1
ePDA	1.87(±0.04) − 0.351 (±0.09)i	5.1 ± 0.4	35 ± 2	63 ± 1

As mentioned, Fig. 5c and d show the polycatecholamines upon incubation in a laccase solution followed by thorough rinsing. Since the size of enzyme molecules, imaged by AFM, are similar to those of polymer clusters, it is difficult to clearly discriminate between individual laccase and polymer globules. Besides, their mechanical properties are also alike, precluding distinguishing them by phase imaging. However, the presence of enzyme can still be depicted by the globular features distributed on top of the polymer layers, especially for ePNE, as pinpointed by the white circles. The different topography of ePNE and ePNE/Lac is further highlighted in the three-dimensional images presented in Fig. S1 (Supplementary Material). The images suggest a good enzyme distribution on the surface, since no large agglomerates could be depicted upon laccase immobilization. The fact that the modified electrodes are thoroughly washed after enzyme adsorption step ensures a robust interaction between the enzyme and the polymer, probably to covalent and non-covalent interactions.

Further confirmation of enzyme immobilization is provided by water contact angle measurements (Table 2). The WCA for ePNE is lower than that of ePDA, as expected for a monomer containing an extra hydroxyl group, which should be kept upon polymerization, making ePNE a more hydrophilic material. After laccase incubation, the WCA values increase, due to the presence of protein on the polymeric film [42].

3.3. Homogeneous catalysis of gallic acid and catechin by laccase

The assessment of the catalytic performance of the bacterial laccase towards the oxidation of the selected phenolic compounds, gallic acid (Fig. 6a and b) and catechin (Fig. 6c and d), was carried out by UV–Vis absorption spectroscopy, in CPB pH 5.0 and 7.0. The phenolic compound bearing a carboxyl group (gallic acid) showed a significant oxidation rate at both acidic and neutral pH values (Fig. 6a and b), even though, a higher laccase concentration was required to obtain similar oxidation rates to catechin. It is worth noting that the peak at 460 nm, assigned to oxidation products, is higher at pH 7.0 than at pH 5.0, indicating a more promising catalysis at neutral pH. Catechin oxidation by laccase is significantly greater at neutral pH, than at pH 5.0, as evidenced by the appearance of novel absorption bands, at 440 and 473 nm, characteristic of the formation of catechin radical species, such as dimers or trimers, which absorb at higher wavelengths [43]. This behaviour is somehow expected for extremophilic laccases that usually have their optimal pH higher than that of fungal laccases [44]. The

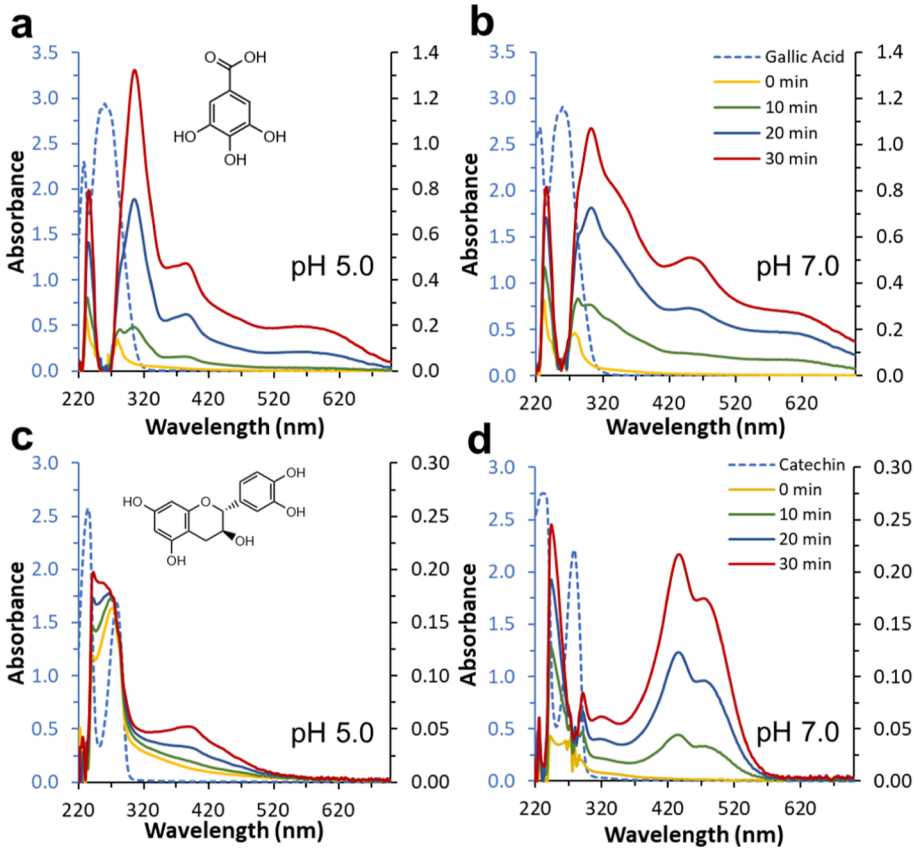

Fig. 6. UV–Vis absorption spectra of (a, b) 0.5 mM gallic acid in the presence of diluted laccase (1.40 mg·mL^{-1}) for 30 min and, (c, d) 0.5 mM catechin, in the presence of diluted laccase (0.28 mg·mL^{-1}) for 30 min. Both phenolic substrates were analysed at pH 5.0 and 7.0. The high absorbance values observed for the substrate (left y axis) were required to clearly detect the changes in the spectra after product formation.

observed catalytic activity is not described in the literature for these enzyme-substrate couples, and it is therefore worth to explore in the biosensing field, since a bacterial laccase biosensor may be able to identify phenolic acids in a complex mixture by changing the measuring pH. Data found regarding catechin-quinone suggests its absorption coefficient (<1300 M^{-1}·cm^{-1}) [45] is lower than that of *o*-benzoquinone (*ca.* 1400 M^{-1}·cm^{-1}) [46] and other simple *o*-quinones [47].

3.4. Heterogeneous catalysis of polycatecholamine-laccase films

Norepinephrine and dopamine have been potentiostatically polymerized on graphite electrodes, since, as mentioned, they have a substantially larger surface area than GC electrodes and a lower cost, granting more immobilized enzyme on the modified electrodes. By using chronoamperometry, the catalytic performance of the laccase-modified electrodes towards the oxidation of gallic acid and catechin could be quantitatively assessed. In the presence of the phenolic substrates, the immobilized laccase will oxidize them, by reducing the molecular oxygen dissolved in solution into water; then, the oxidized substrates will be reduced at an appropriate potential at the modified electrode surfaces. To achieve a good catalytic performance at these new interfaces, there are crucial aspects that must coexist, namely: i) laccase keeps its biological activity upon immobilization, ii) the enzyme is able to catalyse the oxidation reaction of the target phenolic molecules, at the experimental conditions, namely pH, and iii) polycatecholamine films enable the electrochemical conversion of the oxidized phenolic compound at a given electrode potential.

The comparative study of the catalytic performance for both polymeric interfaces (ePNE/Lac and ePDA/Lac) was carried out for a simple phenolic acid: gallic acid, as illustrated in Fig. 7. Firstly, the electrochemical conversion of gallic acid at pH 7.0 was evaluated by cyclic voltammetry (Fig. 7a), revealing an irreversible two-electrons and two-protons oxidation process, at 0.23 V, corresponding to the formation of 3-hydroxy-1,2-benzoquinone as the oxidation product [48,49]. It is known that the quinone species generated upon oxidation go through further chemical processes, thus not being reduced on the reverse cycle, for low sweep rates. Besides, consecutive cycling leads to a film deposition at the electrode surface, especially at neutral or basic pH values [17]. Therefore, by analysing the cyclic voltammogram of Fig. 7a, the potential values of 0.0 and –0.1 V for pH 5.0 and 7.0, respectively, were used for the chronoamperometric detection of gallic acid (Fig. 7b and c). A pulse of 200 s was chosen to ensure that the current obtained was stabilized for the concentration of analyte studied. The chronoamperometric assays performed at such conditions reveal that the ePNE and ePDA modified electrodes with laccase exhibit catalytic responses to gallic acid, as seen by the decrease of currents with the substrate concentration increments, confirming the presence of active immobilized enzyme at the studied interfaces. In particular, using ePNE as matrix for laccase immobilization resulted in great current decreases at pH 7.0 (Fig. 7b), although smaller current variations are observed at pH 5.0 (Fig. 7c). The differences between the assays performed at pH 5.0 and 7.0 can be better depicted in Fig. 7d, where the average of catalytic currents at the same pH is plotted against the gallic acid concentration. The steeper slope of ePNE/Lac at neutral pH agrees with the better catalysis observed in the spectroscopic studies presented in Fig. 6a and b, but is much more pronounced in the amperometric detection of oxidised gallic acid.

To ensure that the catalytic response towards gallic acid was exclusively due to the laccase immobilized in the polymeric matrix, a chronoamperometric assay with a graphite electrode modified only with

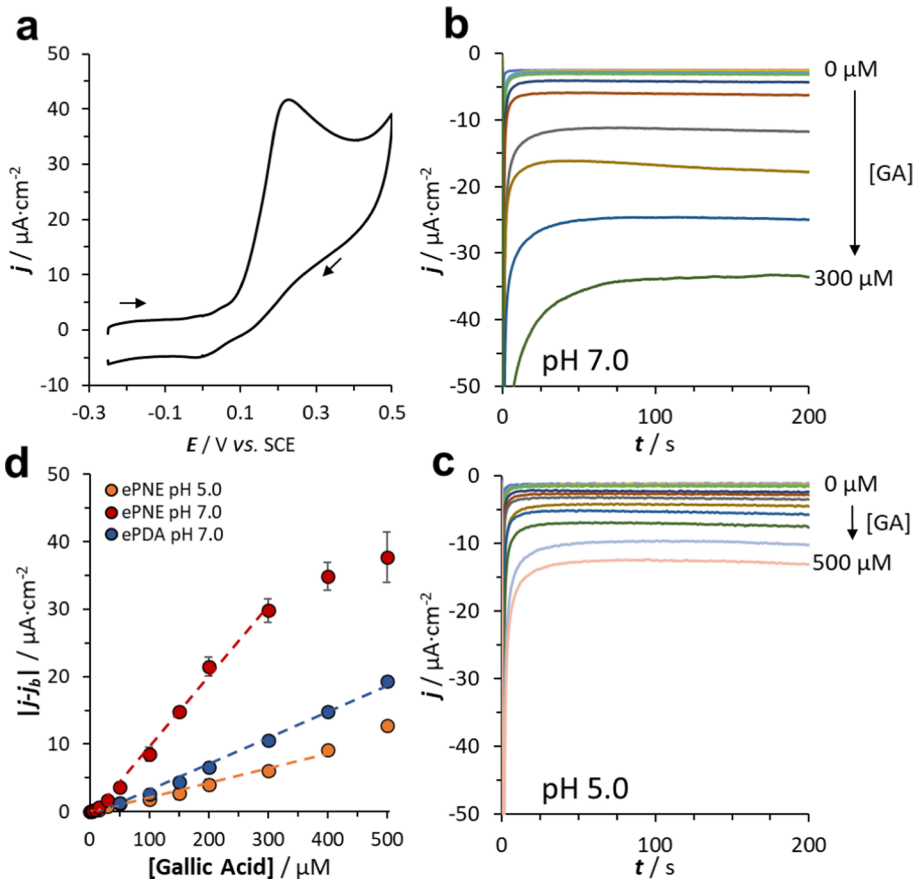

Fig. 7. (a) Cyclic voltammogram of bare GC electrode in deaerated 0.1 mM gallic acid solution in CPB pH 7.0, recorded at 50 mV·s^{-1}. Chronoamperometric responses of graphite electrodes modified with ePNE/Lac, at pH 7.0 (b) and 5.0 (c), with successive additions of gallic acid (corresponding to 1, 3, 5, 10, 15, 30, 50, 100, 150, 200, 300, 400, 500 μM) by applying a potential pulse of 0.0 V (pH 5.0) or −0.1 V (pH 7.0) for 200 s. (d) Average catalytic current densities ($|j\text{-}j_b|$, j_b is the background current density) *versus* gallic acid concentration, obtained from three chronoamperometric assays of different modified electrodes.

Table 3
Analytical parameters for gallic acid and catechin detection by the polycatecholamines-laccase modified electrodes: linear range, sensitivity, and corresponding coefficient of determination (R^2).

Analyte	Film	pH	LOD*/ μM	Linear Range /μM	Sensitivity /μA·cm^{-2}·mM^{-1}	R^2
Gallic acid	ePNE/ Lac	5.0	2.2	3–400	21.58 ± 0.05	0.9902
		7.0	0.7	3–300	104 ± 6	0.9950
	ePDA/ Lac	7.0	5.4	5–500	39 ± 2	0.9945
Catechin	ePNE/ Lac	5.0	11.2	10–1000	1.2 ± 0.3	0.9944
		7.0	1.3	1–1000	14.4 ± 0.3	0.9999

* LOD values were estimated using the expression 3σ/s, where σ is the standard deviation of the background current and s is the sensitivity of the sensor.

polynorepinephrine film was conducted (Fig. S2, Supplementary Material). Clearly, there is no detectable amperometric response to gallic acid at pH 7, applying − 0.1 V, when a graphite/ePNE electrode is used in the absence of enzyme.

The analytical parameters withdrawn from the chronoamperometric assays (Fig. 7) are compiled in Table 3. At pH 7.0, the sensitivity of the ePNE/Lac interface towards gallic acid is exceptionally enhanced (104 μA·cm^{-2}·mM^{-1}), compared to that obtained by us [17], using one-step potentiostatic polydopamine-laccase deposition (19.3 μA·cm^{-2}·mM^{-1}), at pH 4.6 (the optimal pH value for the fungal laccase used). Furthermore, the sensitivity value obtained in this work, at pH 5.0 (21.58 μA·cm^{-2}·mM^{-1}) is slightly higher than that obtained for fungal laccase, at pH 4.6. In addition, much wider linear ranges (3–300 or 3–400 μM) are achieved for the present bacterial laccase at both pH values, compared to that obtained previously (1–150 μM). At least, three assays using distinct modified electrodes were employed to estimate the analytical data, revealing the good reproducibility of the proposed biosensing interfaces.

Table 4
Analytical parameters (LOD, linear range and sensitivity) of other laccase-based amperometric biosensors reported in the literature.

Phenol	pH	Sensitivity /μA·cm^{-2}·mM^{-1}	Linear Range /μM	LOD /μM	Sensor Architecture	Laccase Source	Reference
Gallic acid	4.7	19	1–150	0.29	ePDA/Lac (entrapment)	Fungal	[17]
	6.0	4	–	380	SPE/Ferrocene/Sol-gel with Lac. (drop casted)	Fungal	[53]
	6.5	40	0–500	–	Resin/Lac (immobilized)	Fungal	[51]
	7.0	104	3–300	0.7	ePNE/Lac (immobilized)	Bacterial	This work
Catechin	6.5	283	0–100	–	Resin/Lac (immobilized)	Fungal	[51]
	6.0	41	–	20	SPE/Ferrocene/Sol-gel with Lac. (drop casted)	Fungal	[53]
	7.0	14.4	1–1000	1.3	ePNE/Lac (immobilized)	Bacterial	This work

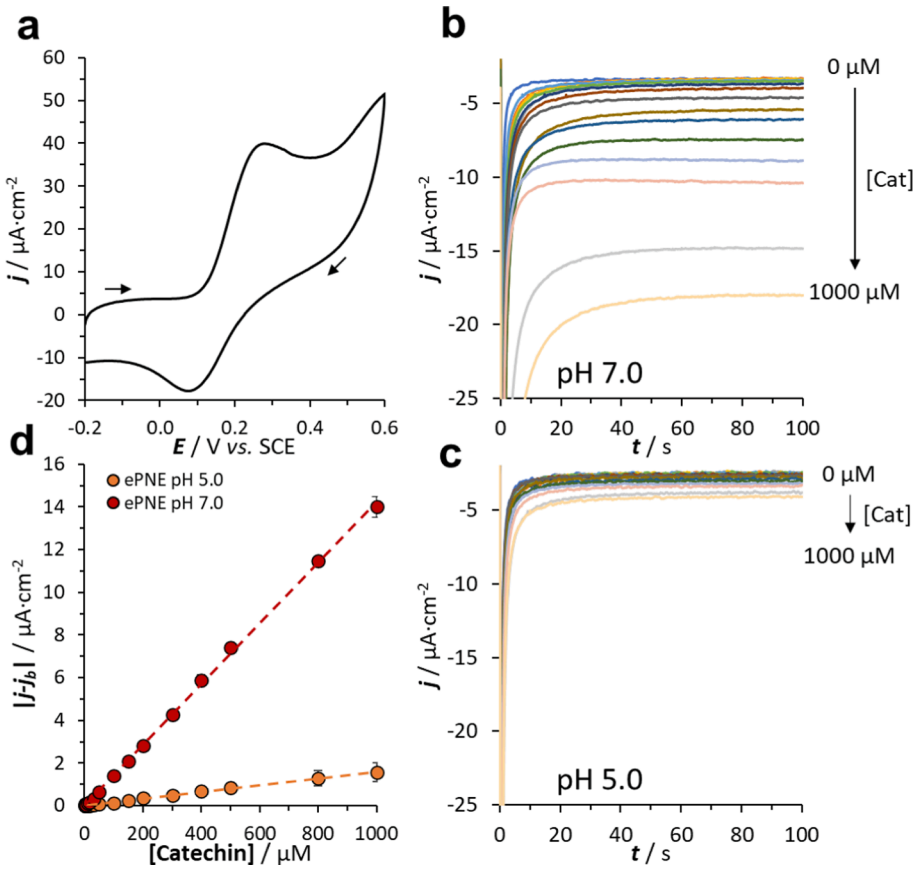

Fig. 8. (a) Cyclic voltammogram of bare GC electrode in deaerated 0.1 mM catechin solution in CPB pH 7.0, recorded at 50 mV·s^{-1}. Chronoamperometric responses of graphite electrodes modified with ePNE/Lac, at pH 7.0 (b) and 5.0 (c), with successive additions of catechin (corresponding to 1, 3, 5, 10, 15, 30,50, 100, 150, 200, 300, 400, 500, 800, 1000 µM) by applying a potential pulse of 0.0 V (pH 5.0) or −0.1 V (pH 7.0) for 200 s. (d) Average catalytic current densities ($|j\text{-}j_b|$, j_b is the background current density) *versus* catechin concentration, obtained from three chronoamperometric assays for different modified electrodes.

Moreover, the modified electrode that displayed the best performance at pH 7.0 was ePNE, with a considerably higher sensitivity than that achieved with ePDA (39 µA·cm^{-2}·mM^{-1}), highlighting the use of norepinephrine as an alternative monomer to dopamine. This result agrees with the higher electroactivity and with the better electrochemical transducing properties disclosed for ePNE regarding ePDA, which can be tentatively explained by ePNE higher organization/porosity. In addition, AFM images (Fig. 5) suggest that there is more enzyme adsorbed onto polynorepinephrine than on polydopamine films.

The representations of the current density *versus* concentration (Fig. 7d) slightly deviate from the typical hyperbolic Michaelis-Menten curve, vaguely denoting a sigmoidal shape, observed for allosteric enzymes with intricate catalytic reaction kinetics, many times leading to detection signal improvements [50]. Since, this bacterial enzyme is poorly explored in amperometric biosensors, it is worth to further investigate this phenomenon towards distinct phenolic compounds in a subsequent study.

Table 4 compares the analytical parameters obtained in this work, using an industrial laccase from bacterial source, with other laccase-based biosensors. The pH-dependent behaviour clearly distinguishes these biosensing interfaces from those constructed using fungal laccases, for example the one in our previous work [17], that display an optimal working pH in the acidic domain. A fungal laccase from *Rigidoporus lignosus* [51], displaying an optimal pH of 6.5, was used before to build a more complex biosensing interface on a screen printed electrode (SPE) modified with ferrocene, incorporating laccase by a sol–gel method. However, a much lower linear range of catechin detection is reported (0 – 100 µM) [51]. In fact, the observed wide linear range of the graphite electrodes modified with ePDA/Lac and ePNE/Lac is unusual, considering the ranges of other enzymatic sensors with amperometric transduction [52].

Fig. 8a shows the electrochemical behaviour of 0.5 mM catechin in CPB pH 7.0 in bare GC, required to select a suitable potential value to carry out the chronoamperometric assays. As major process, a typical quasi-reversible two-electron two-proton redox conversion of an *o*-quinone/*o*-hydroquinone group centred at *ca.* 0.18 V, is identified [54]. Since catechin-quinone is the expected enzymatic oxidation product [55], the potential selected for its amperometric detection was −0.1 V at pH 7.0, and 0.0 V at pH 5.0, as a result of potential shift due the pH change [54]. The imposed potential will prevent any phenolic compound oxidation reaction at the electrode surface, so that the detected cathodic current during the assay only comes from the enzymatic activity. In agreement with the UV–Vis absorption spectra (Fig. 6c and d), considerably more intense amperometric responses were observed at neutral media compared to the acidic conditions, which is due to the pH-dependent catalytic behaviour of the immobilized bacterial laccase. The analytical parameters obtained from the assays in Fig. 8b are also presented in Table 3. As a consequence of the low catechin oxidation rate at pH 5.0, a low sensitivity is observed at this pH for ePNE/Lac modified electrodes (1.2 µA·cm^{-2}·mM^{-1}), whereas at pH 7.0 this parameter is more than 10 times greater (14.4 µA·cm^{-2}·mM^{-1}), as better depicted in Fig. 7c. The examples given in Table 3 show sensors with higher sensitivity, however the linear range for our sensor is much wider. It will be worth to explore a more complex architecture, most probably incorporating catalytic nanomaterials, as shown in the work by Vianello *et al.* [51].

To evaluate the repeatability of the modified electrodes, two consecutive assays were done to detect gallic acid and catechin (Fig. 9),

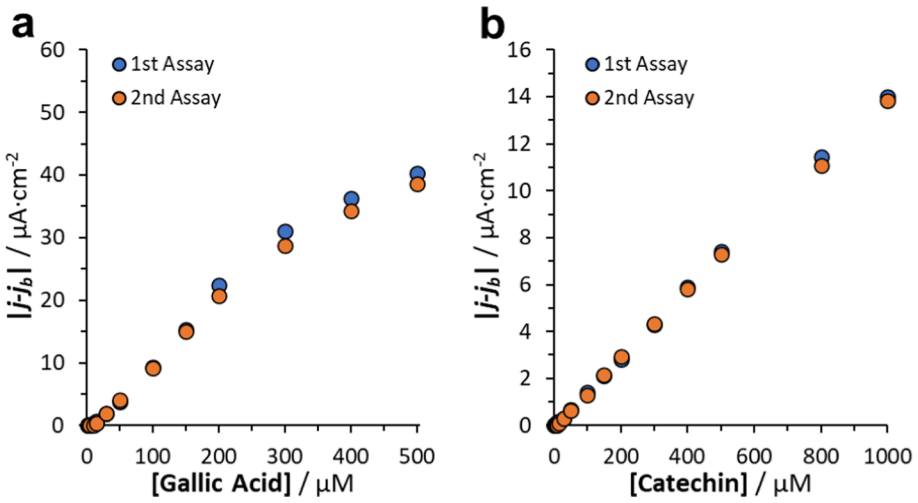

Fig. 9. Current densities ($|j\text{-}j_b|$, j_b is the background current density) *vs.* substrate concentration obtained for two consecutive chronoamperometric responses of graphite electrodes modified with ePNE/laccase at pH 7.0 by applying potential pulses of −0.1 V of 200 s for gallic acid (a) and 100 s for catechin (b).

revealing a minimal deviation between the two assays, with an error of 5 in 105 $\mu A \cdot cm^{-2} \cdot mM^{-1}$ for gallic acid and 1 in 14 $\mu A \cdot cm^{-2} \cdot mM^{-1}$ for catechin. This supports the robustness of the interface and the laccase immobilization on the modified electrode surface.

The facile association between polycatecholamine matrices and active bacterial laccase with an alkaliphilic character reveals the great potential of these interfaces for the direct monitoring of polyphenols in waste samples, that are neutral or slightly basic, without the need for an acidification step. Furthermore, since the bacterial laccases are still poorly explored in the biosensors field, it is imperative to further investigate the analytical properties of the modified interfaces towards distinct classes of phenolic compounds, valuable for the food, cosmetic and pharmaceutical industries, in order to quantify those compounds by direct amperometric measurement in real samples, such as olive mill wastewaters or alkali-pretreated lignocellulosic biomass. Additionally, the shelf-life of the modified electrodes and a systematic study of interferents, that are commonly present in food or agro-industrial waste, *e.g.* glucose, acetic acid, water soluble vitamins (ascorbic acid and vitamin B12), proteins and amino acids among others [56], must be carried on. In particular, proteins are known to strongly interfere with the quantification of phenolic compounds by the reference Folin-Ciocalteu spectroscopic method. It is, thus, relevant to truly validate the biosensor performance to become a viable alternative in the quantification of polyphenols.

4. Conclusions

The objective of this study was to develop enzymatic electrochemical biosensor platforms that fulfil the purpose of detection and quantification of phenolic compounds, common in agro-industrial waste (gallic acid and catechin). To achieve that purpose, we have used polycatecholamines, namely polydopamine and polynorepinephrine, with recognized adhesive properties, to immobilize alkaliphilic bacterial laccase.

The controlled potentiostatic growth of polynorepinephrine and polydopamine has allowed the manipulation of its properties and thickness, making this a more viable technique than chemical oxidation, commonly employed to prepare polycatecholamine coatings. The novel thin polynorepinephrine films are electroactive, owing to the presence of a number of catechol/hydroquinone groups, which are relevant for post-biofunctionalization. In addition, ePNE enables the electron transfer of both cationic and anionic species, at both pH 5.0 and 7.0, in contrast to polydopamine coating with a similar thickness, which hampers the redox conversion of anionic probes at pH 7.0, and cationic ones, at pH 5.0, affecting its application as an amperometric transducer. AFM imaging points to the presence of a greater amount of protein immobilized onto ePNE than on ePDA, which is also consistent with the excellent performance of ePNE/Lac electrodes to detect relevant phenolic compounds, such as gallic acid. Although the sensitivity obtained towards catechin is low when compared with other reported works, the exceptionally wide linear range motivate us to improve the electrode architecture, namely through the incorporation of stable and catalytic nanomaterials in the adhesive polymeric matrix. The use of alkaliphilic laccase enables to extend the detection of polyphenols to neutral conditions, keeping or increasing sensitivity and avoiding the need for acidifying real waste solutions. The bacterial laccases are often overlooked but can be of extreme importance on the development of more selective biosensors, since they can catalyse the oxidation of several compounds at distinct pH values, allowing to distinguish the presence of different polyphenols.

CRediT authorship contribution statement

Luís C. Almeida: Writing – original draft, Investigation, Conceptualization. **Jorge F. Zeferino:** Writing – original draft, Investigation. **Clara Branco:** Investigation. **Guiseppe Squillaci:** Writing – review & editing, Resources. **Alessandra Morana:** Writing – review & editing, Resources. **Romana Santos:** Investigation. **Petri Ihalainen:** Writing – review & editing, Resources. **Liji Sobhana:** Resources. **Jorge P. Correia:** Writing – review & editing, Supervision, Resources. **Ana S. Viana:** Writing – review & editing, Supervision, Resources, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2024.108826.

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