Analyst

RSC Publishing

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View Article Online
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Cite this: DOI: 10.1039/c3an36800a

Pt–Pd bimetallic nanoparticles dispersed in an ionic liquid and peroxidase immobilized on nanoclay applied in the development of a biosensor

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Pt–Pd bimetallic alloy nanoparticles (NPs) dispersed in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (Pt–Pd-BMI·PF_6) were employed together with a peroxidase (PO) enzyme from cauliflower immobilized on nanoclay for the development of a new biosensor for polyphenol determination by square-wave voltammetry (SWV). The biosensor demonstrated good repeatability and reproducibility, low limit of detection (LOD = 3.7×10^{-7} mol L^{-1} for caffeic acid (CA)), and adequate lifetime and stability (maintaining over 80% of the response over 80 days of evaluation, and allowing over 600 measurements by SWV for each electrode). Under optimized conditions, the proposed biosensor was applied in the determination of the bioelectrochemical polyphenolic index (BPI) for samples of commercial white wine, using CA as the phenolic compound standard. The recovery of CA from wine samples ranged from 95.5 to 108.3%. The values for the polyphenolic content obtained using the proposed biosensor showed a good correlation ($r = 0.990$) with those obtained with the reference spectrophotometric method (Folin–Ciocalteu method). Therefore, the proposed biosensor represents a useful tool for the rapid and accurate monitoring of polyphenolic content in wine samples and may also be applicable to other beverage samples, such as juices and teas.

Received 3rd December 2012
Accepted 27th May 2013

DOI: 10.1039/c3an36800a

www.rsc.org/analyst

Introduction

In recent years, with advances in nanotechnology, various new nanomaterials have been investigated for application in the development of electrochemical sensors. The application of nanostructured materials for the immobilization of biomolecules, as well as in the construction of biosensors, represents a highly promising field of research in view of the unique catalytic, electronic, magnetic, optical and electrochemical properties of these materials.[1–5] The analytical performance of sensors and biosensors is notably improved when nanomaterials are used in their composition, bringing significant benefits to the application of these devices in environmental monitoring, food safety, clinical analysis, disease diagnosis, and quality control of pharmaceuticals and food.[1–4]

Metallic nanoparticles (NPs) are among the most studied nanomaterials for the development of biosensors due to their unique properties, such as large active surface area and extraordinary catalytic activity associated with controlled size and composition.[2,6,7] These materials have a high charge transfer capacity, which makes them suitable for achieving higher sensitivity and lower detection limits.[3] Recently, bimetallic NPs have been investigated for their importance in catalysis, since the addition of a second metal causes variations in the particle size, shape, surface morphology, and chemical and physical properties. Due to the bifunctional or synergistic effects, this combination of two metallic elements in a single particle results in a material with interesting properties, including chemical selectivity and catalytic activity superior to those of monometallic NPs.[6,8–10] Among the different architectures of bimetallic NPs, alloy and core–shell structures have been widely employed as catalysts in various types of reactions,[8,9] and in electrocatalysis, particularly in fuel cells.[9,11] In recent years, application of these nanomaterials has also gained significant attention in the development of electrochemical sensors and biosensors.[12–16]

Another fascinating class of compounds that has been used successfully in the development of electrochemical sensors is the ionic liquids (ILs).[17,18] These are composed entirely of ions and they possess very interesting properties, including good chemical and thermal stability, tunable viscosity and miscibility, low volatility, wide electrochemical window and electrolytic conductivity.[19,20] ILs are important stabilizing agents, particularly IL derivatives of 1-*n*-butyl-3-methylimidazolium, which have given very good results as solvents in the

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preparation and stabilization of metallic NPs,[21] and provide a favorable environment for the stabilization of enzymes, protecting these biomolecules from denaturation.[17,22]

Aiming to improve the stability of enzymes, different strategies have been used for the immobilization of these molecules, in particular *via* simple adsorption, encapsulation and covalent attachment.[5,23–25] Recently, various nanostructured materials have been investigated as supports to immobilize enzymes, such as carbon nanotubes,[26] conducting polymer nanomaterials,[27] metallic/polymeric nanoparticles,[5,23] and nanoclays.[28–32] Of these materials, nanoclays are notable due to their interesting characteristics for the immobilization of biomolecules, such as high surface area, thermal and mechanical stability, good biocompatibility, and low cost. Furthermore, they can enhance the biocatalytic efficiency by reducing diffusional limitations.[28,31,32] Montmorillonite, the most important of the smectite nanoclays, has been widely used as a catalyst,[33] as well as a support for the immobilization of (bio)catalysts, such as enzymes.[31,32,34,35] Recently, our group has obtained great success using oxidoreductase enzymes (*e.g.*, polyphenol oxidase, laccase, peroxidase) immobilized on clays for the development of biosensors for the determination of phenolic compounds.[30,35] The peroxidases (POs) are heme enzymes, which exploit the reduction of hydrogen peroxide to catalyze oxidative reactions involving a wide variety of substrates, including polyphenols.[36,37]

Polyphenols are one of the most important groups of plant metabolites and are an integral part of both human and animal diets.[38] They contribute greatly to the sensory and nutritional quality of fruits, vegetables, beverages (*e.g.*, tea, coffee and wine), and other foods derived from plants. These compounds play an important role in human health by acting as protective agents against many chronic diseases associated with oxidative stress, such as cardiovascular diseases, neurodegenerative disorders, and cancer. This beneficial effect is due to the antioxidant activity of these natural compounds, which act by fighting free radicals.[38–40] Several methods have been proposed for the determination of polyphenols, especially using chromatography,[41,42] capillary electrophoresis,[40,43] and spectrophotometry.[42,44] The Folin–Ciocalteu (FC) method[44] is one of the most widely used procedures for the determination of total phenolics in food samples and plant materials. However, this method has low specificity because the FC reagent reacts with other nonphenolic substances, resulting in an overestimation of total phenolic compounds.[45] For this reason, various enzymatic biosensors have been developed to meet the need for simpler and faster methods, with appropriate selectivity and sensitivity for the determination of phenolic compounds.[30,35,46–50]

In this study, Pt–Pd bimetallic alloy NPs dispersed in 1-butyl-3-methylimidazolium hexafluorophosphate IL (Pt–Pd-BMI·PF_6) were employed together with PO obtained from a new enzyme source, cauliflower (*Brassica oleracea* L. var. *botrytis*), and immobilized on nanoclay for the development of a new biosensor for polyphenol determination by square-wave voltammetry (SWV), which was applied in the analysis of white wine samples.

Experimental

Reagents, solutions and samples

All reagents were of analytical grade and employed without further purification. All solutions were prepared with distilled and deionized water. An acetate buffer solution (0.1 mol L^{-1}, pH 5.0) was used as the supporting electrolyte throughout the experiments. The carbon paste was prepared using graphite powder (Fisher Scientific) and mineral oil purchased from Sigma-Aldrich. Montmorillonite (MMT) nanoclay with a modified surface containing 0.5–5 wt% aminopropyltriethoxysilane and 15–35 wt% octadecylamine and montmorillonite K10 (unmodified MMT) were obtained from Sigma-Aldrich. Caffeic acid (CA), chlorogenic acid, gallic acid, quercetin, catechin, hydrogen peroxide, guaiacol and FC reagent were also obtained from Sigma-Aldrich. The cauliflower used as a source of a PO enzyme and the samples of white wine were purchased from a local store in Florianópolis (Santa Catarina, Brazil).

Instrumentation and electrodes

The transmission electron microscopy (TEM) images of the Pt_xPd_{1-x} NP samples were obtained using a JEOL JEM2010 microscope. Spectrophotometric analysis was performed on a Micronal B572 spectrophotometer with a quartz cell (optical path of 1.0 cm). SWV experiments were performed in an electrochemical cell containing a supporting electrolyte, using an Autolab PGSTAT101 potentiostat/galvanostat (Eco Chemie, The Netherlands) operating with data processing software (NOVA, software version 1.6, Eco Chemie). All voltammetry experiments were carried out using a conventional three-electrode system with the biosensor used as the working electrode, a platinum plate as the auxiliary electrode and Ag/AgCl (3.0 mol L^{-1} KCl) as the reference electrode.

Synthesis and characterization of Pt_xPd_{1-x} NPs dispersed in BMI·PF_6

Bimetallic alloy NPs dispersed in BMI·PF_6 IL (Pt–Pd-BMI·PF_6) were synthesized as previously described in the literature.[51] Pt_xPd_{1-x} NPs ($x = 1.0$, 0.7, and 0.5) were prepared by dissolution of the tris(dibenzylideneacetone) diplatinum $Pt_2(dba)_3$ (33 mg, 30 μmol; 23 mg, 21 μmol; and 16 mg, 15 μmol, respectively) and bis(acetylacetonate) palladium ($Pd(acac)_2$) (3 mg, 9 μmol and 5 mg, 15 μmol) precursors in 1 mL of BMI·PF_6. The resulting solution was reacted at 75 °C under 4 atm of molecular hydrogen for 1.5 h. The Pt_xPd_{1-x} NPs formed were washed with dichloromethane (3 × 15 mL) and isolated by centrifugation (3500 rpm) for 3 min, washed with acetone (3 × 15 mL) and dried under reduced pressure. TEM images of the Pt_xPd_{1-x} NPs samples were obtained using a microscope operating at 120 kV. A 20 μm objective aperture and slightly under-focused ($\Delta f \approx$ 300 nm) objective lens were used to obtain the bright field TEM images.

Obtainment of the PO and measurement of activity

The extract of a PO enzyme was obtained by homogenization of 75 g of cauliflower in a blender with 50 mL of a phosphate buffer

solution (0.1 mol L^{-1}; pH 7.0). This extract was filtered and maintained at low temperature (4 °C) in a refrigerator for subsequent use as a PO source in the biosensor construction. The enzymatic activity of the PO present in the cauliflower extract was determined using a spectrophotometric method by measuring the absorbance at 470 nm of the tetraguaiacol produced by guaiacol oxidation. Enzyme activity was measured in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0), 0.2 mL of the enzymatic extract, 2.7 mL of 0.05 mol L^{-1} guaiacol solution and 0.1 mL of a 9.7×10^{-5} mol L^{-1} hydrogen peroxide solution at room temperature, and calculated as the increase in 0.001 units of absorbance per minute.[47]

Immobilization of PO on nanoclay and construction of the biosensor

The immobilization of the PO enzyme in the nanoclay matrix was carried out through physical adsorption using the following methodology:[30,35] in a Petri dish, an aliquot of the cauliflower extract containing the desired concentration of the enzyme (250–2000 units of PO) was added to 10 mg of modified nanoclay (MMT), homogenized, and dried at room temperature (25 °C). The resulting material containing the adsorbed enzyme in nanoclay (PO-MMT) was used for construction of the biosensors. To obtain a control experiment, the same procedure of immobilization has also been carried out using unmodified montmorillonite (MMTK10), for comparative purposes.

The proposed biosensor is based on the modification of a carbon paste electrode (CPE), constructed as follows: firstly, 10 mg of PO-MMT and 150 mg of graphite powder were mixed for 10 min with a mortar and pestle; 20 mg of mineral oil and 30 mg of Pt–Pd-BMI·PF_6 were then added to the mixture and homogenized for 20 min to form the final paste. The modified paste was then tightly packed into a plastic syringe (1.0 mm internal diameter) and a copper wire was inserted to establish the external electrical contact. The obtained biosensor was stored at room temperature when not in use. The other sensors (without PO) containing BMI·PF_6 and Pt_xPd_{1-x} NPs ($x = 1.0$, 0.7, and 0.5) dispersed in IL were constructed similarly. The bare CPE was prepared in the same way but by mixing only graphite powder and mineral oil.

Electrochemical measurements

All of the SWV measurements were carried out at room temperature (25 °C) in an electrochemical cell containing 10.0 mL of an acetate buffer solution (0.1 mol L^{-1}, pH 5.0) with successive additions of a standard solution of caffeic acid or a wine sample. The SWV measurements were performed at a frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 1–10 mV, after successive additions of the analyte. All potential measurements are reported *vs.* Ag/AgCl (3.0 mol L^{-1} KCl), and a stirring time of 60 s was used to homogenize the solutions.

Determination of polyphenolic content in wine

The proposed biosensor was applied in the determination of the bioelectrochemical polyphenolic index (BPI) of wine samples. Five samples of commercial white wine, produced from different grape cultivars (Sauvignon Blanc, Chardonnay and Riesling) from the 2010–2012 vintages were analyzed. Initially, these samples (pH = 3–4) were acidified with hydrochloric acid (pH < 2),[50] and then bubbled with nitrogen in order to eliminate the sulfur dioxide used as a preservative, which is a potential interferent in analysis employing an enzymatic biosensor. Subsequently, the acidified samples were diluted in acetate buffer to maintain the optimum pH for application of the proposed biosensor. The method of standard additions was used for the determination of the BPI of these wine samples, using CA as the phenolic compound standard. Under optimized conditions, aliquots of white wine samples (pretreated) were transferred to the electrochemical cell containing an acetate buffer solution (0.1 mol L^{-1}, pH 5.0), and analyzed using SWV, after successive additions of standard solutions of CA. All measurements were performed in triplicate, and the results for BPI were expressed as milligrams of CA per liter of wine.

For comparison purposes, the wine samples (without pretreatment) were also analyzed by spectrophotometry according to the FC colorimetric method with the microscale protocol[52] for total phenolic compound (TPC) content, which is based on the method reported by Singleton and Rossi,[44] using CA as the phenolic compound standard. All measurements were performed in triplicate, and the results for the TPC content were expressed as milligrams of CA per liter of wine.

Results and discussion

Characterization of Pt_xPd_{1-x} NPs dispersed in BMI·PF_6

$Pt_{0.7}Pd_{0.3}$ and $Pt_{0.5}Pd_{0.5}$ are bimetallic alloy NPs, and $Pt_{1.0}Pd_{0.0}$ are monometallic Pt NPs.[51] The $Pt_{1.0}Pd_{0.0}$, $Pt_{0.7}Pd_{0.3}$, and $Pt_{0.5}Pd_{0.5}$ NPs had a monomodal particle size distribution with mean diameters of 3.6 ± 0.8, 4.2 ± 1.1 and 4.5 ± 1.3, respectively. The size of the Pt_xPd_{1-x} NPs dispersed in BMI·PF_6 was estimated from ensembles of 300 particles found in an arbitrarily chosen area of the enlarged micrographs. Fig. 1 displays the particle size distributions of the NPs obtained, which could be reasonably fitted by a Gaussian curve.

According to the extended X-ray absorption fine structure (EXAFS) results presented previously by Bernardi *et al.*,[51] since TEM indicates a slight diameter increase when x decreases from 1.0 to 0.5, a structural disorder is assumed to be the cause of the oscillation damping. More detailed information regarding the characterization of these nanomaterials can be found in a previous publication.[51]

Immobilization of PO on nanoclay and principle of the enzymatic process

In this study, the PO immobilization consisted of the physical adsorption of the enzyme on the modified nanoclay. The proposed mechanism for the enzymatic immobilization is based on non-covalent interactions, mainly hydrogen bonding, and hydrophobic and electrostatic interactions between the amino acid of the enzyme and the external surfaces and edges of the nanoclay.[31,32] In addition, according to previous studies,[32,34]

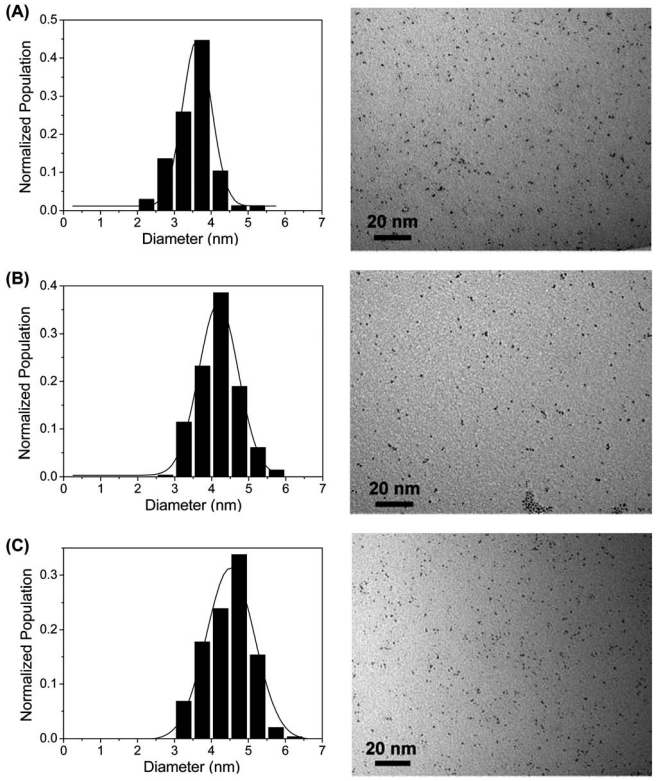

Fig. 1 TEM images and histograms of the (A) $Pt_{1.0}Pd_{0.0}$, (B) $Pt_{0.7}Pd_{0.3}$ and (C) $Pt_{0.5}Pd_{0.5}$ NPs.

the biomolecules may also be adsorbed on the internal surfaces of the layered aluminosilicate mineral, mainly through inter-layer space expansion due to functionalization with amino-propyltriethoxysilane and octadecylamine. However, in this case, it is unlikely that the whole enzyme is intercalated within the inter-layer space of the nanoclay, but instead the protein backbone could be situated at the periphery of the clay matrix while the side chains of the amino acid residues may be involved in intercalation.[34] The experimental results showed that the enzyme immobilized in the nanoclay improved the analytical performance of the biosensor in comparison with that containing free enzyme or PO immobilized on unmodified clay. These results will be discussed in detail below.

The modifier materials used in the construction of the biosensor are shown in Fig. 2, including the PO enzyme immobilized on nanoclay, which acts as a catalyst in the redox reactions occurring on the electrode surface, and the bimetallic NPs stabilized by $BMI{\cdot}PF_6$ IL, which is required to aid the transfer of electrons between the enzyme and the electrode, in view of the high conductivity of this material. The principle of the enzymatic process, on which the proposed sensing mechanism is based, is shown schematically in Fig. 2. In this process, the CA (or other phenolic compound) in contact with the PO (immobilized on the nanoclay), in the presence of hydrogen peroxide, is oxidized to its corresponding *o*-quinone. Subsequently, this quinone is electrochemically reduced on the biosensor surface. The resulting current obtained from the reduction process is quantitatively proportional to the phenolic compound concentration in the electrochemical cell.

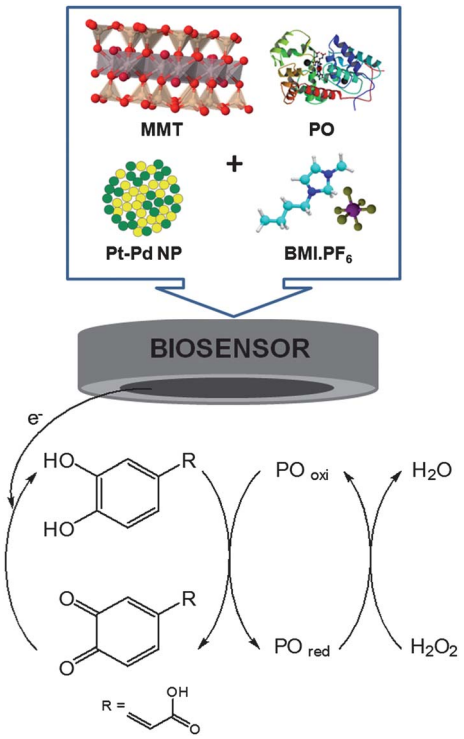

Fig. 2 Schematic representation of the modifier materials used in the construction of the biosensor, and the proposed sensing mechanism based on PO-catalyzed oxidation of CA with its subsequent electrochemical reduction on the electrode surface.

Study on the contribution of the modifiers used in the biosensor construction

Fig. 3 shows the results of a comparative study on electrodes carried out to assess the individual contribution of each modifier, that is, the free enzyme (PO), immobilized enzyme (PO-MMT), $BMI{\cdot}PF_6$ (IL) and Pt_xPd_{1-x} NPs (x = 1.0, 0.7, and 0.5). The electrodes investigated were as follows: (a) bare

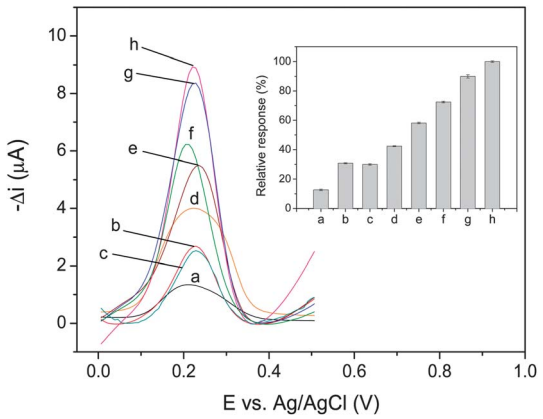

Fig. 3 Square-wave voltammograms obtained using different electrodes: (a) bare CPE, (b) CPE-PO, (c) CPE-PO-MMTK10, (d) CPE-PO-MMT, (e) CPE-PO-MMT-IL, (f) CPE-PO-MMT-IL-$Pt_{1.0}Pd_{0.0}$, (g) CPE-PO-MMT-IL-$Pt_{0.7}Pd_{0.3}$, and (h) CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$ in 0.1 mol L^{-1} acetate buffer solutions (pH 5.0) containing 9.7×10^{-5} mol L^{-1} H_2O_2 and 2.2×10^{-5} mol L^{-1} of CA at a frequency of 50 Hz, pulse amplitude of 100 mV and scan increment of 8 mV. Inset: relative response for bare CPE and different biosensors. Mean ± standard deviation (n = 3).

CPE, (b) CPE-PO, (c) CPE-PO-MMTK10, (d) CPE-PO-MMT, (e) CPE-PO-MMT-IL, (f) CPE-PO-MMT-IL-$Pt_{1.0}Pd_{0.0}$, (g) CPE-PO-MMT-IL-$Pt_{0.7}Pd_{0.3}$, and (h) CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$. All SWV measurements were carried out in a 0.1 mol L^{-1} acetate buffer solution (pH 5.0) containing 9.7×10^{-5} mol L^{-1} H_2O_2 and 2.2×10^{-5} mol L^{-1} CA at a frequency of 50 Hz, pulse amplitude of 100 mV and scan increment of 8 mV. As can be seen, the successive addition of modifier materials to the carbon paste led to changes in the performance of the electrodes, with an improvement in the analytical response. The addition of free PO to the electrode (biosensor "b") caused a significant increase in the peak current for CA, which was almost three times greater than that for the bare CPE (a). This finding can be explained by the enzyme acting as a catalyst in the oxidation of phenolic compounds. The biosensor containing PO immobilized on the unmodified clay (biosensor "c") showed a lower average current than the biosensor containing PO immobilized on modified nanoclay (biosensor "d"). From this observation, it is suggested that the surface modification of the clay (MMT) contributed to a more efficient enzymatic immobilization, possibly due to the presence of a larger quantity of functional groups for the interaction with the enzyme structure, as well as a larger surface area, providing a greater retention of enzymatic activity. Furthermore, the biosensor (d) showed a higher peak current than that of the biosensor containing free enzyme (biosensor "b") and the biosensor containing PO immobilized on the unmodified clay (biosensor "c"), suggesting that the nanoclay functionalized with aminopropyltriethoxysilane and octadecylamine used in the PO immobilization can provide a favorable orientation of the biomolecules, facilitating access of the substrate to the active center of the enzyme. The presence of the IL provided an improvement in the analytical response of the biosensor (d), mainly due to its high conductivity, which contributes to the reduced resistance to charge transfer. An important gain in the response was also obtained with the addition of Pt_xPd_{1-x} NPs ($x =$ 1.0, 0.7, and 0.5) (biosensors "e", "f", and "g"), due to their effect of increasing the rate of electron transfer between the enzyme and the electrode surface, and their intrinsic catalytic ability. The biosensor containing $Pt_{0.5}Pd_{0.5}$ (biosensor "g") presented the highest current for CA, in relation to the biosensors containing $Pt_{1.0}Pd_{0.0}$ (biosensor "e") and $Pt_{0.7}Pd_{0.3}$ (biosensor "f"), and thus the electrode "g" was selected for subsequent studies. This result suggests that the Pt–Pd alloy NPs (in particular $Pt_{0.5}Pd_{0.5}$ NPs) have superior properties in comparison with Pt monometallic NPs, such as better electronic and catalytic properties, which is attributed to the synergistic effect of the bimetallic alloy. According to recent studies, the Pt-containing bimetallic alloys exhibit higher electrocatalytic activity than Pt alone,[14,16,53] reinforcing the results presented in this paper. In summary, the combination of the nanoclay, bimetallic alloy NPs and an IL can provide a suitable microenvironment for the stabilization of the enzyme and to improve the sensitivity of the biosensor.

Study on selectivity of the proposed biosensor

Wine is a beverage widely consumed in Brazil and worldwide, whose composition is rich in phenolic compounds, which are an extremely important component of a healthy diet. Some of the main phenolic compounds present in wine, including chlorogenic acid, gallic acid, CA, quercetin and catechin, were selected to conduct a study on biosensor selectivity. SWV measurements were performed in triplicate in solutions containing 2.2×10^{-5} mol L^{-1} of each phenol compound (individually) in an acetate buffer solution (0.1 mol L^{-1}, pH 5.0), and the responses obtained are shown in Fig. 4. As can be seen, the compounds investigated have observed peak potentials (Ep) with similar values, which suggests that the proposed biosensor (CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$) is not specific for a single compound, but is selective for this group of phenolic compounds. Therefore, the biosensor is not able to separately detect these compounds when present in a mixture, but it allows the determination of the total phenolic content. The responses in terms of peak current followed the order: quercetin > CA > chlorogenic acid > catechin > gallic acid, and CA was selected as the standard for the optimization studies and evaluation of the proposed method, as well as for the determination of the polyphenolic content in wine, because it is found in larger quantities in these samples.

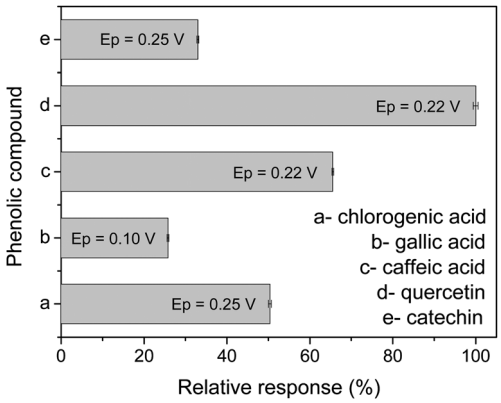

Fig. 4 Relative response and peak potential obtained for different phenolic compounds (chlorogenic acid, gallic acid, caffeic acid, quercetin and catechin), using the proposed biosensor (CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$) in solutions containing 2.2×10^{-5} mol L^{-1} of each phenol compound in an acetate buffer solution (0.1 mol L^{-1}, pH 5.0).

Optimization of the biosensor construction and experimental conditions

In order to optimize the biosensor construction and experimental conditions of the proposed method, several parameters were investigated in triplicate using SWV. The effect of the PO concentration in the paste preparation was investigated from 1.25 to 10.0 units of enzyme (immobilized in nanoclay) per mg of a carbon paste. The best analytic result was observed at 2.50 units of PO per mg of carbon paste, and this enzyme concentration was thus used for the construction of the biosensors. Subsequently, the SWV parameters were studied with the aim of obtaining better sensitivity and peak definition in the CA determination. The frequency (10–100 Hz), pulse amplitude (10–100 mV) and scan increment (1–10 mV) were investigated. The best analytical response was obtained with

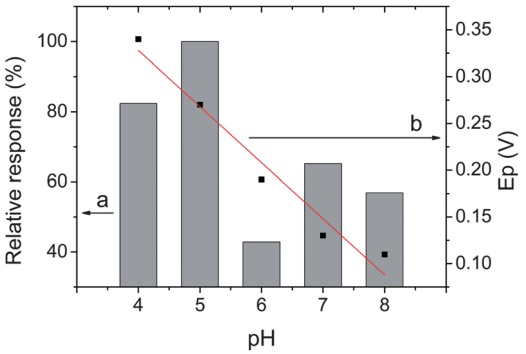

Fig. 5 Influence of pH on (a) resulting peak current (expressed in relative response) and (b) peak potential of the proposed biosensor (CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$) in a supporting electrolyte solution (0.1 mol L^{-1} acetate buffer solution at pH 4–5 and 0.1 mol L^{-1} phosphate buffer solution at pH 6–8) containing 9.7×10^{-5} mol L^{-1} H_2O_2 and 2.2×10^{-5} mol L^{-1} CA estimated by SWV at a frequency of 50 Hz, a pulse amplitude of 100 mV and a scan increment of 8 mV.

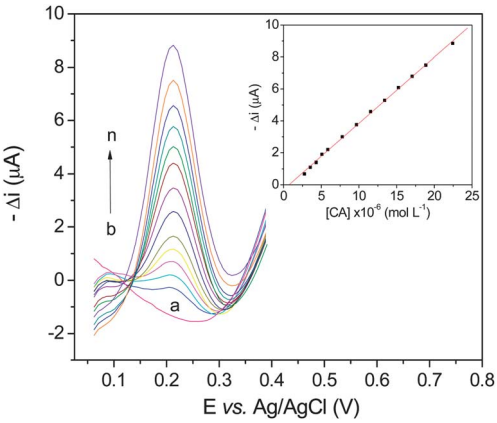

Fig. 6 Square-wave voltammograms obtained using the proposed biosensor (CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$) in (a) an acetate buffer solution (0.1 mol L^{-1}, pH 5.0) containing 9.7×10^{-5} mol L^{-1} H_2O_2, and a CA standard at concentrations of (b) 2.7×10^{-6} to (*n*) 2.2×10^{-5} mol L^{-1}, at 50 Hz frequency, 100 mV pulse amplitude and 8 mV scan increment. Inset: analytical curve of CA.

50 Hz of frequency, 100 mV of pulse amplitude and 8 mV of scan increment. Thus, these instrumental conditions were selected for all further voltammetric determinations of CA. To determine the influence of the pH of the electrolyte support on the biosensor response, a range of 4.0–8.0 (acetate buffer solution at pH 4.0–5.0, and phosphate buffer solution at pH 6–8) was studied. According to the results shown in Fig. 5(a), the highest voltammetric response for CA was registered with a 0.1 mol L^{-1} acetate buffer solution at pH 5.0. Therefore, this pH value was used in further studies. In addition, Fig. 5(b) shows the linear variation between the peak potential and the pH of the electrolyte support with a slope of −56.0 mV/pH ($r = 0.993$). This value is close to that expected for Nernstian systems with two electrons and two protons involved, as described in various studies on CA redox processes reported in the literature.[54,55]

Analytical performance of the biosensor

The repeatability, reproducibility, lifetime and stability of the proposed biosensor were examined by measuring the current response using SWV in an acetate buffer solution (0.1 mol L^{-1}; pH 5.0) containing 9.7×10^{-5} mol L^{-1} H_2O_2 and 2.2×10^{-5} mol L^{-1} CA. For the repeatability study, the relative standard deviation was 2.19% for 10 successive measurements using the same biosensor. The reproducibility was investigated by preparing three biosensors, independently and applying the same procedure. The results showed acceptable reproducibility with a relative standard deviation of 2.45%. The lifetime and stability of the biosensor were investigated at different time intervals over a period of around 80 days (over 600 measurements for each quantity of the carbon paste used in the syringe), the electrode surface being renewed as needed, and stored at room temperature. A relative response of over 80% was obtained after 80 days of evaluation, in relation to that obtained on the day of construction.

The analytical curve for CA was constructed using the proposed biosensor and SWV, in triplicate, under the previously optimized experimental conditions. The electrochemical reduction of CA was obtained at a potential of approximately +0.22 V *vs.* Ag/AgCl at the biosensor surface. The square-wave voltammograms obtained for the calibration curve of CA are shown in Fig. 6. The calibration curve was linear from 2.7×10^{-6} to 2.2×10^{-5} mol L^{-1}, and presented the following regression equation: $-\Delta i = -0.29(\pm 0.051) + 4.14(\pm 0.042) \times 10^5$ [CA], with a correlation coefficient (r) of 0.999, where Δi is the resulting peak current in μA, and [CA] is the concentration of caffeic acid in mol L^{-1}. The limits of detection (LOD) and quantification (LOQ), considered as three and ten times the standard deviation of the intercept/slope, respectively, were calculated as 3.7×10^{-7} and 1.2×10^{-6} mol L^{-1}, respectively.

The apparent Michaelis–Menten constant (K_m^{app}) was obtained from the electrochemical version of the Lineweaver–Burk model (double reciprocal – $1/\Delta i$ *vs.* 1/[CA]), where the intercept with the *x*-axis is equal to $-1/K_m^{app}$.[56] When the CA concentration was higher than 2.2×10^{-5} mol L^{-1}, a plateau was observed (data not shown), indicating that the enzyme–substrate reaction is associated with a Michaelis–Menten kinetic mechanism. The K_m^{app} value obtained for PO immobilized in nanoclay was 6.26×10^{-5} mol L^{-1}. This small K_m^{app} value indicates that the biosensor CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$ has high catalytic efficiency in the CA determination.

Some analytical characteristics observed in the CA determination in this study and in others reported previously in the literature using biosensors are shown in Table 1. The biosensor has a detection limit better than or comparable to those of other studies cited; however, the linear range does not compare favorably with those observed for the other methods described in Table 1.[46–50]

Study on interfering compounds

Sulfurous anhydride is widely employed as a preservative to avoid oxidation and microbial interference during the fermentation and storage of wines. Some compounds present in wine samples may act as potential interferents in phenolic compound determination using an enzymatic biosensor,

Table 1 Some analytical characteristics of different biosensors for CA determination

Electrode	Enzyme[a]	Immobilization method	Sample	Linear range (mol L^{-1})	LOD (mol L^{-1})	Ref.
Glassy carbon modified with electrodeposited gold nanoparticles	Tyr	Crosslinking with glutaraldehyde	Wines	2.0×10^{-6} – 2.0×10^{-4}	6.6×10^{-7}	46
Carbon paste electrode	PO	Chitin chemically crosslinked with epichlorohydrin and glutaraldehyde	Wines	2.0×10^{-5} – 2.0×10^{-4}	2.0×10^{-6}	47
Graphite screen-printed	Lac	Entrapment in a polyvinyl alcohol film	Teas	5.0×10^{-7} – 1.3×10^{-4}	5.2×10^{-7}	48
Carbon nanotube composite	PO	Entrapment in the composite	Wines and teas	3.3×10^{-7} – 3.8×10^{-4}	1.1×10^{-7}	49
Graphite screen-printed modified with ferrocene	Lac Tyr Lac-Tyr	Sol–gel matrix of diglycerysilane	Must and wines	—	6.0×10^{-6} 7.8×10^{-5} 2.4×10^{-5}	50
Carbon paste electrode modified with $Pt_{0.5}Pd_{0.5}$-BMI·PF_6	PO	Adsorption on nanoclay	Wines	2.7×10^{-6} – 2.2×10^{-5}	3.7×10^{-7}	This work

[a] Tyr: tyrosinase; PO: peroxidase; Lac: laccase.

especially sulfur dioxide and its conjugate species, since these substances act as inhibitors of the catalytic activity of enzymes, such as the PO applied in the biosensor construction. Thus, the influence of some potentially interfering species on the biosensor response, including sulfite, bisulfite, ascorbic acid, glucose and sucrose, was investigated. SWV measurements were taken in solutions containing 2.2×10^{-5} mol L^{-1} of CA in an acetate buffer solution (0.1 mol L^{-1}, pH 5.0), on adding different concentrations of the potential interferent (1 : 1, 1 : 5 and 1 : 10 CA : interferent). The variation in the current caused by the presence of the potential interferent was calculated by comparing the biosensor current readings for solutions containing CA with and without the interferent. The studies demonstrated that the ascorbic acid, glucose and sucrose did not show significant interference in the CA determination in a 1 : 10 molar ratio, leading to changes in the analytical signal for catechol of less than ±6.0%. However, the presence of sulfite and bisulfite caused significant interference in the response of the biosensor, reaching a change in the analytical signal of CA, in a 1 : 10 molar ratio, of around 50%. To eliminate this problem, the solutions containing the interferent were acidified, as previously described by Montereali *et al.*,[50] and then nitrogen was bubbled into the solution in order to eliminate interfering species. This procedure resulted in excellent results, reducing the interference of such compounds in a 1 : 10 molar ratio, to less than ±5.0%. Therefore, this pre-treatment was applied to all wine samples before performing voltammetric analysis. Thus, this biosensor can be used for the determination of phenolic compounds in the presence of these potentially interfering compounds without significant interference in the analytical response.

Recovery study and analytical application

The proposed biosensor was applied in the determination of bioelectrochemical polyphenolic index (BPI) of samples of commercial white wine. The recovery study and the determination of BPI were performed by SWV with the standard addition method, in triplicate, using CA as the phenolic compound standard.

Recovery measurements were obtained by adding different standard concentrations of CA to each wine sample. The percentage recovery values were calculated by comparing the concentrations detected in samples with and without the addition of known concentrations of the CA standard solution. The recoveries of 95.5 to 108.3% obtained for these samples demonstrate the satisfactory accuracy of the proposed biosensor (Table 2).

To verify the efficiency of the proposed biosensor, these white wine samples were used for the determination of BPI by the electroanalytical method developed. For comparison purposes, these wine samples were also analyzed for total phenolic compound content (TPC) using the FC colorimetric

Table 2 Recovery of CA in white wine samples using the proposed biosensor

Sample[a]	Grape variety	Vintage	CA (10^{-6} mol L^{-1}) Added	CA (10^{-6} mol L^{-1}) Found[b]	Recovery[c] (%)
S1	Sauvignon Blanc	2011	1.2	1.3 ± 0.05	108.3
			2.5	2.4 ± 0.03	96.0
S2	Chardonnay	2010	2.2	2.1 ± 0.05	95.5
			4.1	4.2 ± 0.02	102.4
			6.1	6.2 ± 0.06	101.6
S3	—	2011	1.8	1.9 ± 0.02	105.6
			2.7	2.7 ± 0.04	100.0
S4	Chardonnay	2011	1.9	2.0 ± 0.06	105.3
			3.8	3.7 ± 0.03	97.4
S5	Riesling	2012	1.2	1.3 ± 0.04	108.3
			2.2	2.2 ± 0.02	100.0
			3.3	3.2 ± 0.03	97.0

[a] Samples of commercial white wine. [b] Mean ± standard deviation; $n = 3$. [c] Recovery = (mean found value/added value) × 100.

Table 3 Bioelectrochemical polyphenolic index (BPI) of white wine samples estimated using the proposed biosensor, and total phenolic compound content (TPC) obtained by the FC method,[52] using the CA as the phenolic compound standard

Sample[a]	Grape variety	Vintage	Biosensor BPI (mg L^{-1} CA)[b]	FC method TPC (mg L^{-1} CA)[b]
S1	Sauvignon Blanc	2011	14.8 ± 0.4	326.9 ± 1.8
S2	Chardonnay	2010	9.1 ± 0.8	227.0 ± 1.5
S3	—	2011	7.2 ± 0.3	186.0 ± 1.3
S4	Chardonnay	2011	14.3 ± 0.6	308.0 ± 1.7
S5	Riesling	2012	5.0 ± 0.7	176.0 ± 1.2

[a] Samples of commercial white wine. [b] Mean ± standard deviation; $n = 3$.

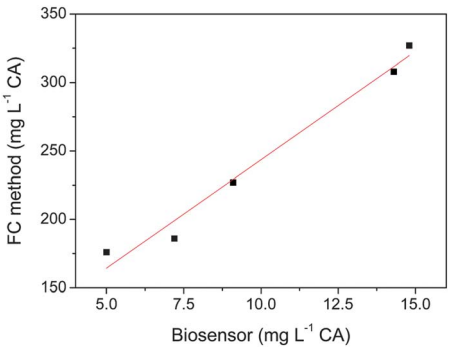

Fig. 7 Correlation between the results obtained for the content of phenolic compounds in wines estimated by using the proposed biosensor (CPE-PO-MMT-IL-$Pt_{0.5}Pd_{0.5}$) and the FC method (data shown in Table 3).

method.[52] The BPI and TPC values (expressed as milligrams of CA per liter of wine) determined by the proposed electroanalytical method and FC method, respectively, are shown in Table 3. As reported in papers published earlier,[46,48–50] the concentration values obtained using the FC method are higher than those obtained using the proposed biosensor. This behavior can be ascribed to different oxidizing agents used in each case, *i.e.*, an enzyme used in the biosensor construction (PO) and a chemical compound used in the FC method (phosphotungstic-phosphomolybdic acid).[48] Thus, the PO enzyme selectively oxidizes diphenolic compounds, while the FC reagent can oxidize not only phenolic compounds but also other nonphenolic species in the sample (*e.g.*, aromatic amines, glucose, ascorbic acid and sulfur dioxide).[48,49,52] However, despite the differences between these two methods, a good correlation was found ($r = 0.990$) when the results obtained using the biosensor were plotted against the results obtained with the FC method for the five wine samples analyzed (Fig. 7), demonstrating that the proposed electrochemical method is useful for estimating the content of phenolic compounds in wines.

Conclusions

It is concluded that this new biosensor based on $Pt_{0.5}Pd_{0.5}$ bimetallic NPs dispersed in IL (BMI·PF_6) and PO enzyme from cauliflower immobilized on nanoclay exhibited good analytical performance for the determination of CA (which can be extended to other diphenolic compounds). The results obtained suggest that the good performance of the proposed method is due to the efficient immobilization of the PO on the nanoclay modified with aminopropyltriethoxysilane and octadecylamine, and the facilitation of electron transfer between the biomolecules and the electrode surface due to the presence of the bimetallic NPs and an IL. In addition, it is assumed that the modifiers used in biosensor construction provide a suitable microenvironment for the enzyme stabilization. The proposed biosensor allowed the determination of the phenolic compound contents of white wines using a very fast and simple experimental procedure, providing a good correlation with the results obtained with the reference spectrophotometric method. Therefore, the proposed biosensor can be used as a useful tool for the rapid and accurate monitoring of total polyphenolic content in wine samples, and may also be applied to other beverage samples, such as juices and teas.

Acknowledgements

Financial support from CNPq (Processes 470430/2009-5 and 470505/2011-7), MCT/CNPq/PADCT and also the scholarship granted by CNPq to JMEP and LL, as well as financial support from CAPES-Brazil to DB, are gratefully acknowledged.

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