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PAPER

Halloysite clay nanotubes and platinum nanoparticles dispersed in ionic liquid applied in the development of a catecholamine biosensor

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Halloysite clay nanotubes were used as a support for the immobilization of the enzyme peroxidase from clover sprouts (*Trifolium*), and employed together with platinum nanoparticles in 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid (Pt-BMI·PF_6) in the development of a new biosensor for the determination of catecholamines by square-wave voltammetry. Under optimized conditions, the analytical curves showed detection limits of 0.05, 0.06, 0.07, 0.12 μM for dopamine, isoproterenol, dobutamine and epinephrine, respectively. The biosensor demonstrated high sensitivity, good repeatability and reproducibility, and long-term stability (18% decrease in response over 150 days). A recovery study of dopamine in pharmaceutical samples gave values from 97.5 to 101.4%. The proposed biosensor was successfully applied to the determination of dopamine in pharmaceutical samples, with a maximum relative error of ±1.0% in relation to the standard (spectrophotometric) method. The good analytical performance of the proposed method can be attributed to the efficient immobilization of the peroxidase in the nanoclay, and the facilitation of electron transfer between the protein and the electrode surface due to the presence of the Pt nanoparticles and ionic liquid.

Introduction

Halloysite nanotubes (HNTs), naturally occurring aluminosilicate nanotubes, have recently resurfaced in the scientific area. HNTs ($Al_2Si_2O_5(OH)_4 \cdot nH_2O$) are two-layered aluminosilicates, chemically similar to kaolin, which have a predominantly hollow tubular structure in the submicron range.[1,2] Chemically, the outer surface of this nanoclay has properties similar to tetrahedral SiO_2 while the inner region of the cylinder is related to octahedral Al_2O_3. The clay sheets are curved and form multilayered tubes due to the strain caused by the lattice mismatch between adjacent silicon dioxide and aluminum oxide layers.[1–3] HNTs occur widely in soils of wet tropical and subtropical regions and in the weathered rocks of many types of igneous and non-igneous structures.[1] Therefore, this is an economically viable material that can be mined from the corresponding deposit as a raw mineral, with minimal risk to the environment. Due to its specific characteristics of high surface area, good biocompatibility and low cost, this nanomineral has recently been investigated as a promising material for the immobilization of enzymes[4,5] and metalloporphyrins,[3] the preparation of supported metal nanoparticles[6] and in drug delivery systems.[2]

Great attention has been given to the application of nanostructured materials in biosensing systems, such as carbon nanomaterials (*e.g.*, nanotubes and graphene)[7–13] and metallic nanoparticles (NPs).[8,14,15] These promising materials present unique chemical and physical properties, such as catalytic activity, high active surface area, surface tenability and chemical stability. The application of these nanomaterials for the immobilization of enzymes has been successful[8–13,15] mainly because they provide a favorable microenvironment for these biomolecules, preserving their native structure, and also they facilitate electron transfer between the enzymes and the electrode. Moreover, their properties have contributed to enhancing performance in terms of miniaturization, biocompatibility, sensitivity, accuracy and stability of novel biosensors.[7–15]

Another class of novel materials known as ionic liquids (ILs) has been widely applied in the development of biosensors.[16,17] These materials exhibit excellent properties such as good chemical and thermal stability, negligible vapor pressure, wide potential window, high ionic conductivity and catalytic ability.[18] The use of enzymes in ILs offers many advantages, including better enzyme stability, because it protects these biomolecules from denaturation.[19–21] It is assumed that electrostatic interactions occur between the IL and the enzyme, resulting in a more rigid biomolecule, which needs to overcome a higher kinetic barrier to unfold in comparison with the enzyme in a non-ionic organic solvent.[19] In addition, ILs have been used in the preparation and stabilization of metallic NPs,[22] and dispersions of metal NPs in ILs have been applied in the development of high

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performance biosensors for the determination of several phenolic compounds.[16,23,24]

Catecholamine drugs, such as dopamine, isoproterenol (isoprenaline), dobutamine and epinephrine (adrenaline), are widely used in the treatment of heart disease, bronchial asthma, allergic emergencies and also in cardiac surgery and myocardial infarction.[25–27] Catecholamines are produced in humans through activation of the sympathetic nervous system and act as hormones and neurotransmitters to monitor the heart rate, glycogenolysis, fatty acid mobilization, body temperature and brain muscles activity.[27] Dopamine, one of the most important neurotransmitters, is involved in motor control, endocrine function, reward, emotion and cognition. The dopaminergic system is implicated in disorders such as Parkinson's and Alzheimer's diseases and addiction, and is the major target for antipsychotic medication in the treatment of schizophrenia.[28]

Various techniques have been proposed in the literature for catecholamine determination in pharmaceuticals and biological fluids including spectrophotometry,[21] chromatography,[29] chemiluminescence,[30] capillary electrophoresis[31] and voltammetry.[27,32–34] However, many of these conventional analytical methods, such as chromatography, are very expensive and require long execution times. The development of electrochemical biosensors, especially those using voltammetric techniques, has proven to be an attractive alternative for the determination of phenolic compounds in several types of samples. Some advantages of using voltammetry, especially square-wave voltammetry, rather than conventional techniques, are their fast response, good sensitivity, low cost of equipment and reagents and, in most cases, no need for sample pretreatment.

In this context, the use of biosensors based on oxidoreductase enzymes, such as laccase,[35] tyrosinase[36] and peroxidase,[37] has also been reported. Peroxidases (POs) are heme enzymes found in several plants, microorganisms and animals, which exploit the reduction of hydrogen peroxide to catalyze a number of oxidative reactions involving a wide variety of substrates, for instance, phenolic compounds.[38,39]

Enzyme immobilization can improve the stability of these biomolecules, maintaining their catalytic activity for a longer period of time compared to free enzymes.[40] Among the several methods available for the immobilization of biomolecules, adsorption is notable for its simplicity and relatively low cost.[41] Various materials that offer a large surface area for enzyme–support interactions can be used in this procedure, such as agarose beads, clay minerals, porous glass and epoxy resins.[40] Recent advances in nanotechnology have provided a diversity of nanostructured materials with a great potential for application in the immobilization of enzymes, in particular carbon nanotubes,[42] polymeric nanofibers and nanowires,[43,44] metallic/polymeric nanoparticles,[43,45] mesoporous silica,[43] and nanoclays.[4,5,46]

In this study, HNTs were used as a support for the immobilization of peroxidase (PO) obtained from a new enzyme source, clover sprouts (*Trifolium*), and employed together with platinum NPs dispersed in 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid (Pt-BMI·PF_6) in the development of a new biosensor for the determination of catecholamines by square-wave voltammetry (SWV).

Experimental

Reagents, solutions and samples

Halloysite nanotubes (HNTs) ($Al_2Si_2O_5(OH)_4 \cdot 2H_2O$) were purchased from Sigma-Aldrich. This material has an average tube diameter of 50 nm and inner lumen diameter of 15 nm, a typical specific surface area of 65 $m^2\ g^{-1}$ and pore volume of ~1.25 mL g^{-1}. The HNTs were used as the support for the immobilization of a clover sprout (*Trifolium*) homogenate as a source of the PO enzyme. The clover sprouts were purchased from a local supermarket in the city of Florianópolis (Santa Catarina, Brazil), washed and cooled in a refrigerator at 4 °C. The platinum NPs dispersed in 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid (Pt-BMI·PF_6) were synthesized as previously described in the literature.[47,48] The metal NPs obtained with an average size of 2.3 ± 0.4 nm were characterized by XRD and TEM analysis, as described in a previous paper published by our group.[23] The carbon paste was prepared using graphite powder (Acheson 38, Fisher Scientific) and Nujol purchased from Aldrich. Dopamine, isoproterenol, dobutamine, epinephrine, ascorbic acid, uric acid and hydrogen peroxide were obtained from Sigma-Aldrich. Phosphate buffer solution (0.1 M) at various pH values was prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4, adjusting the pH with H_3PO_4 or NaOH, and an acetate buffer solution (0.1 M) was similarly prepared using CH_3COOH and CH_3COONa. These buffer solutions were used as supporting electrolytes in the biosensor optimization studies. Samples of dopamine hydrochloride injection (5 mg mL^{-1}) of different brands (denoted herein by A, B and C) were supplied by the University Hospital of the Federal University of Santa Catarina (Santa Catarina, Brazil). All reagents were of analytical grade and were used without further purification, and all solutions were prepared with double-distilled water.

Instrumentation and electrodes

The PO activity determination was carried out using a B572 spectrophotometer (Micronal) with an optical path of 1.0 cm (quartz cell). Solutions were homogenized using a Unique 1400A ultrasonic bath. The electrochemical measurements using SWV were performed on an Autolab PGSTAT101 potentiostat/galvanostat (Metrohm Autolab, The Netherlands) interfaced with a microcomputer, and the data treated using NOVA software (version 1.6.013). The SWV measurements were carried out with a system of three electrodes: the proposed biosensor as the working electrode, a platinum plate as the auxiliary electrode, and Ag/AgCl (3.0 M KCl) as the reference electrode.

Obtainment of the PO and measurement of activity

The PO enzyme was obtained with the following methodology: 25 g of the clover sprouts were homogenized in a blender with 50 mL of phosphate buffer solution (0.1 M, pH 7.0) until total homogenization of the extract, at low temperature (4 °C) to preserve the enzymatic activity. The homogenate was filtered to eliminate the solid tissue and stored in a refrigerator for subsequent use as the PO source. PO activity was calculated as the increase in 0.001 units of absorbance per min at 470 nm. The

activity of PO present in the clover sprouts homogenate was determined in triplicate by measuring the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.2 mL of plant extract, 2.7 mL of 0.05 M guaiacol solution and 0.1 mL of 9.8 mM hydrogen peroxide solution in phosphate buffer solution (0.1 M, pH 7.0) at room temperature.[49]

Immobilization of PO on HNTs

The immobilization of the PO enzyme on the HNTs matrix was carried out through physical adsorption using the following methodology:[24] 20 mg of HNTs were added to an aliquot of the clover sprouts homogenate containing the desired concentration of PO enzyme (500–2000 units). A paste was obtained after homogenization with a spatula (5 min) which was dried at room temperature. The resulting material containing the adsorbed enzyme (PO-HNT) was used for construction of the biosensor.

Construction of the biosensor

The procedure used to construct the biosensor followed a methodology developed by our group.[49] Firstly, 140 mg of graphite powder (70%, w/w) and 20 mg of the HNTs containing 1500 units of PO immobilized (10%, w/w) were homogenized for 15 min with a mortar and pestle. Secondly, 20 mg of Nujol (10%, w/w) and 20 mg of Pt-BMI·PF_6 (10%, w/w) were added to the mixture which was then homogenized for a total of 15 min. After complete homogenization, the paste obtained was compacted into the point of a 1 mL plastic syringe with 1.0 mm internal diameter. Finally, a copper wire of dimensions 0.4 cm × 10.0 cm was inserted to obtain the electrical contact and the biosensor was then used as the working electrode. The sensors with or without PO containing HNT, HNT-BMI·PF_6 and HNT-Pt-BMI·PF_6 were prepared following the same steps. The bare carbon paste electrode (CPE) was prepared in a similar way by mixing only graphite powder and Nujol in a mortar.

Electrochemical measurements

All of the SWV measurements were carried out at room temperature (25 ± 0.5 °C) in an electrochemical cell containing a 10.0 mL aliquot of phosphate buffer solution (0.1 M, pH 7.0), and successive additions of a standard solution of dopamine (or other catecholamine: isoproterenol, dobutamine or epinephrine) or a sample of a pharmaceutical formulation added using a micropipette. The SWV measurements were performed at a frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 1.0–10.0 mV, after successive additions of the analyte. All potential measurements are reported *vs.* Ag/AgCl (3.0 M KCl), and a stirring time of 60 s was used to homogenize the solutions. All measurements were performed in triplicate for each analyte concentration and the error bars represent the standard error of the arithmetic mean for three replicates.

Preparation of pharmaceutical samples and determination of the dopamine

Samples of dopamine hydrochloride injection (A, B and C) were appropriately diluted in phosphate buffer solution (0.1 M, pH 7.0) and quantified using the proposed biosensor applying a standard method. The method of standard addition was used for the determination of dopamine using the biosensor. Under the optimized conditions, aliquots of the previously diluted sample were transferred to the electrochemical cell and quantified using SWV, after successive additions of standard solutions of dopamine. All measurements were performed in triplicate. The standard (spectrophotometric) method for dopamine determination recommended in the United States Pharmacopoeia[50] was used to compare the analytical results obtained with the proposed biosensor.

Results and discussion

Immobilization of PO on HNT

In this study, the PO immobilization was carried out by physical adsorption using the HNTs as a solid support. The proposed mechanism for the immobilization of the PO enzyme on the HNTs is based on non-covalent interactions, mainly hydrogen interactions and electrostatic interactions between the amino acid of the enzyme and the surface of nanoclay. The immobilization was efficient mainly due to the characteristics of the selected support, especially biocompatibility and large surface area. The experimental results showed that the enzyme maintained its activity when immobilized and applied in the construction of the biosensor. In addition, the enzymatic immobilization increased the stability of the sensor and, hence, allowed its application for a longer period of time.

Principle of the enzymatic process

Fig. 1A shows the components used to fabricate the biosensor, with particular attention given to the Pt-BMI·PF_6 and the PO immobilized on HNTs, which act as a catalyst in the enzymatic reaction occurring on the electrode surface. As seen in the schematic representation (Fig. 1B), the process begins with the oxidation of PO molecules in the presence of hydrogen peroxide on the biosensor surface with the subsequent reduction of the enzymes in the presence of dopamine. This catecholamine is then converted to its corresponding *o*-quinone by PO and reduced electrochemically on the biosensor surface at a potential of approximately +0.14 V *vs.* Ag/AgCl. The resulting current of the reduction process is proportional to the concentration of dopamine and is used for the quantification of this analyte. The same process can be extended to the other catecholamines studied (isoproterenol, dobutamine and epinephrine), changing only the peak potential.

A study on the contribution of the modifiers used in the biosensor construction

A study on different electrodes was carried out to evaluate the contribution of POs immobilized on HNTs and the ILs containing Pt NPs to the analytical response of the proposed biosensor. Fig. 2A shows the square-wave voltammograms obtained using the (a) bare CPE, and the modified CPEs: (b) CPE-PO, (c) CPE-HNT, (d) CPE-HNT-PO, (e) CPE-HNT-BMI·PF_6, (f) CPE-HNT-BMI·PF_6-PO, (g) CPE-HNT-Pt-BMI·PF_6 and (h) CPE-HNT-Pt-BMI·PF_6-PO. All SWV measurements were carried out in 0.1 M phosphate buffer solution (pH 7.0) containing 98.0 μM hydrogen peroxide and 18.87

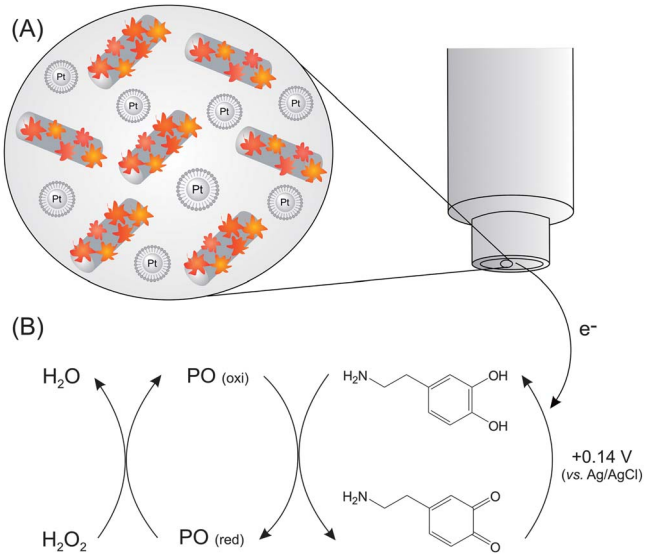

Fig. 1 Schematic representation of (A) carbon paste modified with Pt-BMI·PF_6 and PO immobilized in HNTs, and (B) enzymatic reaction between dopamine and PO with electrochemical reduction of the quinone formed at the biosensor surface.

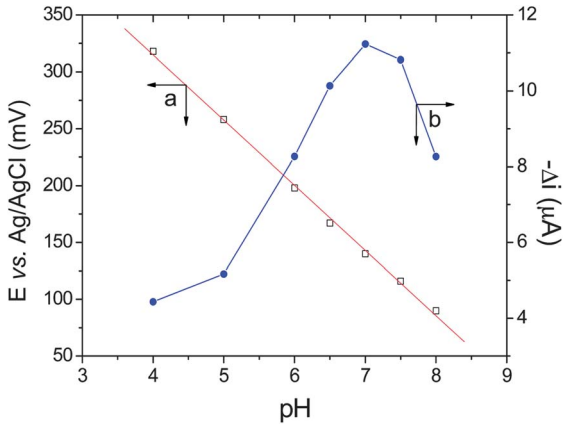

Fig. 3 Influence of pH on peak potential (a) and resulting peak current (b) of the proposed biosensor in the supporting electrolyte solution (0.1 M acetate buffer solution pH 4–5 and 0.1 M phosphate buffer solution pH 6–8) containing 3.92 μM dopamine and 98.0 μM hydrogen peroxide estimated by SWV at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5 mV.

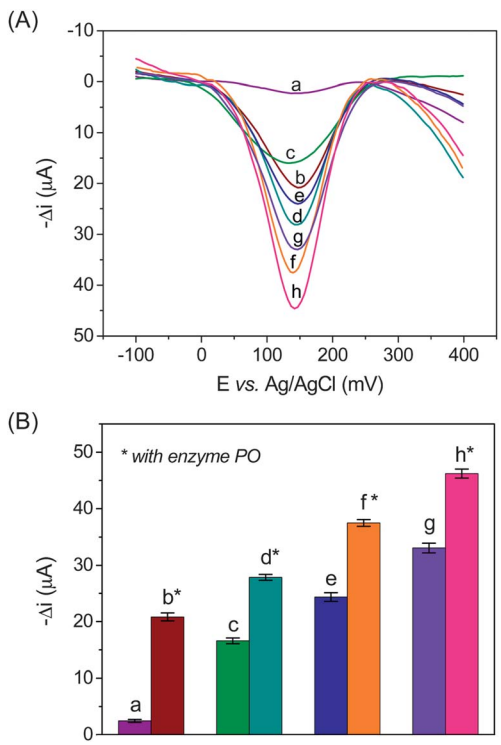

Fig. 2 (A) Square-wave voltammograms obtained using different electrodes: (a) bare CPE, (b) CPE-PO, (c) CPE-HNT, (d) CPE-HNT-PO, (e) CPE-HNT-BMI·PF_6, (f) CPE-HNT-BMI·PF_6-PO, (g) CPE-HNT-Pt-BMI·PF_6, (h) CPE-HNT-Pt-BMI·PF_6-PO in 0.1 M phosphate buffer solution (pH 7.0) containing 98.0 μM hydrogen peroxide and 18.87 μM of dopamine at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5 mV. (B) Arithmetic mean of current values for each electrode, and error bars represent the standard error of the mean ($n = 3$).

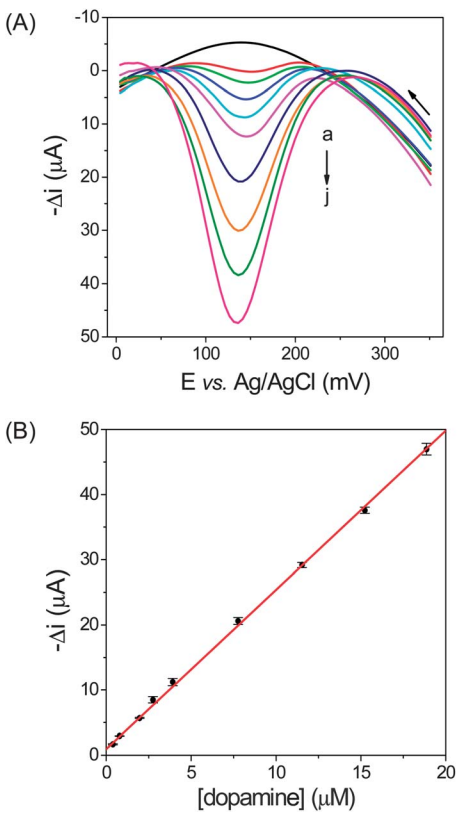

Fig. 4 (A) Square-wave voltammograms obtained using the biosensor proposed in (a) phosphate buffer solution (0.1 M, pH 7.0) containing 98.0 μM hydrogen peroxide and dopamine standard solutions in the following concentrations: (b) 0.40, (c) 0.79, (d) 1.97, (e) 2.75, (f) 3.92, (g) 7.77, (h) 11.54, (i) 15.24, (j) 18.87 μM at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5 mV. (B) Calibration curve for dopamine. All error bars represent the standard error of the mean ($n = 3$).

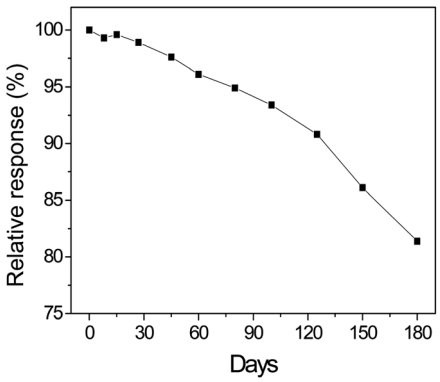

Fig. 5 Study on the proposed biosensor stability by SWV in phosphate buffer solution (0.1 M, pH 7.0) containing 18.87 μM dopamine and 98.0 μM hydrogen peroxide.

μM of dopamine at a frequency of 50 Hz, a pulse amplitude of 100 mV and a scan increment of 5 mV. Fig. 2B shows the arithmetic mean of current values for each electrode, and error bars represent the standard error of the mean ($n = 3$). As can be seen, the successive addition of modifier materials to the carbon paste caused changes in the performance of the electrodes, leading to an improvement in the analytical response. The addition of POs to the electrode (biosensor "b") causes a significant increase in the peak current for dopamine, which is approximately eight times larger than that of the bare CPE (a). This finding can be explained by the enzyme acting as a catalyst in the oxidation of this catecholamine on the electrode surface. The addition of HNTs to the electrode (sensor "c") also provides a notable increase in the current for dopamine (around six times larger than that of the bare CPE), due to the increase in the active surface area of the sensor, in view of the high porosity of this nanomaterial, which provides a "pre-concentration" of dopamine molecules on the electrode surface. The biosensor (d) constructed with POs immobilized on nanoclay had a peak current larger than that of the electrodes (b) and (c), which contain only the enzyme or HNTs, and the immobilization also leads to increased stability of the enzyme. The electrodes (e) and (f) presented an improvement in the response promoted by the high electrical conductivity of the IL, which decreases the charge transfer resistance. An important gain in the response was also obtained with the addition of Pt NPs (sensor "g") due to their effect of increasing the electron transfer rate, which arises from the redox processes. Also, the use of an immobilized enzyme (PO-HNT) in the construction of a biosensor (h) caused a significant increase in the peak current for dopamine, due to the high catalytic activity of PO in the oxidation of this catecholamine, which was preserved through the efficient enzymatic immobilization on a biocompatible support with a high surface area. The HNTs used in the PO immobilization can facilitate the heterogeneous electron transfer between the protein and the electrode surface, due to a favorable orientation of the biomolecules. In addition, the combination of the metal NPs and ILs can also provide a suitable microenvironment for the enzyme stabilization, prolonging its useful life and permitting direct electron transfer between the enzyme and the electrode surface.[16] Therefore, the present study confirms the advantages of the use of modifier materials, such as metal NPs, ILs and nanoclays (HNTs), in the development of biosensors with improved analytical performance.

Table 2 Dopamine recovery from pharmaceutical samples using the proposed biosensor

	Dopamine (μM)		
Sample[a]	Added	Found[b]	Recovery (%)[c]
A	5.61	5.47 ± 0.04	97.5
	7.34	7.34 ± 0.07	100.0
	9.01	9.09 ± 0.08	100.9
B	5.61	5.58 ± 0.04	99.5
	7.34	7.25 ± 0.08	98.8
	9.01	9.10 ± 0.06	101.0
C	5.61	5.52 ± 0.08	98.4
	7.34	7.44 ± 0.05	101.4
	9.01	8.99 ± 0.06	99.8

[a] Dopamine hydrochloride injection (A, B and C). [b] Mean ± standard deviation; $n = 3$. [c] Recovery = (mean found value/added value) × 100%.

Table 3 Determination of dopamine in pharmaceutical samples using a standard method[50] and the proposed biosensor

	Dopamine (mg mL^{-1})			Relative error (%)[c]	
Sample[a]	Label	Standard method[b]	Biosensor[b]	RE_1	RE_2
A	5.0	4.94 ± 0.12	4.91 ± 0.08	−1.8	−0.6
B	5.0	5.15 ± 0.03	5.16 ± 0.05	+3.2	+0.2
C	5.0	4.98 ± 0.06	5.03 ± 0.06	+0.6	+1.0

[a] Dopamine hydrochloride injection (A, B and C). [b] Mean ± standard deviation; $n = 3$. [c] RE_1 = biosensor *vs.* label value; RE_2 = biosensor *vs.* standard method.

Effect of pH on the voltammetric response of dopamine at the biosensor

The pH of the supporting electrolyte solution has a significant influence on the electrochemical behavior of dopamine by

Table 1 Analytical parameters of the biosensor in the determination of catecholamines

Catecholamine	E^a	Intercept (μA)	Slope (μA μM^{-1})	r^2	Linear range (μM)	LOD (μM)
Dopamine	+140	0.93 ± 0.044	2.44 ± 0.020	0.9990	0.40–18.87	0.05
Isoproterenol	−230	−0.12 ± 0.045	2.12 ± 0.006	0.9995	0.79–17.42	0.06
Dobutamine	−290	1.75 ± 0.051	2.19 ± 0.005	0.9990	0.28–25.93	0.07
Epinephrine	−240	1.68 ± 0.079	1.97 ± 0.006	0.9992	0.79–17.06	0.12

[a] Peak potential values in mV *vs.* Ag/AgCl.

varying both the peak current and peak potential. Therefore, an investigation into the effect of the pH of the electrolyte solution on the peak current and peak potential was carried out by SWV using the biosensor in a series of buffer solutions of varying pH in the range of 4–8 (acetate buffer solution pH 4–5 and phosphate buffer solution pH 6–8), containing 3.92 μM of dopamine and 98.0 μM hydrogen peroxide. According to the results shown in Fig. 3(a), the peak potential for dopamine indicated a linear variation with the pH, shifting to a more negative potential at higher pH. There is a linear relationship between the peak potential and pH of the solution with a slope of around −57.4 mV/pH unit and $r^2 = 0.9984$, which is very close to the expected Nernstian value (59.2 mV/pH) for a two electron–two proton reaction, suggesting that dopamine oxidation–reduction is a process involving two electrons and two protons, as described in several studies reported in the literature.[51–53]

In addition, this study was used to select the best supporting electrolyte solution for the proposed method. The response of the peak current at different pH values is shown in Fig. 3(b). The resulting peak current for dopamine was highest at pH 7 and decreased gradually with increasing pH. Consequently, phosphate buffer solution (0.1 M, pH 7.0) was selected as the supporting electrolyte in this study.

Optimization of the biosensor construction and experimental conditions

In order to optimize the biosensor construction and the experimental conditions of the proposed method, several parameters were investigated using SWV. Initially, the effect of enzyme concentration from 2.5 to 10.0 PO units per mg of carbon paste was studied in electrodes with a composition of graphite powder : binder : PO immobilized on HNTs of 70 : 20 : 10% (w/w/w). The analytical responses (resulting peak currents) of the biosensor in 0.1 M phosphate buffer solution (pH 7.0) containing 18.87 μM dopamine and 98.0 μM hydrogen peroxide increased with enzyme concentrations of up to 5.0 PO units per mg of carbon paste. This enzyme concentration was then used for further construction of the biosensor.

The application of an IL containing metallic NPs as a binder to replace Nujol in the construction of electrodes has also been investigated in previous studies carried out by our group.[23,49] According to these investigations, the Nujol : NPs–IL proportion of 50 : 50% (w/w) results in the best analytical response, and this composition was therefore used for subsequent biosensor construction. In agreement with reports in the literature,[16,17,23,24,49] the addition of ILs to a CPE modifies the microstructure of the paste and increases the rate of electron transfer, mainly due to the high conductivity of the IL in comparison with other binders (*e.g.*, Nujol, paraffin, *etc.*). In addition, the intrinsic catalytic properties of metallic NPs contribute to improving the analytical performance of the biosensors.[16,17,23,24]

The presence of hydrogen peroxide is essential for the biosensor response because it is the natural substrate of the PO enzyme. However, high concentrations of peroxide can cause enzyme inactivation and, consequently, decrease the sensitivity and lifetime of the biosensor.[54] Thus, the influence of hydrogen peroxide concentration (from 9.8 to 192.0 μM) on the proposed biosensor response was investigated and the optimum concentration was found to be 98.0 μM. Therefore, this concentration was selected for subsequent studies.

The SWV parameters were also investigated in the search for better sensitivity and peak definition in the dopamine determination. The parameters and ranges investigated were: frequency (10–100 Hz), pulse amplitude (10–100 mV) and scan increment (1–10 mV). This study was carried out in 0.1 M phosphate buffer solution (pH 7.0) containing 18.87 μM dopamine and 98.0 μM hydrogen peroxide, and the best analytical response was obtained using a frequency of 50 Hz, pulse amplitude of 100 mV and scan increment of 5 mV. Thus, these instrumental conditions were selected for all subsequent determinations of dopamine and other catecholamines.

Analytical performance of the biosensor

Under the conditions previously optimized using SWV, the proposed biosensor showed a linear response range of 0.40 to 18.87 μM of dopamine, which can be expressed according to the following regression equation: $-\Delta i = 0.93(\pm 0.044) + 2.44(\pm 0.020)$ [dopamine], with a correlation coefficient (r^2) of 0.9990 for $n = 3$, where Δi is the resultant peak current (μA) and [dopamine] is the dopamine concentration (μM). The calculated limit of detection (LOD = three times the standard deviation of the intercept/slope) and limit of quantification (LOQ = ten times the standard deviation of the intercept/slope) were found to be 0.05 and 0.18 μM, respectively. Fig. 4 shows the square-wave voltammograms and the inset gives the calibration curve for dopamine, constructed from a plot of the resultant current peak *vs.* dopamine concentration. The electrochemical reduction of dopamine was obtained at a potential of approximately +140 mV *vs.* Ag/AgCl.

A repeatability study was carried out through ten successive measurements by SWV, using the proposed biosensor in 0.1 M phosphate buffer solution (pH 7.0) containing 18.87 μM dopamine and 98.0 μM hydrogen peroxide. A relative standard deviation (RSD) of 3.9% was obtained in this study, showing a good accuracy between measurements. The biosensor-to-biosensor reproducibility was also investigated considering six biosensors prepared independently. A good reproducibility was obtained, with RSD values of 5.5% between the biosensors analyzed.

The stability and lifetime of the biosensor are also important parameters in analytical determinations. Therefore, the biosensor was tested for dopamine determination at different intervals over a period of around 180 days (over 600 determinations for each quantity of carbon paste used in the syringe), being stored at room temperature. These analyses were performed in triplicate, and the average results obtained are shown in Fig. 5. As can be observed, a relative response of over 95% was obtained for the first 60 days of evaluation, in relation to that obtained on the day of construction. Upon reaching 180 days, the biosensor exhibited a relative response of over 80%. These results are considered to be suitable and can be attributed to the conservation of the enzymatic activity, due to the effective immobilization of PO on a biocompatible support, along with the "protective sphere" formed by the IL. The favorable microenvironment formed around the enzyme makes this method efficient for maintaining the stability and lifetime of this biomolecule.[16]

The influence of potentially interfering species on the biosensor response is another important parameter that was studied in relation to the application of the biosensor to biological samples. Dopamine, ascorbic acid and uric acid coexist in the extracellular fluid of the central nervous system, and the concentration levels of both ascorbic and uric acid are much higher than that of dopamine. In these biological fluids, the dopamine concentration levels are very low (0.01–1 μM), while the ascorbic acid, for example, is present at relatively high concentrations (around 0.1 mM) and oxidizes at a potential similar to that of dopamine.[55] Thus, the effect of the interference of ascorbic and uric acids on the analytical response of dopamine was investigated, since these species can be directly oxidized on the biosensor surface or even react with the hydrogen peroxide required for the enzymatic process, decreasing the biosensor response. The studies demonstrated that the ascorbic and uric acids did not show significant interference in the dopamine determination in a 1 : 100 molar ratio (dopamine : interfering compound), leading to changes in the analytical signal for dopamine of less than 7%. Therefore, this biosensor can be used for dopamine determination in the presence of these potentially interfering compounds with suitable selectivity.

Response of biosensor to other catecholamines

The use of enzymes as biocatalysts in the construction of biosensors with the objective of determining phenolic compounds has been widely described in the literature.[23,24,35–37] The PO catalyzes the oxidation of mono and diphenols to their corresponding quinones in the presence of hydrogen peroxide.[38,39] In this regard, although the biosensor response was optimized as a function of the dopamine, it is expected that the proposed biosensor can detect other catecholamines, as mentioned before. Therefore, the analytical behavior of the biosensor in the determination of catecholamines other than dopamine, such as isoproterenol, dobutamine and epinephrine, were studied in detail. Table 1 shows the analytical parameters for the catecholamines determined, including dopamine. The responses in terms of detectability (LOD) followed the order: dopamine > isoproterenol > dobutamine > epinephrine. As can be observed, the levels of sensitivity of the calibrations (slope of calibration curve) obtained for the catecholamines analyzed were similar, since their chemical structures are very similar.

Recovery study and analytical application

A recovery study and the determination of dopamine were performed with the standard addition method, in triplicate, using three pharmaceutical samples (A, B and C = dopamine hydrochloride injection). Recovery measurements were obtained by adding different standard concentrations (5.61, 7.34 and 9.01 μM) of dopamine to each pharmaceutical sample. The percentage recovery values were calculated by comparing the concentrations detected in samples with and without the addition of known concentrations of the dopamine standard solution. The recoveries of 97.5 to 101.4% obtained for these samples demonstrate the satisfactory accuracy of the proposed biosensor (Table 2).

In addition, to verify the efficiency of the method developed, these pharmaceutical samples were also used in the dopamine quantification. The dopamine concentrations in three samples of injectable formulations were determined, in triplicate, using the proposed biosensor and the standard (spectrophotometric) method.[50] The results of the quantifications using the biosensor, the standard method and label values were compared (shown in Table 3). The contents of dopamine determined using the biosensor are consistent with the content specified on the label of the pharmaceutical samples, with a relative error of less than ±3.3%, and in relation to the standard method a maximum relative error of ±1.0% was obtained. It can thus be concluded that the proposed method is suitable for the determination of dopamine in pharmaceuticals, and is also appropriate for the quantification of other catecholamines (epinephrine, dobutamine and isoproterenol).

Conclusions

This paper describes the development of a novel biosensor containing the PO enzyme obtained from clover sprouts immobilized on HNTs, together with Pt NPs dispersed in a hydrophobic IL (Pt-BMI·PF_6). The proposed biosensor showed good stability and lifetime, adequate reproducibility and a low detection limit for the determination of dopamine and other catecholamines (epinephrine, dobutamine and isoproterenol). The use of the enzyme obtained from a natural source as a biocatalyst provided high efficiency, low cost and suitable selectivity in the construction of a biosensor for the determination of catecholamines. It is suggested that the excellent analytical performance of the proposed method is due to the efficient immobilization of the PO in the nanoclay, and the facilitation of electron transfer between the protein and the electrode surface due to the presence of the Pt NPs and ILs. Also, the modifiers used provide a suitable microenvironment for the enzyme stabilization. The results obtained show that the method is simple and presents high sensitivity for the determination of dopamine in pharmaceuticals with suitable accuracy, and is also appropriate for the quantification of other catecholamines in pharmaceutical and/or biological samples.

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