Sensors and Actuators B 239 (2017) 1016–1025

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

One-pot preparation of SBP-PANI-PAA-ethylene glycol diglycidyl ether sensor for electrochemical detection of H_2O_2

Dimas Ignacio Torres [a], María Victoria Miranda [b], Viviana Campo Dall' Orto [a,*]

[a] Universidad de Buenos Aires, CONICET, Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Facultad de Farmacia y Bioquímica, Junín 956, Ciudad Autónoma de Buenos Aires, 1113, Argentina
[b] Universidad de Buenos Aires, CONICET, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Ciudad Autónoma de Buenos Aires, 1113, Argentina

ARTICLE INFO

Article history:
Received 26 April 2016
Received in revised form 16 August 2016
Accepted 19 August 2016
Available online 20 August 2016

Keywords:
Polyaniline
Soybean seed coat peroxidase
Epoxy
Poly(acrylic acid)
Hydrogen peroxide

ABSTRACT

Soybean seed coat peroxidase (SBP) was used in combination with aniline (ANI), acrylic acid (AA), ethylene glycol diglycidyl ether (EGDE) and H_2O_2, to prepare a biosensor in one-pot synthesis strategy. SBP catalyzed the chemical polymerization of ANI initiated by H_2O_2, to give the conductive polymer (PANI) in acidic medium where acrylate acted as dopant. Further reactions between free radicals, AA and the diepoxy EGDE determined the entrapment of the SBP together with PANI in a hydrogel on the electrode surface, becoming wired for the amperometric detection of H_2O_2 at neutral pH and −100 mV vs Ag/AgCl. On the other hand, the absence of AA and EGDE in the reaction mixture led to an unstable sensor modifier and an erratic response to H_2O_2. The apparent K_m for immobilized SBP in the flow system was 0.0763 mM, lower than the parameter for the free enzyme at pH 7.0. Quantitative analysis of H_2O_2 was carried out in a flow injection system (FIA); the calibration plot was linear in the range of 5.0–50 μM, with a limit of detection of 2.2 μM. A dental bleaching sample was successfully analyzed and the catalase test results demonstrated the specificity of the response.

1. Introduction

Intrinsically conducting organic polymers such as polyaniline, polypyrrole and polythiophene have been studied intensively due to their high electrical conductivity and good environmental stability for biosensor development. In the family of conducting polymers, polyaniline (PANI) is unique because of its ease of synthesis and its doping/de-doping chemistry: the electronic state of the polymer can be changed through variation of either the number of electrons or the number of protons [1]. Among the various oxidation states of PANI, the imine sites from emeraldine base (EB) can be protonated to the bipolaron form (dication salt, ES) which undergoes further rearrangement to form the delocalized polaron lattice (a polysemiquinone radical cation salt) with a conductivity of a semiconductor level [2,3]. In this way, when the chemical polymerization of aniline (ANI) is performed at pH lower than 3.0, the acidic condition minimizes the formation of undesired branched products and the conductivity is enhanced [4]. Instead, PANI films produced in non-acidic media are usually non-conducting [5].

ANI can be polymerized using H_2O_2 as oxidant and horseradish peroxidase (HRP) as catalyst, to obtain PANI in its conducting ES form at pH 4.3 in a medium containing sulphonated polystyrene (SPS) [6]. The negatively charged SPS assists the polymerization by in some way pre-organising the reacting monomers and intermediates, and by acting as counter ion (dopant) through the formation of a soluble complex with the positively charged PANI [7,8]. On the other hand, the chemically synthesized PANI is a suitable polymeric support for enzyme immobilization. Fernandes et al. reported the immobilization of HRP made by adding the enzyme solution at pH 6.0 to PANI previously activated with glutaraldehyde, and the enzymatic activity of the solid was tested for the oxidation of phenolic compounds with H_2O_2 [9].

The enzyme immobilization in film assemblies organized on electrode supports is a key step in the development of biochemical modifiers for electroanalysis. Redox enzymes usually lack direct electrical communication with electrode supports, often overcome by using conductive polymers as "wiring" matrixes that transport the electrons between the enzyme redox center and the conducting surface. The earliest sensor developments with PANI presented redox activity only in acidic solutions at pH values below 3.0,

* Corresponding author.
E-mail addresses: vcdall@ffyb.uba.ar, vivianacdall@gmail.com (V. Campo Dall' Orto).

http://dx.doi.org/10.1016/j.snb.2016.08.110
0925-4005/

thus preventing their integration with redox enzymes that usually operate in neutral pH regions. This limitation was resolved by using composite PANI polymers doped with *Poly*(acrylic acid) (*Poly*(AA)), which switched the redox activity to pH 7.0 [10]. The PANI-*Poly*(AA)-coated electrodes for biosensor assemblies offer an outstanding signal amplification and a suitable environment for immobilization of biomolecules [4].

Determination of H_2O_2 and other organic peroxides is of practical importance in clinical, environmental, and many other fields [11]. The fabrication of H_2O_2 sensors has attracted great interest since the measurement of H_2O_2 is the basis of detecting many biologically active compounds such as glucose and cholesterol [12]. Ultrasensitive potentiometric approach of H_2O_2 sensing was carried out using a membrane electrode doped with the cation exchanger dinonylnaphthalene sulfonate [13]. The sensitive determination of H_2O_2 may also be achieved in the amperometric mode by the use of peroxidase-modified electrodes, and in this context the combination of PANI with HRP was thoroughly explored, the enzyme being either electrostatically attached to PANI at pH 6.8 [14], or cross–linked with glutaraldehyde [15].

The approach was also tested with seed coat soybean peroxidase (SBP), obtained from a non expensive food industry by–product [16,17]. SBP was immobilized on various PANI-based polymers activated with glutaraldehyde and was tested for pyrogallol oxidation with H_2O_2 [18]. A sol–gel thin–film SBP biosensor was constructed for the detection of H_2O_2 in acid medium, with methylene blue as redox mediator [19]. A patent also claims the cross–linking of SBP with the diepoxide *Poly*(ethylene glycol diglycidyl ether) (PEGDGE) and the poly(4-viny1pyridine)–Os(II) complex to develop an electrochemical biosensor for H_2O_2 [20].

This paper reports the fabrication and characterization of a PANI–based biosensor incorporating SBP for the detection of H_2O_2. The novelty resides in the use of a different protecting environment for the enzyme on the electrode surface, and the synthesis of PANI and of *Poly*(AA) catalyzed by SBP which is also the recognition element of the sensor. In this case, AA was combined with ANI, the monomeric diepoxy cross–linker ethylene glycol diglycidyl ether (EGDE) and the enzyme to catalyze the ANI polymerization on the electrode surface by addition of H_2O_2. The SBP molecules got entrapped in the resulting matrix, staying attached on the electrode surface and electrically wired at neutral pH. The combination of AA with EGDE was chosen for this application on the basis of their known aptitude in the development of hydrophilic networks to immobilize SBP [21,22].

2. Experimental

2.1. Materials and instrumental

Acrylic acid (AA; 99 wt%), catalase from Aspergillus niger, $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ were purchased from Sigma–Aldrich. Ethylene glycol diglycidyl ether (EGDE; 50 wt% in ethylene glycol dimethyl ether) was from TCI America. Hydrogen peroxide H_2O_2 30% for analysis was from Merck. Aniline (ANI) was distilled under vacuum. Acetone was from Anedra. Hydrochloric acid, sodium hydroxide and potassium dihydrogen phosphate were analytical grade reagents. Deionized water was used for all aqueous solutions. Soybean seed coats were kindly donated by Molinos Río de la Plata, Argentina.

UV–vis experiments were made with an Evolution Arrays Spectrophotometer from Thermo Scientific, using a conventional quartz cuvette. ATR-FTIR (diamond attenuated total reflectance) spectrum was recorded using a Nicolet iS50 Advanced Spectrometer (Thermo Scientific) with 32 scans and a resolution of 4 cm^{-1}; the sample was deposited on a glass substrate for this experiment.

The flow system consisted of an HPLC SpectraSYSTEM Isocratic Pump P100, a Rheodyne injection valve (Model 7125) with a 20 μL sample loop, a guard column and the flow cell for amperometric detection.

The amperometric detection of H_2O_2 was performed with a home-made microprocessor-controlled electrochemical analyser with electrochemical impedance spectroscopy module. A thin-layer cell (7-μL nominal volume), equipped with a glassy carbon working electrode (GCE, Bioanalytical Systems, 7-mm^2 area), which was properly modified with the enzymatic layer, and a stainless steel block as auxiliary electrode, operated at a constant potential vs. Ag/AgCl. Stainless steel and polyether ether ketone were used for the flow system connections.

The carrier was an aqueous solution containing 50 mM KH_2PO_4 brought to different pH values (PBS), properly filtered and degassed. The H_2O_2 was detected amperometrically with the novel biosensor in the flow system at a reductive potential. All the samples were diluted properly with carrier, and filtered through a 0.45-μm pore diameter nylon membrane before injection. Most of the experiments were performed at a flow rate of 0.3 mL min^{-1}.

Batch experiments were performed in a 10-mL cell with a 7-mm^2 GC working electrode, a graphite auxiliary electrode and a Ag/AgCl reference electrode (BAS), using 500 mM KNO_3 solution brought to pH 6.0.

2.2. Extraction and purification of soybean seed-coat peroxidase (SBP)

The crude extract of SBP was obtained by contact of seed coats with a solution containing 50 mM sodium acetate/acetic acid and 5 mM $CaCl_2$ [18]. The homogenate was centrifuged at 2000 × *g* for 15 min and the supernatant was separated. Then it was purified as described elsewhere [23]. The catalytic activity of the enzyme in the purified extract was 46539 units mL^{-1} (5900 units mg^{-1}) determined by comparison with the standard purchased from Sigma: Peroxidase from Glycine max (soybean) P-1432, 50–150 units per mg of solid. One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C. For the sensor assembly, a diluted solution of SBP was used, containing 1551 units mL^{-1} (0.2629 mg SBP mL^{-1}).

2.3. Preparation of the modified electrode

A mixture of monomers was freshly obtained by mixing 1 μL of EGDE, 1 μL of AA and 5 μL of ANI with 5.0 mL of acetone. The adequate amount of monomers in the acetone solution depended on their miscibility with the solvent.

Then, the corresponding GCE was polished with alumina, rinsed with distilled water and dried. The biopolymerization was performed on the clean electrode surface by adding 0.5 μL of SBP diluted solution, 0.5 μL of monomers mixture and 0.5 μL of 100 mM H_2O_2. The acetone evaporated and after 15 min a film was formed. Then, it was hydrated with 100 mM KNO_3 solution during 30 min before use.

As a control, the GCE was modified with 0.5 μL of SBP diluted solution, 0.5 μL of solution of ANI in acetone, and 0.5 μL of 100 mM H_2O_2.

2.4. Sensor performance

Electrochemical impedance spectroscopy (EIS) experiments were performed in 500 mM KNO_3 solution over a frequency range of 100 kHz – 0.1 Hz at an applied voltage of +200 mV vs Ag/AgCl, with 10 mV amplitude and a total of 40 data point. When necessary, the redox couple used as probe was 50 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$.

H_2O_2 detection with the chemically modified working electrode was performed in batch and in a flow system, in triplicate. The study of sensor response in batch was performed by H_2O_2 addition in a concentration range between 2.0 mM and 11 mM (final concentration), with the biosensor polarized at −100 mV vs Ag/AgCl. The study of interferences performed in batch was carried out with urea, glucose and ascorbic acid added to the 500 mM KNO_3 solution at different final concentration levels (in triplicate for each level): 0.1 mM; 0.3 mM; 0.5 mM; 1.0 mM and 10 mM.

The commercial sample for in-office dental bleaching system was purchased in a local store, and properly diluted in 50 mM PBS, pH 7.0 for the analysis in the flow system.

Catalase was used to demonstrate the specificity of the biosensor, by decreasing the H_2O_2 response when present in the solution. The experiment was performed in batch with the biosensor polarized at −100 mV vs Ag/Ag, by adding 48 units of catalase to 10,00 mL of 500 mM KNO_3 solution, then making three additions of 0.410 mM H_2O_2 (final concentration) and three additions of diluted sample containing 0.408 mM H_2O_2 (final concentration).

3. Results and discussion

3.1. Design of SBP-Poly(EGDE-AA-ANI)/GCE

This biopolymerization took place when the SBP solution was mixed with 100 mM H_2O_2 and the monomers (ANI, EGDE and AA) diluted in acetone, on the GCE surface (Scheme 1). The adequate amount of monomers in the acetone solution depended on their miscibility with the solvent. The acetone evaporated after 15 min of reaction giving place to a homogeneous film. The modified surface was then hydrated with 100 mM KNO_3 solution for 30 min before use.

In this experiment, the enzyme SBP catalyses the non-selective oxidation of a substrate (in this case, the monomer ANI) to a free radical product by the electron-acceptor H_2O_2 in a well known ping-pong mechanism. The catalytic cycle of peroxidases involves the oxidation of Fe(III) in the active site by H_2O_2 forming a reactive Fe(IV) = O species and a radical intermediate of the heme group (compound I). One-electron reduction of compound I by ANI gives compound II, in which the FeIV = O species remains intact and porphyrin radical is reduced, releasing the radical cation $ANI^{+\bullet}$. Then another molecule of the reducing substrate (ANI) regenerates the Fe(III) ion and releases $ANI^{+\bullet}$. This oxidized product initiates the radical polymerization on the electrode surface, giving PANI. Now, in the presence of PANI, the reduction of compound I on the GCE is achieved by this conductive polymer (Scheme 2), and the reaction between SPB and H_2O_2 becomes "wired" with the electrode for electrochemical detection [24].

The H_2O_2 reduction charge is propagated along the polymer chain to the GCE surface polarized at −100 mV vs Ag/AgCl, by fast electron transfer reactions involving $PANI^{+/0}$ redox species. Pekmez et al. [25] have explained that a partial reduction of PANI film occurs under this condition, and the polymer is thus a mixture of reduced (polyleucoemeraldine) and oxidized (polyemeraldine) species and their cation radicals protonated to various degrees, depending on the reaction medium.

In this way, the oxidized SBP regenerates the conductive form of PANI, the emeraldine salt (ES). If PANI is synthesized at pH higher than 4.0, the electroactivity can be lost due to the failure in formation of ES.

The presence of AA in the reaction medium contributes to lower the pH value, and the acrylate formed (A^-) acts as counter ion (dopant) to achieve the electrical neutrality of the oxidized ES. The question is whether AA and A^- remain as monomers or polymerize by vinyl groups when $ANI^{+\bullet}$ radicals are released.

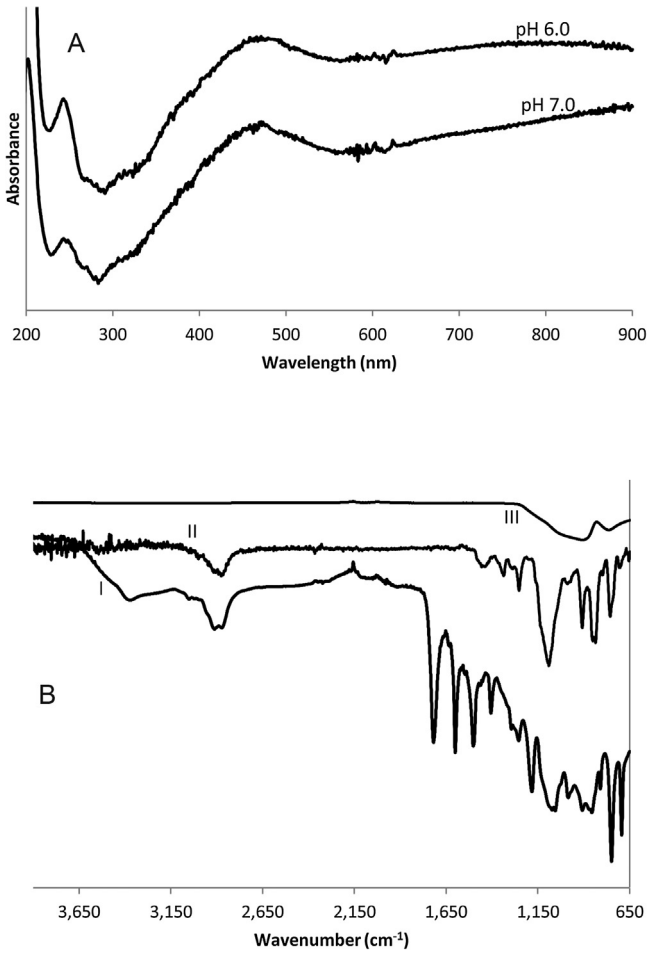
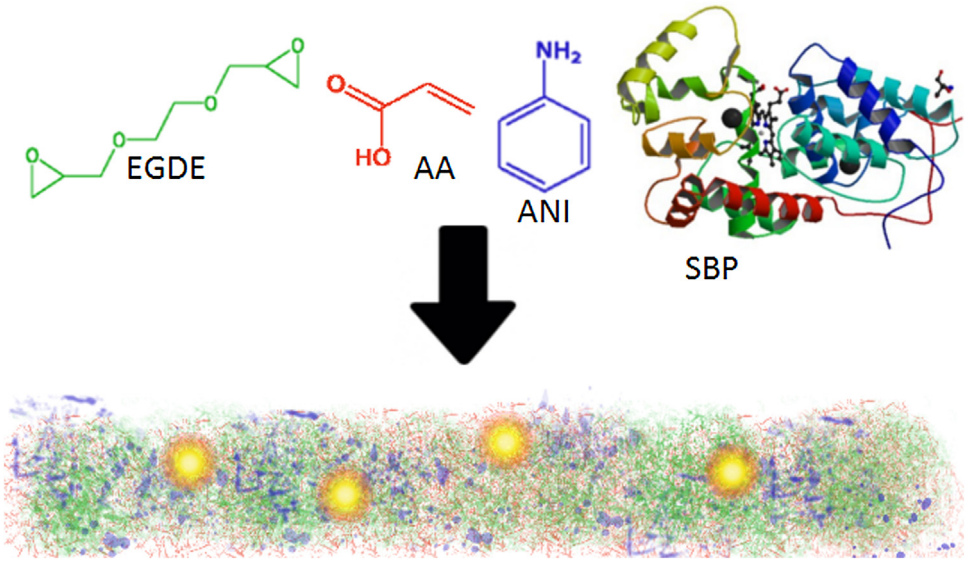


Fig. 1. UV–vis spectra at pH 6.0 and 7.0 of SBP-*Poly*(EGDE-AA-ANI) deposited on quartz substrate (A); ATR-FTIR spectra of SBP-*Poly*(EGDE-AA-ANI) (I), EGDE(II) and glass substrate (III) (B).

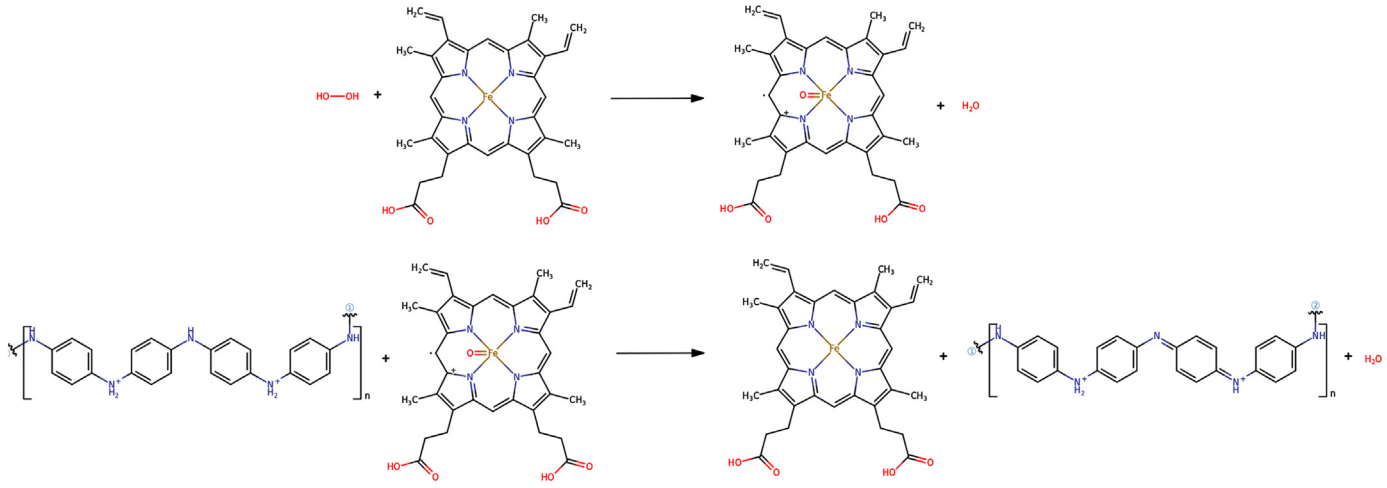
In parallel, EGDE monomers can react with AA forming ester groups, and with the histidine residues of SBP binding covalently by epoxy opening. Thus, EGDE and AA would be the cross-linkers than retain both the enzyme and PANI closely entrapped in a hydrogel matrix on the GCE surface, forming SBP-*Poly*(EGDE-AA-ANI) in a one-pot synthesis (Scheme 1). In control tests, it was observed that no film was formed in the absence of the enzyme. Besides, when EGDE and AA were omitted, the film was not stable enough to be used as sensor.

The SBP-*Poly*(EGDE-AA-ANI) composite film was deposited on quartz surface and studied by UV–vis spectroscopy. It was put in contact with solutions at pH 6.0 and 7.0, producing the spectra shown in Fig. 1A. Two main bands attributable to the conducting polymer, were detected: a broad adsorption around 460 nm corresponding to the radical cation (semiquinone structure) and the very broad band around 750 nm due to imine moieties (quinone structure, dication or bipolaron). The band at 440 nm indicated the presence of the lowest oxidized state of PANI, and the band at 750 nm corresponded to the delocalized state from proton-doped polyemeraldine. From these results we evidenced the presence of the emeraldine state protonated (ES) even at pH 6.0 and 7.0 in the SBP-*Poly*(EGDE-AA-ANI) composite film [26], responsible of SBP wiring on sensor surfaces.

The ATR-FTIR spectrum of SBP-*Poly*(EGDE-AA-ANI) in Fig. 1B evidenced the surface coverage. The absorption band at 1722 cm^{-1} was due to C=O symmetric stretching, and the band at 1604 cm^{-1} could be attributed to asymmetric stretching of —COO^- in a



Scheme 1. Schematic illustration for the synthesis of the SBP-*Poly*(EGDE-AA-ANI)/GCE.



Scheme 2. Mechanism of reaction of H_2O_2 and PANI with SBP.

polyether environment, in similitude with *Poly*(EGDE-MAA) [27]. The symmetric stretching of —COO^- was detected at 1410 cm^{-1}, and the band around 1100 cm^{-1} corresponded to ether groups from EGDE. The bands at 940 and 1630 cm^{-1} expected for vinyl groups were not detected, indicating that AA was polymerized. The amide I band between 1600 and 1700 cm^{-1} expected for SBP was very weak, indicating that the relative amount of enzyme in the composite was low. The formation of PANI was revealed by the absorption bands at 1603, 1183, and 837 cm^{-1}, which were attributed to the vibrations of, —C═C–, Ph–NH–Q and C—N in the ANI unit [28]. The band at 1503 cm^{-1} with a shoulder at 1497 cm^{-1} could be assigned to benzenoid ring stretching from EB and ES forms, respectively [29].

Fig. 2A shows the amperometric response in batch of SBP-*Poly*(EGDE-AA-ANI)/GCE to H_2O_2 in a concentration range of 2.0–11 mM, compared with the performance of SBP-PANI/GCE in Fig. 2B: clearly, the response to H_2O_2 followed a linear tendency with concentration in the first case (see insert in Fig. 2A), and was lower and erratic in the second case. The mean slope of regression line for H_2O_2 obtained with SBP-*Poly*(EGDE-AA-ANI)/GCE was 18.90 ± 0.59 nA mM^{-1}, and with SBP-PANI/GCE was 2.13 ± 0.30 nA mM^{-1}. According with the *t*-test for comparing means, the slope values differed significantly at the 1% level and 6 degrees of freedom. In order to evaluate the selectivity of the proposed method, additional experiments were performed in batch. Three metabolic compounds were selected to analyze possible interferences because they usually coexist with H_2O_2 in the diffusive layer of bioenzymatic sensors developed for clinical use. In this way, a 500 mM KNO_3 solution at pH 6.0 was spiked with urea, glucose or ascorbic acid in a concentration range between 0.1 mM and 10 mM, considering that a 11 mM H_2O_2 solution can produce a signal step of 0.26 μA with the modified electrode polarized at −100 mV vs Ag/AgCl (Fig. 2C). The absence of faradaic current in the range of μA indicated that these compounds caused negligible interference in the detection of H_2O_2 with this biosensor.

This evidence would support the hypothesis of a protective matrix for SBP and PANI, formed by radical polymerization of AA initiated by $ANI^{+\bullet}$, in combination with a probable esterification of AA with EGDE, and SBP covalent binding to EGDE. These reactions were previously found by MALDI-TOF in matrixes formed by these monomers and methacrylic acid instead of AA [22]. In the present work, the replacement of AA by MAA was not satisfactory.

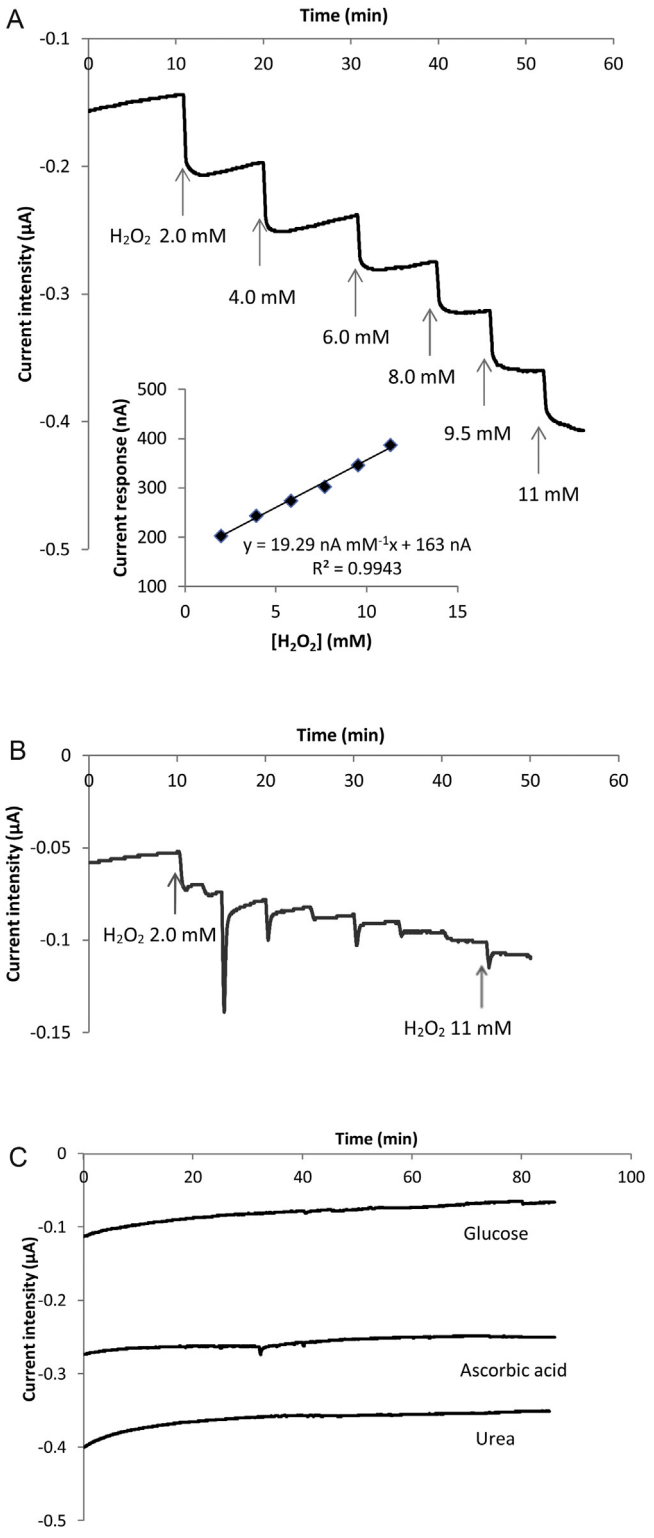


Fig. 2. Amperometric response in batch to H_2O_2 in 500 mM KNO_3, pH 6.0 at −100 mV vs Ag/AgCl with SBP-*Poly*(EGDE-AA-ANI)/GCE (A); the insert presents the calibration plot. The same experiment was performed with SBP-PANI/GCE (B). The electrochemical response of potential interfering substances (glucose, ascorbic acid, urea) in batch with SBP-*Poly*(EGDE-AA-ANI)/GCE (C).

Table 1
Circuit modeling results of SBP-*Poly*(EGDE-AA-ANI)/GCE and of bare electrode with EIS Spectrum Analyser 1.0 simulating programme. The redox couple used as probe was 50 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 500 mM KNO_3 solution, pH 6.0. The equivalent circuits for the modified and bare electrodes are represented in the inserts of Fig. 3A.

Parameters	Modified electrode		Bare electrode	
	Mean value	S.E. (%)	Mean value	S.E. (%)
R_S (Ω)	96.45	0.99	95.5	2.5
R_{CT} (Ω)	786.4	1.5	150.7	6.6
C_{DL} (F)	–	–	7.8×10^{-7}	8.1
P[a] (F)	2.1×10^{-6}	1.3	–	–
n	0.86	0.18	–	–
A_W ($\Omega\, s^{-0.5}$)	387.4	3.4	658.2	3.4

[a] Parameter of constant phase element (CPE), pre-exponential factor.

3.2. *Interfacial properties of SBP-*Poly*(EGDE-AA-ANI)/GCE*

In general, the electrocatalytic activity and electron transfer properties of the immobilized enzymes on the electrodes are significantly affected by the conformation and structure of enzymes on the electrode surfaces. The electrochemical impedance spectroscopy (EIS) is a powerful tool that can provide information about the electrode processes and the interfacial properties of a modified electrode surface.

The Nyquist plots of EIS for the bare GCE and the modified GCE using the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple as a probe, are shown in Fig. 3A, and the estimated parameters for each equivalent circuit are presented in Table 1.

The experimental data obtained with bare GCE fitted well to the Randles equivalent circuit. Instead, the results with the modified electrode fitted to an equivalent circuit in which the double layer capacitance (C_{DL}) was replaced by a constant-phase element (CPE) (inserts in Fig. 3A). This CPE usually arises due to the interface electrode-film defects such as local charge inhomogeneities and different adsorbed species; the average effects determine the macroscopic impedance value, which depends on the reaction rate distribution [30].

The electron transfer resistance (R_{CT}) of a redox probe at the electrode surface can be estimated by the Nyquist diameter of the EIS spectrum. The estimated R_{CT} in the presence of the 50 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ probe using the bare electrode was 150.7 Ω, and 786.4 Ω using SBP-*Poly*(EGDE-AA-ANI)/GCE. This difference could be attributed to the amount of covered area of the modified electrode and/or the structural properties of the SBP layer on the GCE surface. Here, it can be assumed that the electro-active species (in this case, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple) could directly diffuse to the electrode surface through pores and defects of the immobilized SBP layer, or the electro-active species could permeate through the immobilized SBP layer and react at the electrode surface [31].

On the other hand, the Nyquist plot of EIS for the SBP-*Poly*(EGDE-AA-ANI)/GCE was obtained in the absence of the probe redox couple at pH 6.0 (Fig. 3B). The profile found at DC voltage of +200 mV vs Ag/AgCl resulted similar to the profiles for conductive PANI films electropolymerized on GCE and measured in acid medium (1.0 M H_2SO_4, 0.5 M Na_2SO_4) [32].

PANI obtained and tested in acid medium behaves as a good electronic conductor at intermediate oxidation levels [33,34]. It has been described that in the reduced state (– 100 mV vs SCE) and in the highly oxidized state (+1000 mV vs SCE) higher resistances are expected [32]. At intermediate potentials the resistance of PANI is expected to decrease, so that the film tends to behave as a capacitor in series with a resistance even for relatively high frequencies (*f*). As *f* decreases, the capacitance tends to a value (C_{SP}) which is *f*-independent.

In our system, the PANI was synthesized in acid medium provided by AA but the EIS experiments were performed in 500 mM

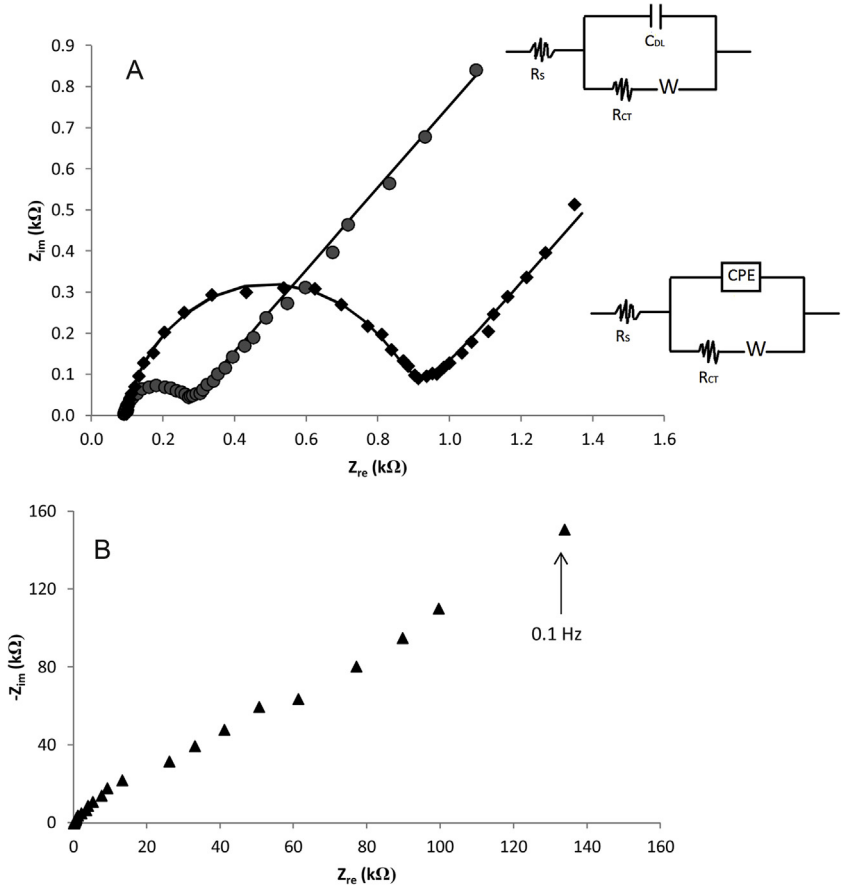


Fig. 3. The Nyquist plots of electrochemical impedance spectroscopy (EIS) for the bare GCE (●) and the SBP-*Poly*(EGDE-AA-ANI)/GCE (♦); the redox couple used as probe was 50 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 500 mM KNO_3, pH 6.0, and the inserts correspond to the equivalent electrical circuit model used in each simulation (A). The Nyquist plot was also obtained for SBP-*Poly*(EGDE-AA-ANI)/GCE in the absence of the redox probe (▲) (B).

KNO_3 brought to pH 6.0. Calculation of C_{SP}, through the expression $Z_{im} = 1/(2\pi f C_{SP})$, gave a specific capacitance of about 150 μF cm^{-2}. This result was consistent with the values reported by Sarac et al: C_{SP} of 38.9 μF cm^{-2} for PANI/GCE and 239.1 μF cm^{-2} for PANI/CFME (carbon fiber microelectrode) [35].

3.3. Optimization of experimental variables

The typical hydrodynamic voltammograms of SBP-*Poly*(EGDE-AA-ANI)/GCE were performed to investigate the effect of the different experimental conditions on the amperometric detection of H_2O_2.

The effect of the applied potential on the current responses of 50 μM H_2O_2 was investigated in a 50 mM PBS pH 7.0 carrier solution at a flow of 0.3 mL min^{-1}. Fig. 4A exhibits that the peak currents increased when changing the electrode potential from +100 mV to −200 mV vs Ag/AgCl, particularly from −100 mV.

The applied potential chosen for H_2O_2 detection was −100 mV vs Ag/AgCl, a compromise between the optimization of signal *to* noise ratio and the minimization of eventual interferences from the matrix species such as dissolved molecular oxygen.

The effect of pH on the current intensity of 50 μM H_2O_2 was studied in 50 mM PBS carrier solution at the applied potential of −100 mV vs Ag/AgCl. The tested pH range was between 6.0 and 8.0, considering the optimal pH expected for immobilized SBP. Fig. 4B clearly indicates that the best pH value was 7.0, beyond which the response was remarkably lower. Even though the optimal pH value for the free enzyme in solution is 4.0–5.0 [18,23], it changed upon SBP immobilization.

Fig. 4C exhibits the dependence of the hydrodynamic response of H_2O_2 on the flow rate of the PBS carrier in the flow injection system. When the flow rate of the substrate (H_2O_2) is limited by mass transport, the diffusion layer at the electrode surface becomes thinner and the diffusion time becomes shorter, which leads to an increase of the diffusion current with the flow rate of the carrier. Instead, in our system the residence time of the substrate in the enzymatic layer was a limiting factor when the flow rate of the carrier increased, reason by which the current peak decreased from 0.3 to 0.6 mL min^{-1}. For the quantitative experiments, the flow rate was set in 0.3 mL min^{-1}.

3.4. Analytical performance of SBP-Poly(EGDE-AA-ANI)/GCE

The amperometric measurement using SBP-*Poly*(EGDE-AA-ANI)/GCE was investigated by injections of H_2O_2 at different concentration levels in the flow system under the optimized conditions: 50 mM PBS pH 7.0; working electrode potential: −100 mV vs Ag/AgCl; and flow rate: 0.3 mL min^{-1}.

The relationship between the amperometric peak current intensity and the concentration of H_2O_2 depicted in Fig. 5A shows a typical Michaelis-Menten (M-M) plot. The data were analyzed by applying a M–M type kinetic model (Eq. (1)).

$$I = I_{max}\frac{[H_2O_2]}{K_m^{app} + [H_2O_2]} \quad (1)$$

and

$$I_{max} = nFAk_{cat}^{app}[E] \quad (2)$$

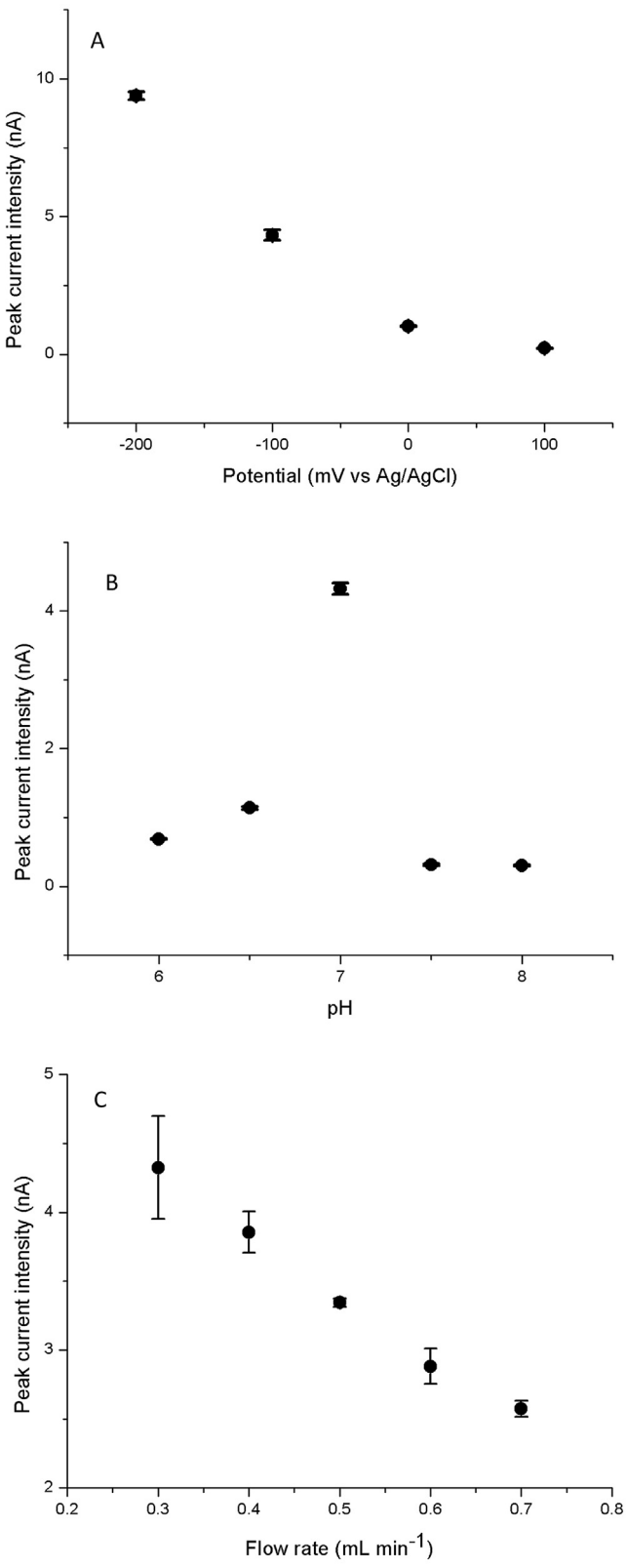


Fig. 4. Determination of the optimal operational parameters in the flow system for H_2O_2 detection with SBP-*Poly*(EGDE-AA-ANI)/GCE: working electrode potential (A), pH of the PBS solution used as carrier (B), and flow rate (C).

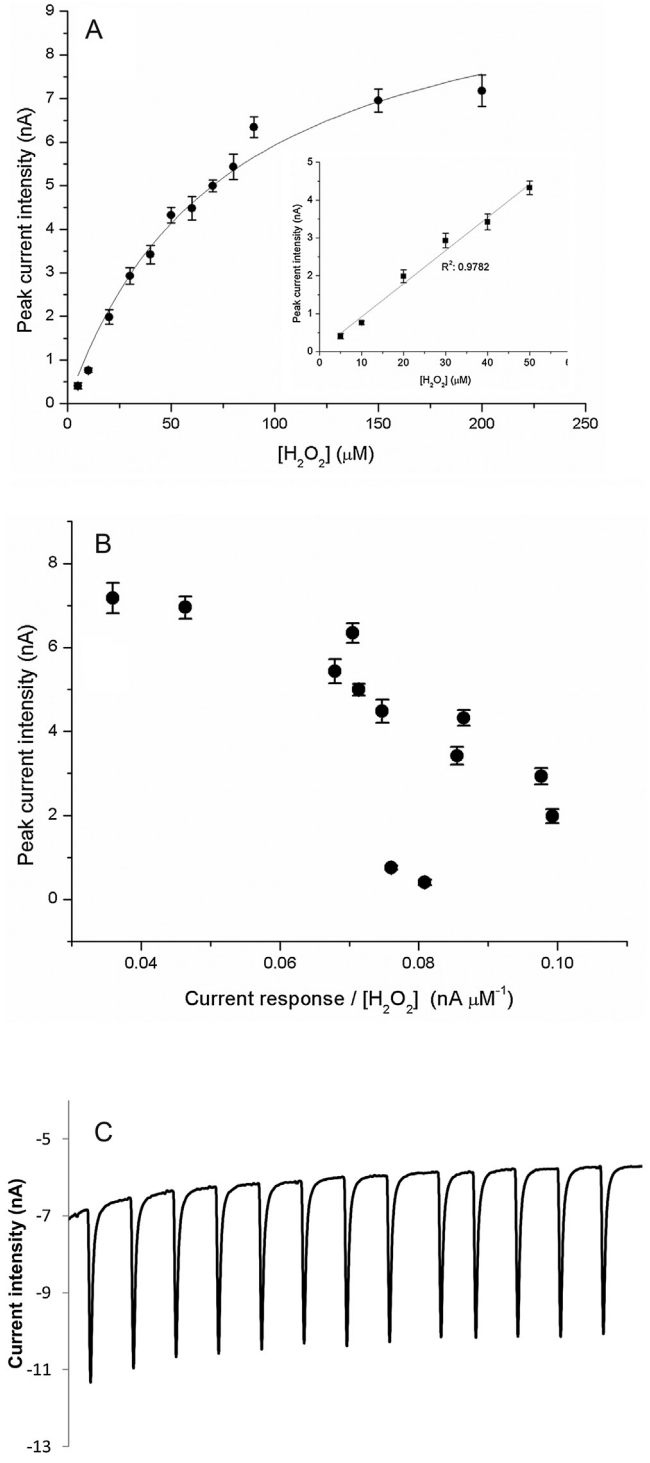


Fig. 5. The relationship between the amperometric peak current intensity and the concentration of H_2O_2 with SBP-*Poly*(EGDE-AA-ANI)/GCE in the flow system using 50 mM PBS pH 7.0, the working electrode polarized at −100 mV vs Ag/AgCl, and a flow rate of 0.3 mL min^{-1}. The insert shows the linear section (A). The conversion of the experimental data to an Eadie-Hoftee plot (B). The repeatability using one modified electrode estimated from the current response to 50 μM H_2O_2 produced by 13 successive injections (C).

where [*E*] is concentration of enzyme SBP at the modifier layer on the electrode surface. The apparent K_m was estimated in 76.3 μM (S.E.: 6.7 μM), and the I_{max} in 10.45 nA (S.E.: 0.44 nA). The apparent k_{cat} was estimated in 1.647×10^{-2} s^{-1} (or 0.9885 min^{-1}); and apparent k_{cat}/K_m was 216 M^{-1} s^{-1}.

Table 2
Reported values of apparent K_m for SBP or HRP, with H_2O_2 as substrate, different reducing agents, pH and assemblies.

Enzyme	co-substrate	pH	*app* K_m ^{H_2O_2}	Reference
free SBP	phenol	6.0	1.092 mM	[17]
free SBP	ABTS	6.8	0.173 mM	[16]
free SBP	ABTS	5.0	0.045 mM	[16]
free HRP	PANI	6.0	11 mM	[18]
HRP-PANI	PANI	6.0	27.11 mM	[18]
HRP-PANI-PVS	PANI	6.9	3.461 mM	[25]
SBP-*Poly*(EGDE-AA-ANI)	PANI	7.0	0.0763 mM	this work

The apparent K_m is the equilibrium constant for the dissociation of the enzyme-substrate complex. The values presented in Table 2 indicate that the pH value and also the process of enzyme immobilization particularly increased the affinity between H_2O_2 and the peroxidase in this novel sensor.

The k_{cat} of free SBP under physiological conditions in batch was reported in 1230 s^{-1}, with a catalytic efficiency k_{cat}/K_m of 7.1 μM^{-1} s^{-1} for the free enzyme in batch. The catalytic efficiency found for immobilized SBP in a flow system resulted low when was compared with the efficiencies reported for batch systems. This could be explained by the fact that using a thin-layer cell in amperometric detection mode, only a 10% of the H_2O_2 passing parallel to the electrode surface can be reduced. Other aspects to be considered are the amount of entrapped SBP together with the amount of active/available biomolecules in the hydrogel matrix.

The conversion of M–M plot to an Eadie-Hofstee (E-H) plot is a strategy to put in evidence diffusive restrictions of the substrate inside the enzymatic layer, because a linear profile in E-H plot is expected when the reaction rate obeys the M–M law. In Fig. 5B, a departure from linearity was exhibited only at extreme values of H_2O_2 concentration, indicating that the diffusive restrictions were minimal under the optimized conditions of detection [36,37].

A calibration plot is presented in the insert of Fig. 5A. The linear range of the biosensor for H_2O_2 concentration was obtained between 5.0 and 50 μM, with a sensitivity of 0.0875 nA μM^{-1} (S.E.: 0.0033), an *y*-intercept of 0.043 nA (S.E.: 0.099) and a correlation coefficient (R^2) of 0.9782. The limits of the *y*-intercept confidence interval at 95% were 0.412 and −0.327, containing zero as expected [38].

The repeatability using one modified electrode was estimated from the current response to 50 μM H_2O_2 (Fig. 5C), yielding a mean current response of 4.310 nA with a R.S.D. of 1.5% (n: 13). The detection limit of 2.2 μM was calculated considering the S.D. of the current response produced by 13 injections of 50 μM H_2O_2 (S.D.: 0.064 nA) and the signal-*to*-noise ratio of 3.

3.5. Practical application

The analytical applicability of the biosensor was evaluated for the quantification of H_2O_2 in a sample of an in-office dental bleaching system for professional use. The sample of bleaching gel was mixed and properly diluted with PBS for the electrochemical detection of H_2O_2 in the flow system under the optimal analytical conditions. Besides, H_2O_2 standard solution was added to the sample in a final concentration level of 24.0 μM, and then analyzed (Fig. 6A). The experimental results are presented in Table 3.

The H_2O_2 level in this sample was calculated in 37.6 wt% by external standard quantification using the linear regression equation. In parallel, the H_2O_2 level in this sample was calculated in 35.0 wt% by standard addition. The ANOVA test indicated that the population means were not significantly different at the 0.05 level. To further verify the reliability, the H_2O_2 concentration in the sample determined by titration was 35 wt%. The standard added to the diluted sample in a concentration level of 24.0 μM was recovered in a 107% (found: 25.7 μM).

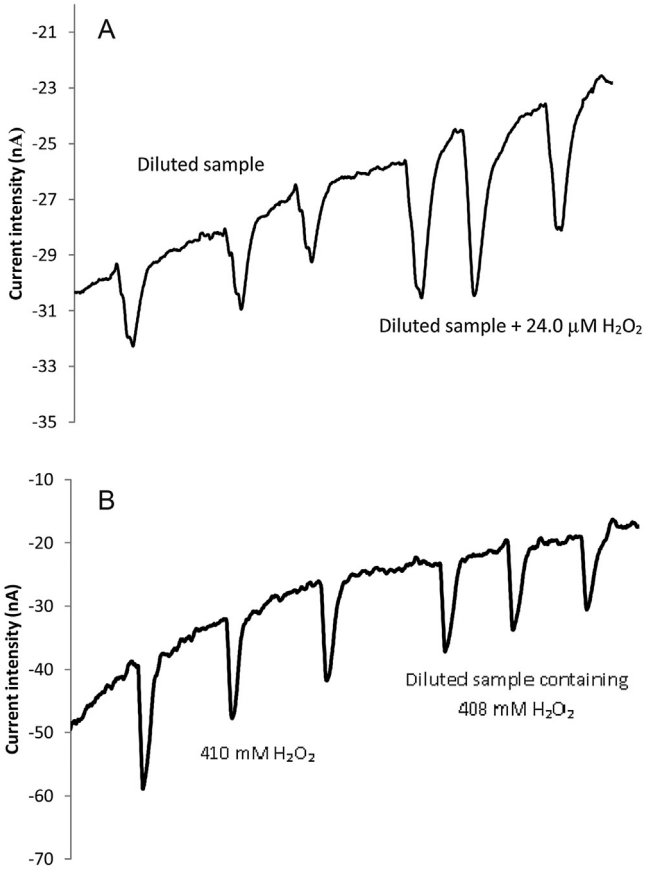


Fig. 6. Amperometric response of the diluted dental bleaching sample, and the diluted sample spiked with 24.0 μM H_2O_2, in the flow system with SBP-*Poly*(EGDE-AA-ANI)/GCE polarized at −100 mV vs Ag/AgCl, using 50 mM PBS pH 7.0 and a flow rate of 0.3 mL min^{-1} (A). Specificity study of the electrochemical response with SBP-*Poly*(EGDE-AA-ANI)/GCE, in batch, using a 10-mL solution of catalase (48 units) in 500 mM KNO_3. Successive 50-μL additions of H_2O_2 standard solution and of diluted sample were made (B).

The specificity of the electrochemical response of this biosensor was also studied in batch in the presence of catalase. The biosensor was polarized at −100 mV vs Ag/AgCl in 10.00 mL of 500 mM KNO_3 solution containing 48 units of catalase, and aliquots of H_2O_2 standard solution in a final concentration of 0.410 mM were added. The current increased during the first 40 s after the standard addition, and returned to the background value one minute later. The experiment was then repeated with diluted sample reaching a final H_2O_2 concentration of 0.408 mM and registering the same pattern. In these cases, the immobilized SBP first reacted with the specific substrate, till it was consumed by the alternative dismutation reaction catalyzed by soluble catalase (Fig. 6B).

3.6. Sensor stability and reproducibility

The sensor storage stability was examined by storing it at 4° C in a wet chamber preventing from contact with water, with intermittent measurements of the response to H_2O_2 standard solution (Fig. 7A). The mean current response of the tested sensor for the detection of 50 μM H_2O_2 over 15 days was 4.53 nA and the S.D. was 0.23 nA (R.S.D.: 5.1%).

The modified electrode fabrication reproducibility was estimated from the current response to 50 μM H_2O_2 of eight different biosensors (Fig. 7B). A mean current response of 4.39 nA was obtained, with a S.D. of 0.27 nA and a R.S.D. of 6.0%. The mean cur-

Table 3
Experimental results of H_2O_2 measurement in dental bleaching sample, using the flow injection system.

	peak current intensity of replicates (nA)			mean peak current intensity (nA)	S.D. (R.S.D.)
Diluted sample	2.357	2.427	2.239	2.341	0.095 (4.1%)
Diluted sample + 24.0 μM H_2O_2	4.753	4.877	4.277	4.636	0.32 (6.8%)

	Quantification by external calibration	Quantification by standard addition
Average H_2O_2 concentration (wt%)	37.6	35.0
S.D. (R.S.D.)	1.6 (4.1)	1.4 (4.1)
Confidence interval upper limit	41.4	38.6
Confidence interval lower limit	33.7	31.5

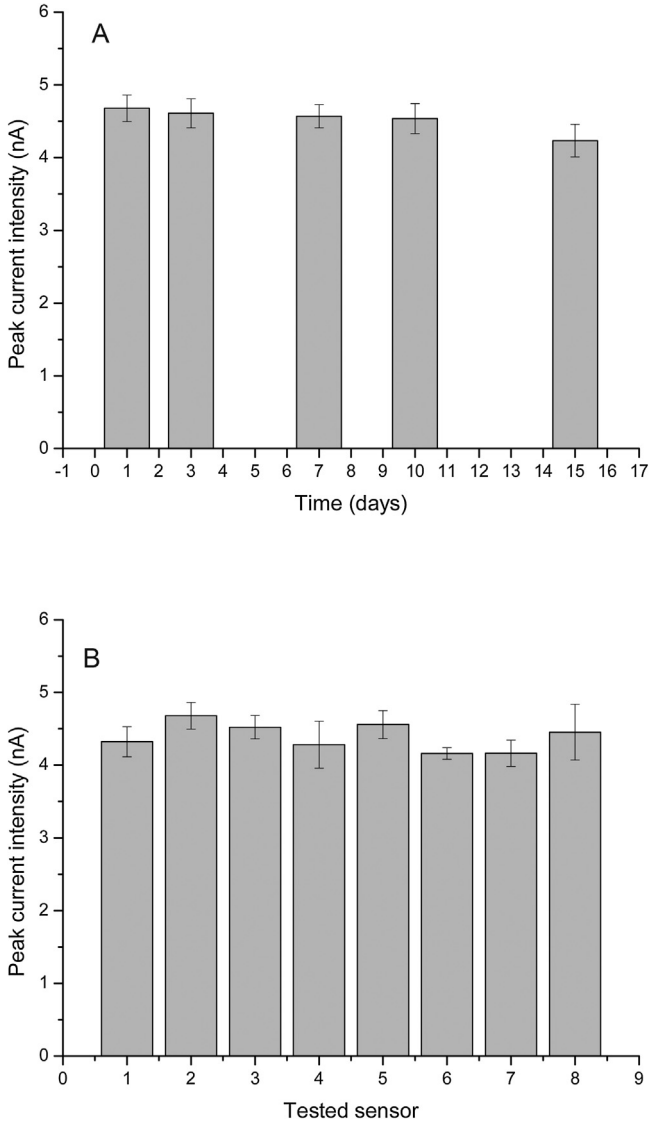


Fig. 7. Reproducibility of one biosensor response as function of time to 50 μM H_2O_2 in the flow system (A), and reproducibility studied on different biosensors (B).

rent response of a sensor inter-days was not significantly different from the mean current response inter-electrodes (*P*: 0.01).

4. Conclusions

In this procedure, the radical polymerization of ANI in acidic medium provided by AA was achieved with H_2O_2, catalyzed by SBP. The presence of EGDE and AA acting as cross-linkers was relevant to create an environment capable of immobilizing both SBP and PANI on the electrode surface. AA underwent polymerization initiated by free radicals from ANI, and some extent of covalent binding to EGDE molecules was expected. Experimental evidence of PANI formation in the presence of polyacrylate and EGDE adducts was obtained from UV–vis and ATR-FTIR spectroscopy.

The electrocatalytic reaction mechanism of H_2O_2 on SBP-*Poly*(EGDE-AA-ANI)/GCE could be resumed as follows. The H_2O_2 reached the electrode modifier where it was enzymatically reduced by SBP to give H_2O and to oxidize the enzyme. SBP was regenerated to the native form by accepting electrons from the reduction state of the polymer, while *Poly*(EGDE-AA-ANI)$_{red}$ itself changed into the oxidation state (*Poly*(EGDE-AA-ANI)$_{ox}$). The oxidized form of the polymer could be recycled to the reduced state at the electrode surface, generating a current signal. In this process, PANI experimented the transformation between the reduced leucoemeraldine (LE) state to the partly oxidized emeraldine state in the salt form (ES), exhibiting high conductivity due to previous protonation by AA, and presenting delocalization of charge all over the chain (polaron lattice).

The SBP exhibited a shift in the optimal pH value from 4.0-5.0 (free) to 7.0 (immobilized). Also the apparent K_m resulted lower, indicating higher affinity for the substrate H_2O_2. Glucose, urea and ascorbic acid did not interfere in H_2O_2 analysis with the modified electrode polarized at −100 mV vs Ag/AgCl.

A commercial sample for dental bleaching could be analyzed either by external calibration or by standard addition. The catalase test indicated that the electrochemical response given by this sample was specific for H_2O_2.

In this way, we demonstrated that a non-expensive enzyme (SBP) could be used as a recognition element and, at the same time, to synthesize both a conducting polymer and a hydrogel, in the assembly of the modifier on an electrode surface for H_2O_2 detection.

Acknowledgements

The authors thank the financial support from Universidad de Buenos Aires (UBACyT 10-12/237, 11-14/915 and 13-16/021), CONICET (10-12/PIP 076), ANPCyT (BID Bicentenario/PICT 1957; PICT 2012 0151 and PICT 2012 0716). Dimas I. Torres thanks the CONICET for his doctoral fellowship.

References

[1] V.F. Lvovich, A perspective on electrochemical impedance analysis of polyaniline films on electrodes, Electrochem. Soc. Interface Fall (2009) 62–66.
[2] G. Inzelt, Conducting Polymers A New Era in Electrochemistry, Springer-Verlag, Berlin Heidelberg, 2008, pp. 15–18.
[3] K.M. Molapo, P.M. Ndangili, R.F. Ajayi, G. Mbambisa, S.M. Mailu, N. Njomo, M. Masikini, P. Baker, E.I. Iwuoha, Electronics of conjugated polymers (I): polyaniline, Int. J. Electrochem. Sci. 7 (2012) 11859–11875.
[4] O.A. Raitman, E. Katz, A.F. Buckmann, I. Willner, Integration of polyaniline/poly(acrylic acid) films and redox enzymes on electrode supports: an *in situ* electrochemical/surface plasmon resonance study of the bioelectrocatalyzed oxidation of glucose or lactate in the integrated bioelectrocatalytic systems, J. Am. Chem. Soc. 124 (2002) 6487–6496.
[5] H.H.S. Javadi, F. Zuo, K.R. Cromack, M. Angelopoulos, A.G. MacDiarmid, A.J. Epstein, Charge transport in the emeraldine form of polyaniline, Synth. Met. 29 (1989) 409–416.

[6] L.A. Samuelson, A. Anagnostopoulos, K.S. Alva, J. Kumar, S.K. Tripathy, Biologically derived conducting and water soluble polyaniline, Macromolecules 31 (1998) 4376–4378.
[7] P. Walde, Z. Guo, Enzyme-catalyzed chemical structure-controlling template polymerisation, Soft Matter 7 (2011) 316–331.
[8] K. Junker, G. Zandomeneghi, Z. Guo, R. Kissner, T. Ishikawa, J. Kohlbrecher, P. Walde, Mechanistic aspects of the horseradish peroxidase-catalysed polymerisation of aniline in the presence of AOT vesicles as templates, RSC Adv. 2 (2012) 6478–6495.
[9] K.F. Fernandes, C.S. Lima, F.M. Lopes, C.H. Collins, Properties of horseradish peroxidase immobilised onto polyaniline, Process Biochem. 39 (2004) 957–962.
[10] P.N. Bartlett, E. Simon, Poly(aniline)-poly(acrylate) composite films as modified electrodes for the oxidation of NADH, Phys. Chem. Chem. Phys. 2 (2000) 2599–2606.
[11] W. Chen, S. Cai, Q.-Q. Ren, W. Wen, Y.-D. Zhao, Recent advances in electrochemical sensing for hydrogen peroxide: a review, Analyst 137 (2012) 49–58.
[12] C. Karunakaran, T. Madasamy, N.K. Sethy, Ed., in: Biosensors and Bioelectronics, Elsevier, Amsterdam, 2015.
[13] X. Wang, W. Qin, Reactive intermediates-induced potential responses of a polymeric membrane electrode for ultrasensitive potentiometric biosensing, Chem. Commun. 48 (34) (2012) 4073–4075.
[14] N.G.R. Mathebe, A. Morrin, E.I. Iwuoha, Electrochemistry and scanning electron microscopy of polyaniline/peroxidase-based biosensor, Talanta 64 (2004) 115–120.
[15] P. Wang, S. Li, J. Kan, A hydrogen peroxide biosensor based on polyaniline/FTO, Sensor. Actuat. B Chem. 137 (2009) 662–668.
[16] J.K. Amisha Kamal, D.V. Behere, Activity, stability and conformational flexibility of seed coat soybean peroxidase, J. Inorg. Biochem. 94 (2003) 236–242.
[17] F. Ghaemmaghami, I. Alemzadeh, S. Motamed, Seed coat soybean peroxidase: extraction and biocatalytic properties determination, Iran. J. Chem. Chem. Eng. 7 (2) (2010) 28–38.
[18] M.L. Magri, M.V. Miranda, O. Cascone, Immobilization of soybean seed coat peroxidase on polyaniline: synthesis optimization and catalytic properties, Biocatal. Biotransform. 23 (2005) 339–346.
[19] B. Wang, B. Li, Z. Wang, G. Xu, Q. Wang, S. Dong, Sol-gel thin-film immobilized soybean peroxidase biosensor for the amperometric determination of hydrogen peroxide in acid medium, Anal. Chem. 71 (10) (1999) 1935–1939.
[20] A. Heller, M.S. Vreeke, Soybean peroxidase electrochemical sensor. Patent US 5665222 A, Date of patent: 9 Sept 1997.
[21] L.R. Denaday, M.V. Miranda, R.M. Torres Sánchez, J.M. Lázaro Martínez, L.V. Lombardo Lupano, V. Campo Dall' Orto, Development and characterization of a polyampholyte-based reactor immobilizing soybean seed coat peroxidase for analytical applications in a flow system, Biochem. Eng. J. 58–59 (2011) 57–68.
[22] L.V. Lombardo Lupano, J.M. Laízaro Martínez, N.M. Vizioli, D.I. Torres, V. Campo Dall' Orto, Synthesis of water-soluble oligomers from imidazole, ethylene glycol diglycidyl ether, and methacrylic acid. An insight into the chemical structure, aggregation behavior and formation of hollow spheres, Macromol. Mater. Eng. 301 (2) (2016) 167–181.
[23] M.V. Miranda, M.L. Magri, R.B. Cabrera, H.M. Fernández Lahore, O. Cascone, Optimisation of preoxidase adsorption on concanavalin A-agarose, Lat. Am. Appl. Res. 33 (2003) 67–71.
[24] E.I. Iwuoha, D. Saenz de Villaverde, N.P. Garcia, M.R. Smyth, J.M. Pingarron, Reactivities of organic phase biosensors. 2. The amperometric behaviour of horseradish peroxidase immobilised on a platinum electrode modified with an electrosynthetic polyaniline film, Biosens. Bioelectron. 12 (8) (1997) 749–761.
[25] N. Pekmez, K. Pekmez, A. Yildiz, Electrochemical behaviour of polyaniline films in acetonitrile, J. Electroanal. Chem. 370 (1994) 223–229.
[26] P.N. Bartlett, E. Simon, Poly(aniline)-poly(acrylate) composite films as modified electrodes for the oxidation of NADH, Phys. Chem. Chem. Phys. 2 (2000) 2599–2606.
[27] J.M. Lázaro Martínez, M.F. Leal Denis, V. Campo Dall' Orto, G.Y. Buldain, F.T.-I.R. Synthesis, solid state NMR and SEM studies of novel polyampholytes or polyelectrolytes obtained from EGDE, MAA and imidazoles, Eur. Polym. J. 44 (2008) 392–407.
[28] H.-Y. Lee, S.-P. Rwei, L. Wang, P.-H. Chen, Preparation and characterization of core-shell polyaniline-polystyrene sulfonate@Fe_3O_4 nanoparticles, Mater. Chem. Phys. 112 (2008) 805–809.
[29] M. Trchová, J. Stejskal, Polyaniline The infrared spectroscopy of conducting polymer nanotubes (IUPAC Technical Report), Pure Appl. Chem. 83 (10) (2011) 1803–1817.
[30] H. Cesiulis, N. Tsyntsaru, A. Ramanavicius, G. Ragoisha, NanoScience and Tecnology, in: P. Avouris, B. Bhushan, D. Bimberg, C.-Z. Ning, K. von Klitzing, R. Wiesendanger, I. Tiginyanu, P. Topala, V. Ursaki (Eds.), Nanostructures and Thin Films for Multifunctional Applications Technology, Properties and Devices, Springer, 2016, 2016, pp. 3–42.
[31] Y. Wang, Y. Hasebe, Carbon felt-based bioelectrocatalytic flow-through detectors: highly sensitive amperometric determination of H_2O_2 based on a direct electrochemistry of covalently modified horseradish peroxidase using cyanuric chloride as a linking agent, Sensor. Actuat. B Chem. 155 (2011) 722–729.
[32] P. Fiordiponti, G. Pistoia, An impedance study of polyaniline films in aqueous and organic solutions, Electrochim. Acta 34 (2) (1989) 215–221.
[33] S.H. Glarum, J.H. Marshall, The impedance of poly(aniline) electrode films, J. Electrochem. Soc. 134 (1987) 142–147.
[34] E.W. Paul, A.J. Ricco, M.S. Wrighton, Resistance of polyaniline films as a function of electrochemical potential and the fabrication of polyaniline-based microelectronic devices, J. Phys. Chem. 89 (1985) 1441–1447.
[35] A.S. Sarac, M. Ates, B. Kilic, Electrochemical impedance spectroscopic study of polyaniline on platinum, glassy carbon and carbon fiber microelectrodes, Int. J. Electrochem. Sci. 3 (2008) 777–786.
[36] D. Yu, B. Blankert, E. Bodoki, S. Bollo, J.-C. Vire, R. Sandulescu, A. Nomura, J.-M. Kauffmann, Sensor. Actuat. B-Chem. 113 (2006) 749–754.
[37] L. Goldstein, in: S.P. Colowick, N.O. Kaplan (Eds.-in-Chief), Methods in Enzymology, K. Mosbach (Ed.), vol. 44: Immobilized Enzymes, Academic Press Inc New York, 1976, pp. 397–450.
[38] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, sixth edition, Pearson Education Limited, 2010.

Biographies

Dimas Ignacio Torres obtained his degree in Pharmacy from University of Buenos Aires, Argentina, in 2014. Currently, he is a PhD student at the Institute of Chemistry and Biological Physicochemistry (IQUIFIB), CONICET, University of Buenos Aires, on a doctoral fellowship from the National Research Council (CONICET). His research is based on the development of novel polymeric materials based on epoxy resins and heterocycles, to be applied as modifiers for electrochemical sensors and as reactants for microencapsulation.

María Victoria Miranda obtained her degree in Biochemistry in 1990. She is Full Professor of Biotechnology at the Faculty of Pharmacy and Biochemistry (FFyB), of the University of Buenos Aires (UBA), and research scientist at the NANOBIOTEC Institute of CONICET. At present, one of her research interests deals with the extraction and purification of soybean peroxidase, a non-expensive by-product of food industry, for multiple applications.

Viviana Campo Dall'Orto obtained her degree in Biochemistry in 1992, and earned a doctoral fellowship from UBA. In 1998 stayed at the University of Florence for eight months to work on electrochemical sensors development. She obtained the PhD title at UBA in 1999 for her research in chemical sensors based on porphyrin films. She is Professor of Analytical Chemistry at FFyB-UBA, and research scientist at IQUIFIB, CONICET. One of her research projects is based on the synthesis of novel polymeric networks as platforms for enzymatic sensors and free-label immunosensors.