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# Amperometric detection of phenolic compounds by polypyrrole-based composite carbon paste electrodes

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**Abstract**

This contribution describes new composite carbon paste electrodes (CPEs) for the determination of phenolic compounds. The composite CPEs were prepared by in situ generation of polypyrrole (PPy) within a paste containing the enzyme polyphenol oxidase (PPO). The best paste composition (enzyme/pyrrole monomer/carbon particles/Nujol) was determined for a model enzyme, glucose oxidase (GOx) according to the enzymatic activity of the resulting electrodes and to the enzyme leakage from the paste during storage in phosphate buffer. The in situ electrogenerated PPy improves the enzyme immobilisation within the paste since practically no enzyme was lost in solution after 72 h of immersion. Moreover, the enzyme activity remains particularly stable under storage since the biocomposite structure conserves 80% of its activity after 1 month of storage. Following the optimisation of the paste composition, PPO-based carbon paste biosensors were prepared and presented excellent analytical properties toward catechol detection with a sensitivity of 4.7 A $M^{-1}$ $cm^{-2}$ and a response time lower than 20 s. The resulting biosensors were applied to the determination of polyphenolic compounds (e.g., epicatechin and ferulic acid).


*Keywords:* Carbon paste electrodes; Polypyrrole; Enzymatic biosensors; Polyphenol oxidase; Flavonols

Carbon paste electrodes (CPEs) have been extensively used for electroanalytical applications since their introduction by Adams in 1958 [1] due to their intrinsic advantages including low background current, renewable or disposable electrochemical interface and low cost of fabrication [2]. More especially, these electrode materials, owing to their mild fabrication process consisting basically in blending intimately at room temperature a carbon powder with a hydrophobic binder, have been involved in the design of amperometric biosensors through the incorporation of biological redox catalysts such as plant tissues [3] or enzymes [4]. In the latter case, a wide variety of additive materials or molecules can also be incorporated within the paste during the fabrication process to mediate the enzyme reaction, to stabilise the biomolecule activity or to improve the sensor selectivity. However, CPEs, based on the physical entrapment of enzyme molecules, exhibit severe limitations due mainly to their weak mechanical stability, which produces enzyme leakage in the solution of analysis, and to their fabrication reproducibility [5]. In an effort to maintain the carbon paste integrity, some works have proposed the coating of the carbon paste interface by a semi-permeable membrane allowing substrate exchange such as Nafion gel [6] or a poly(*o*-phenylene diamine) film [7] for example. Besides the extension of the biosensor dynamic range, owing to substrate diffusional control, this methodology eliminates the interface renewability and lowers dramatically the biosensor sensitivity. Then the in situ modification of the carbon paste by polymeric structures acting as rigid binders [8] has been developed to circumvent these drawbacks. These composite biomaterials present an improved mechanical stability of the paste as well as the intrinsic advantage of surface regeneration of classical carbon pastes [9]. However, the fabrication of these materials involves chemical reactions or heating processes that can deactivate

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the biological activity of the enzyme or denature the protein. In this context, we report here a new approach for the soft fabrication of rigid biocomposites based on the in situ electrochemical generation of a polymer network. The rigidifying process results from the electrochemical polymerisation of pyrrole monomer previously incorporated within the paste. This new carbon paste hardening concept has been applied to the entrapment of glucose oxidase (GOx) as enzyme model within the biocomposite structure to assess the efficiency of the immobilisation process and to investigate the influence of an immobilised protein on the physico-chemical properties of the composite. Then, tyrosinase, a polyphenol oxidase (PPO) enzyme, was incorporated within the paste for the design of a phenolic biosensor focused on the determination of flavonols.

## 1. Experimental section

### 1.1. Chemicals

All chemicals are of the highest analytical grade and used as received. Aqueous solutions were prepared in deionised water prepared by passing distilled water through an Elga Prima/Maxima purification system. Carbon pastes are made with graphite powder (BDH) and Nujol (BDH). Pyrrole monomer and ferrocene carboxylic acid were purchased from Aldrich. GOx (E.C.1.1.3.4, type VII, from *Aspergillus niger*, 168 Sigma units/mg), PPO (EC1.14.18.1, from mushroom, 123 units/mg), phenol, catechol, ferulic acid and (−)-epicatechin were purchased from Sigma. All buffers were prepared from potassium monobasic and dibasic phosphate salts (Aldrich).

### 1.2. Apparatus

Electrochemical characterisation and analytical testing were carried out in a one-compartment electrochemical cell using a carbon paste electrode as the working electrode and a platinum foil as the counter electrode. All potentials were measured and reported with respect to a saturated calomel electrode (SCE). A water jacket, thermostatically controlled (thermostat liquitherm F) at $25 \pm 0.2$ °C, was used for the characterisation and analytical evaluation of the biosensor. All other electrochemical measurements were carried out at room temperature.

An Autolab PGStat 20 Ecochemie was used for AC Impedance and other electrochemical measurements. Impedance spectra were fitted using the software EQUIVCRT. An electrochemical equipment constituted by a PAR Model 173 potentiostat equipped with a model 179 digital coulombmeter and a model 175 programmer in conjunction with a Sefram TRP recorder was also used for the in situ electrochemical polymerisation of pyrrole. The electropolymerisation processes were performed in a three-electrode cell of 5 ml.

### 1.3. Methods

#### 1.3.1. CPEs fabrication

The carbon pastes employed for the fabrication of CPE, polypyrrole CPE (PPy-CPE) and biocomposite CPE were prepared as follows: Carbon paste was prepared by mixing graphite powder with Nujol in a mortar until it was uniformly wetted using a graphite/Nujol ratio of 4/1 in weight. This ratio was employed as it provides convenient analytical properties [3]. Pyrrole monomer was incorporated in the carbon paste using a pyrrole ratio of 10% in weight. The enzymes (GOx or PPO) were mixed with the pure and pyrrole modified carbon pastes with ratios of protein ranging from 1% to 6% in weight.

After blending, the pastes were packed into a Teflon electrode holder (geometric surface area 0.07 $cm^2$) with electrical contact made via a vitreous carbon rod. The electrode surface was smoothed on a paper to produce a reproducible working surface.

PPy-CPE electrodes were prepared by electropolymerisation of a polymer membrane on the aforementioned pyrrole-free carbon paste enzyme mixture. The electrosynthesis was performed in a solution containing $LiClO_4$ (0.1 M) and pyrrole (25 mM) by cycling the potential seven times between $-0.3$ and 0.9 V/SCE at a scan rate of 50 mV $s^{-1}$. Biocomposite electrodes were fabricated by in situ electropolymerisation of the pyrrole monomer mixed within the paste in a $LiClO_4$ (0.1 M) solution. The electropolymerisation process was performed either by cycling the potential between $-0.3$ and 0.9 V/SCE at a scan rate of 50 mV $s^{-1}$, or by applying a fixed potential of 0.77 V/SCE to zero current.

#### 1.3.2. AC impedance

Impedance characterisations were carried out in a 1-mM ferrocene carboxylic acid potassium phosphate buffer solution (0.05 M, pH 7.4). A frequency range of 65,000–0.1 Hz was utilised with a potential amplitude of 5 mV rms. A bias of 0.3 V/SCE was used in order to study the electrode reaction at the half wave potential of the oxidation of ferrocene carboxylic acid.

#### 1.3.3. Scanning electron microscopy (SEM)

Microstructure characterisation of the carbon paste electrode surface was performed using a Hitachi S-3200 N low vacuum scanning electron microscope. A working distance of 8 mm was used with 20.0 kV at a magnification of 1200×.

#### 1.3.4. Determination of the amount of enzyme released under storage

The three enzyme electrodes (CPE, PPy-CPE and biocomposite CPE) were stored in a 0.1 M phosphate buffer (pH 7) solution during 72 h. The quantity of enzyme released from the paste in the storage solution was estimated periodically through the enzymatic activity of this solution

measured by spectrophotometry as previously described [10]. The release experiments were carried out with GOx as an enzyme model because of its robustness.

*1.3.5. Amperometric analysis*

The amperometric measurements of PPO substrates were performed in stirred air-saturated phosphate buffer 25 $cm^3$ (0.05 M, pH 7.4) by holding the carbon paste electrodes at a potential of $-0.2$ V that has been reported as the optimum operating potential for PPO based biosensors [11]. Various phenolic compounds from $10^{-3}$ to 1 M stock solutions were added to this stirred solution and the current corresponding to the reduction of the enzymatically generated *o*-quinones was recorded.

## 2. Results and discussion

In situ modification of the carbon paste structure was effected via the electropolymerisation of previously mixed pyrrole monomer (10% in weight) with the carbon powder, enzyme and Nujol blend. Then, we have examined the polymer electrogeneration by cycling repeatedly the potential between $-0.2$ and 0.85 V/SCE (not shown). Despite the dissolution of the monomer units within the pasting liquid, the electropolymerisation process took place at the surface and in the subsurface of the paste leading to the generation of a rigid carbon road. This electropolymerisation process was electrochemically visualised through the growth of the polypyrrole redox wave over cycle repetition. Then electropolymerisation process was optimised and he PPy generation was carried out by applying a fixed potential of 0.77 V/SCE to zero current. The obtained composite structures were assessed in terms of electrical and structural properties by impedance spectroscopy and SEM. Fig. 1 displays the impedance spectra of an unmodified and of the composite carbon pastes. Moreover, in order to proof the presence of the polymeric structure within the carbon paste, the aforementioned material was compared to a carbon paste coated by a polypyrrole film.

The analysis of the impedance data was performed using an equivalent circuit model where the total series resistance of the system ($R_s$) is in series with a parallel combination of a charge-transfer resistance ($R_{CT}$) and a Constant Phase Angle impedance ($Z_{CPA}$). The resistance, $R_{CT}$, is thought to represent charge-transfer resistance across the interface. Due to the irregular structure and surface roughness of carbon paste electrodes, an empirical Constant Phase Angle impedance ($Z_{CPA} = K(J\omega)^{-\beta}$) is used to represent the double layer capacitance [12], where $K$ is a measure of the magnitude of the non-faradic impedance and the exponent, $\beta$, reflects the extent of deviation from an ideal capacitive behaviour.

After modelling the low-frequency arc by the equivalent circuit model described above, the results indicate a decrease in charge-transfer resistance from CPE (14.4 kΩ) to biocomposite CPE (6.65 kΩ). Furthermore, an increase in $K$ was

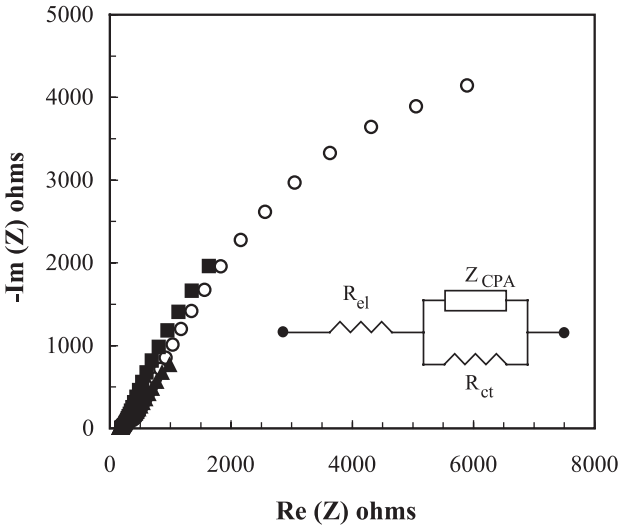


Fig. 1. AC impedance spectra of GOx-based CPEs : (○) CPE, (■) PPy-CPE and (▲) biocomposite CPE obtained for CPEs containing 6% in weight of enzyme. The impedance spectra was recorded within the frequency range 65 kHz to 0.1 Hz at a potential bias of 0.3 V/SCE in $10^{-3}$ M ferrocene-buffered solution.

observed from bare to biocomposite CPEs, 2.7 and 22 μF, respectively, with $\beta$ indicating a mixture of Warburg-like diffusion and capacitive behaviour. The impedance results would suggest that the presence of the conducting polymer has improved the electrical properties of the fabricated electrodes, since a decrease in charge-transfer resistance was noted. Such a decrease in charge-transfer resistance can be related to an improvement in electrical connection between the graphite grains and an enhancement in electroactive surface area due to the presence of the polymer. Otherwise, for the biocomposite CPE, the phase angle decreased. This decrease in phase angle can be related to the presence of the polymer throughout the paste, which provides a conducting pathway between the graphite grains. This hypothesis is corroborated by the comparison of SEM morphologies of the different enzyme electrode configurations (Fig. 2). The surface topography for a CPE (Fig. 2A) shows a granular surface with areas of high charging. This charging effect decreases for PPy-CPE (Fig. 2B) and is virtually absent for the biocomposite CPE (Fig. 2C), which exhibits a homogeneous surface. As charging can be related to the insulating properties of the sample under observation [13], the resistance of each sample can be qualified and compared. Thus, a CPE (Fig. 2A) is more resistive compared to a biocomposite CPE (Fig. 2C), which is in agreement with impedance measurements.

Since polypyrrole allows to improve the electrical properties of the carbon paste material, we have assessed the impact of the in situ generated polymer onto the enzyme retention for GOx as model enzyme. Fig. 3 presents the evolution of the enzyme release with time for the three biomaterials, soaking in phosphate buffer solution, in the case of an enzyme loading of 6% in weight.

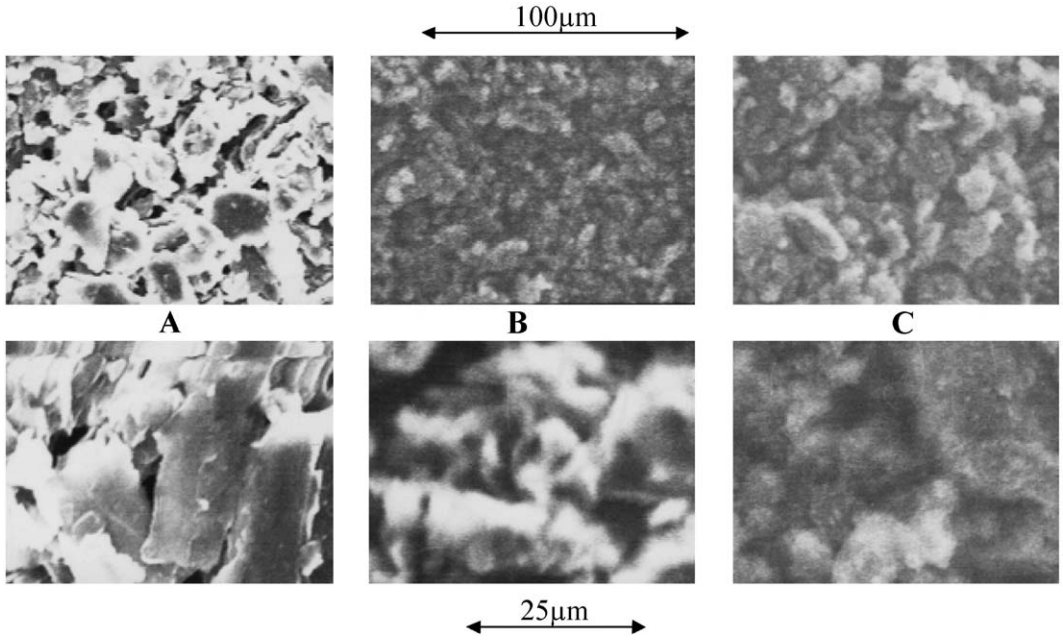


Fig. 2. SEM micrographies of CPE (A), PPy-CPE (B) and biocomposite CPE (C) containing 6% in weight of GOx.

It clearly appears in Fig. 3 that the presence of polypyrrole limits in a large extent the enzyme release since after 2 h of soaking the released quantity of GOx determined for the biocomposite CPE and the PPy-CPE are respectively equal to 0 and 3.5 µg, whereas the level of released enzyme reaches 45 µg for the unmodified CPE. For longer soaking duration, the amount of GOx lost in solution by the biocomposite CPE remains extremely low reaching only a level of 0.1 µg after 72 h soaking, whereas the PPy-CPE configuration leads to 150 µg. Such result highlights the improvement in the enzyme retention brought by the in situ electropolymerised polypyrrole compared to PPy film generated at the electrode surface. This correlates the electrochemical and structural characterisations that demonstrated the presence of electrogenerated PPy in the bulk of the carbon paste material. In addition, the storage stability of the biocomposite CPE based on an enzyme ratio of 6% and stored dry at −4 °C was investigated. The electrode activity remained stable 3 days without any loss of activity while a loss of activity lower than 20% was recorded after 30 days of storage.

The aforementioned characterisations have demonstrated the reliability of this new biocomposite material in terms of

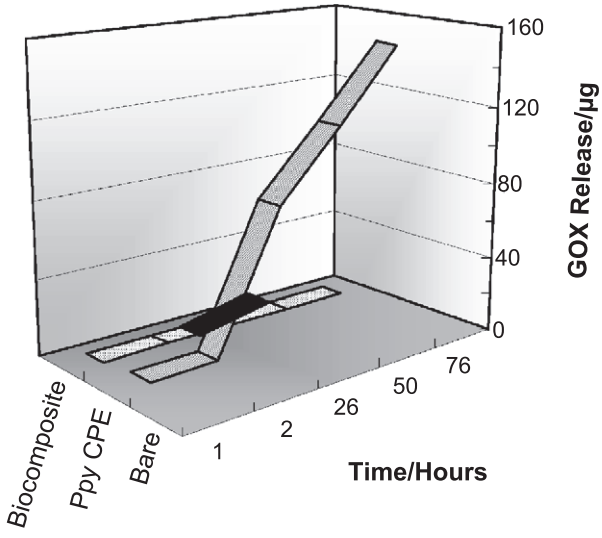


Fig. 3. Estimated quantities of GOx released in phosphate buffer versus time for the CPE (A), PPy-CPE (B) and biocomposite CPE (C). The experiments were performed at room temperature in 3 ml of 0.1 M phosphate-buffered solution (pH 7).

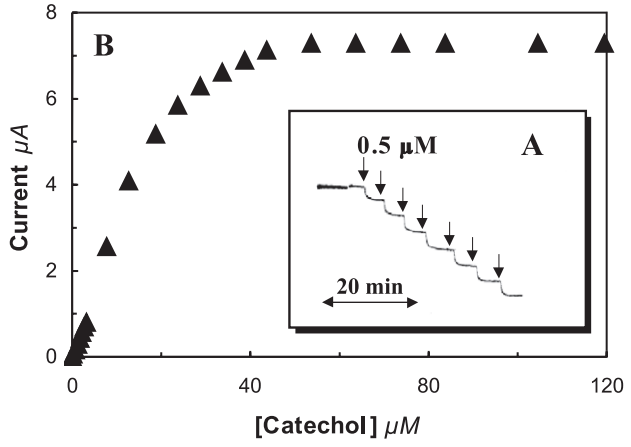


Fig. 4. Steady-state current-time response of the biocomposite CPE (A) for increasing catechol concentrations in $5 \times 10^{-7}$ M steps. Calibration curves for catechol obtained biocomposite CPE (B, ▲). Amperometric responses recorded at −0.2 V/SCE in stirred air-saturated 0.1 M phosphate buffer at 25 °C with CPEs containing 6% in weight of PPO.

electrical properties and enzyme retention. Then, such a material has been utilised for the fabrication of a PPO-based biosensor dedicated to the detection of flavonols.

PPO catalyses the oxidation of phenol and catechol derivatives, such as flavonols, in the presence of oxygen. Then, we have fabricated a PPO biosensor using an enzyme load of 6% in weight. The detection of catechol was effected in stirred air saturated 0.1 M pH 7 PBS at a potential of − 0.2 V/SCE through the electrochemical reduction of the enzymatically produced quinone. It is important to note that following this electrochemical reduction, the obtained hydroquinones may enter in a new biocatalytic cycle leading to a possible signal amplification. Fig. 4B presents the calibration curve for catechol obtained at the biocomposite CPE as well as the steady-state current-time response of the biocomposite CPE to successive injection of catechol (Fig. 4A). It appears in Fig. 4A that the amperometric response of the obtained biosensor is rapid since 95% of the steady-state deviation was reached within 20 s. These increments in catechol concentration are within the concentration range corresponding to the linear part of the calibration curve and hence illustrate the repeatability of the recorded signal (RSD = 2.1%). The electroanalytical characteristics of the biocomposite CPE were extracted from Fig. 4B. Then, the sensitivity of the biocomposite CPE to catechol is equal to 4.75 A $M^{-1}$ $cm^{-2}$ and is 5.2 times higher than such recorded for PPy-CPE configuration (data not shown). This sensitivity is comparable to and three times higher than such obtained for biosensors based on the entrapment of PPO within PPy films generated at the surface of carbon screen-printed electrodes [14] and platinum electrodes[15], respectively. Otherwise, $J_{max}$, estimated to 103 μA $cm^{-2}$, is 3.3 times larger than such obtained for the PPy-CPE electrode. On the contrary, the dynamic range of the PPy-CPE electrode is about two times larger than such obtained for the biocomposite CPE. These results correlate with the ac impedance data proving that in situ PPy was generated evenly in the carbon paste and acts as a polymeric binder. The recorded $K_M^{app}$, estimated by considering the catechol concentration at which the biosensor response is half of the saturation value, is lower than that obtained for the free enzyme [16]. Such behaviour is classically observed in the case of PPO based biosensors and could be related to the aforementioned amplification phenomenon. Finally, the obtained biosensor was utilised for the detection of different substrates including phenol, epicatechin, as flavonol model, and ferulic acid as polyphenol model. The obtained relative sensitivities, reported for catechol, are displayed in Fig. 5.

Indeed, the oxidation rate of phenol is approximately 40% lower than that of catechol for the free enzyme. The sensitivity of the biosensor is lowered for phenol compared to catechol from approximately 38%. Such behaviour shows that the immobilised enzyme retains its substrate specificity when entrapped within a biocomposite CPE. The substitution on the phenolic ring lowers the biosensor performances for ferulic acid and epicatechin determination as it reduces

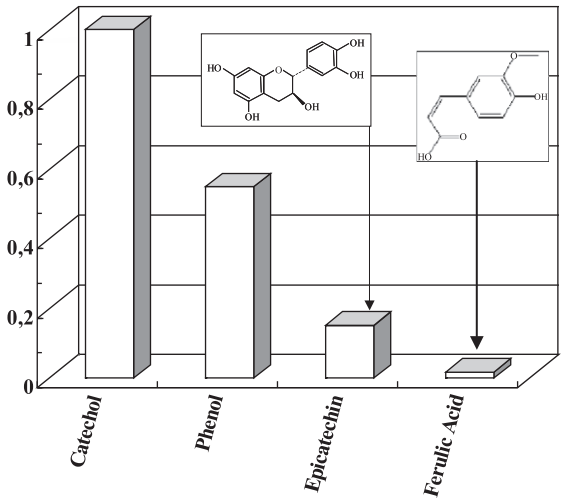


Fig. 5. Relative sensitivities of the biocomposite CPE to phenol, epicatechin and ferulic acid reported to catechol as standard. Experimental conditions as in Fig. 4.

the enzyme activity [17]. The biosensor, nevertheless, exhibits fast response time for all substrates and shows sensitive detection limits for epicatechin and ferulic acid, namely 15 and 200 nM, respectively. Fig. 5 clearly highlights the higher sensitivity of the biocomposite CPE toward the flavonol model than toward the polyphenol one. According to these encouraging results, further polyphenol and flavonol present in beers will be tested in order to evaluate the effectiveness of such a biocomposite sensor for the detection in real samples of flavonols that are responsible of cloudiness in beers [18].

## 3. Conclusion

This contribution has described a new concept of carbon paste modification enabling improved enzyme retention. This modification consists on the bulk modification of the carbon paste by an electrogenerated polypyrrole structure. The obtained biosensors are fast responsive and show a good operational stability since its catechol sensitivity remained constant after five consecutive assays (including stabilisation, sensing and washing process). Associated to a good storage stability. Finally, the potentialities of the biosensor for flavonol determination were investigated, and further works are currently under way for the quality control of real beers.

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