



Biosensor based on laccase immobilized on plasma polymerized allylamine/carbon electrode

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ABSTRACT

In this work, a simple and rapid method was used to functionalize carbon electrode in order to efficiently immobilize laccase for biosensor application. A stable allylamine coating was deposited using a low pressure inductively excited RF tubular plasma reactor under mild plasma conditions (low plasma power (10 W), few minutes) to generate high density amine groups (N/C ratio up to 0.18) on rough carbon surface electrodes. The longer was the allylamine plasma deposition time; the better was the surface coverage. Laccase from *Trametes versicolor* was physisorbed and covalently bound to these allylamine modified carbon surfaces. The laccase activities and current outputs measured in the presence of 2,2'-azinobis-(3-ethylbenzothiazole-6-sulfonic acid) (ABTS) showed that the best efficiency was obtained for electrode plasma coated during 30 min. They showed also that for all the tested electrodes, the activities and current outputs of the covalently immobilized laccases were twice higher than the physically adsorbed ones. The sensitivity of these biocompatible bioelectrodes was evaluated by measuring their catalytic efficiency for oxygen reduction in the presence of ABTS as non-phenolic redox substrate and 2,6-dimethoxyphenol (DMP) as phenolic one. Sensitivities of around $4.8 \mu\text{A mg}^{-1} \text{L}$ and $2.7 \mu\text{A mg}^{-1} \text{L}$ were attained for ABTS and DMP respectively. An excellent stability of this laccase biosensor was observed for over 6 months.

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1. Introduction

Biosensors are designed to detect and/or quantify target molecules such as those for medical use and environmental monitoring [1–4]. Various biological recognition elements, including cofactors, enzymes, antibodies, microorganisms, organelles, tissues, and cells from higher organisms, have been used in the fabrication of biosensors [5,6]. Due to their unique specificity and sensitivity, enzymes are the most widely used recognition elements [7].

In the case of enzyme based biosensors, the enzymatic immobilization procedure is an important aspect to enhance the overall operational performance. An optimal immobilization procedure should ensure activity and stability of the protein and, at the same time, provide a good accessibility of substrate to the active site of the enzyme [8–10]. Three immobilization approaches have been mainly reported, including physical adsorption, entrapment and covalent bonding [11]. From the stability and reactivity point of view, immobilization through covalent bonding seems to be the best way of enzyme

attachment [6,9]. However, this immobilization procedure requires proper functional groups on the biosensor material surface. Carboxyl, hydroxyl, amine and aldehyde groups are the main chemically reactive groups suitable for the covalent immobilization of enzymes. To generate such groups, various surface modification techniques such as dipping, spin coating, electrochemical and self-assembled monolayer (SAM) deposition have been reported [12–15]. Among them, the plasma polymerization technique has gained considerable popularity since it is a one-step environmentally friendly dry process which allows the functionalization of the outermost surface layer of the material with a great variety of functional groups whose density can be monitored by tuning the different plasma parameters (plasma power, precursor flow rate, treatment duration, etc.) [16,17]. A number of monomers have been plasma polymerized for biosensor application but the aminated ones are preferred because of their high reactivity [18]. Among them, allylamine is a very popular monomer for providing surface amino groups using plasma methods [19]. Due to their biocompatibility, the plasma-polymerized allylamine (PPAA) coatings are also widely used in biomedical applications [20]. Allylamine contains a carbon-carbon double bond susceptible to polymerization through plasma activation [19,21]. Due to the fact that the plasma polymerization process involves fragmentation and reorganization in the presence of highly energetic active species, the control

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of the plasma parameters during the deposition of allylamine is the central issue for the amount of amine functions grafted to the surface [22]. The aminated surfaces have dual characteristics; one is their hydrophilicity, which allows the dense loading of biological components onto the modified surface and the other is their reactivity, allowing covalent immobilization to prevent leaching of immobilized biocatalysts.

The development and the performance of biosensors depend also on the physicochemical characteristics of the materials employed for the construction of the transducer and the matrices used for the enzyme immobilization. The most common transducers employed are either inert metals, such as platinum or gold [23–25]. Recently, less expensive materials e.g. carbonaceous materials such as graphite, carbon fibers, porous carbon and glassy carbon, carbon spheres and nanotubes have gained increasing interest. These materials have a high chemical inertness and provide a wide range of working potentials with low electrical resistivity. In addition they exhibit reproducible electrochemical behavior, ability for surface regeneration, as well as a very low cost [26]. Many studies dealing with plasma deposition of allylamine on different materials (polymers, metals, etc.), for subsequent biomolecules immobilization, have been published [27–31], however only a few recent papers report on the PPAA deposition on carbon material [16,32–34] mainly for composite material application. In addition, few studies report on the measurement of the enzyme activity covalently immobilized after allylamine plasma deposition on polymeric materials [28–31]. Moreover, the long term durability of the immobilized biocatalyst depends on the plasma deposited carrier material stability. This latter property depends mainly on the allylamine plasma deposition conditions which are generally not fully optimized and rarely tested under biological practical conditions [27,35]. Mainly PPAA aging under air is studied [36]. Few others studied the PPAA stability in solvents [27,37]. In the present study, therefore, plasma polymerization of allylamine has been used to introduce amine groups on spectroscopic carbon electrode surface for subsequent covalent immobilization of laccase from *Trametes versicolor*. The stability in water of the deposited coating has also been optimized. To deposit the allylamine coating, a low pressure inductively excited radio frequency (RF) tubular plasma reactor was employed. The plasma power was first optimized to obtain a stable PPAA coating with good amine retention. Then, the effect of the plasma treatment duration on the PPAA coating thickness, wettability and chemistry was evaluated. Purified laccase from *T. versicolor* was then physically adsorbed or covalently bound to these allylamine modified carbon surfaces. The effect of the laccase immobilization methods (physical adsorption/covalent) on the laccase biosensor efficiency was also investigated by measuring both the biocatalytic laccase activity and the current output in presence of 2,2'-azino-bis-(3-ethylbenzothiazole-6-sulfonic acid) (ABTS) as substrate. The sensitivity of these electrodes towards a non-phenolic mediator (ABTS) and a phenolic one (2,6-dimethoxyphenol (DMP)) was evaluated for covalently immobilized laccase PPAA carbon biosensors by amperometry.

2. Materials and methods

2.1. Materials

In this work, 7 mm diameter spectrographic carbon-graphite rods (Mersen, France) were used as electrode material. Prior to surface modification the carbon electrodes were ground with SiC paper (Buehler, Germany) with grit sizes 80, cleaned with Milli-Q water and dried by filtered compressed air. The obtained carbon surface roughness (R_a) is around 2500 nm (± 200). Polished silicon wafers (100) ($R_a = 1$ nm) were purchased from Siltronix, France and were used after ultrasonically cleaned in acetone bath for 30 min and rinsed with ethanol for 30 min.

Allylamine ($\text{CH}_2=\text{CHCH}_2\text{NH}_2$, purity = 98%, mol.wt. = 57.09) was purchased from Sigma Aldrich, France and used in this study

without further purification. Argon gas (Air Liquide, purity >99.9%) was used as a carrier gas. The reagents 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were purchased from Sigma-Aldrich. N-hydroxysuccinimide (NHS) was from Fluka. Buffer solutions were prepared with sodium phosphate (Acros Organics), sodium dihydrogen phosphate, sodium acetate and acetic acid (Prolabo) in deionized water (Milli-Q grade, Millipore). NaClO_4 (Sigma-Aldrich) was used as electrolyte.

2.2. Enzyme

Laccase was produced from *T. versicolor* (ATCC 32745). The bioreactor (5 L) was inoculated with pounded mats obtained as previously described [38]. Laccase production was induced with 2,5-xyldine (0.2 mM) at the beginning of the culture. Seven-day-old culture liquid was filtered through a glass wool to eliminate the mycelium. Extracellular polysaccharides were precipitated with 10% acetone, separated by successive filtrations (final porosity 0.22 μm). The filtrate was concentrated by ultrafiltration (Millipore YM10, cut-off 10 000 Da), and laccase was recovered in 20 mM citrate-phosphate buffer (CPB) pH 5 by diafiltration using the same membrane. The enzyme was then applied to an anion exchange column (Sephacrose Q, GE Healthcare) equilibrated in the same buffer, and then the active retained fractions onto a phenyl Sepharose column. Combined active retained fractions were pooled and concentrated by ultrafiltration on YM 30, then dialyzed against 50 mM phosphate buffer at pH 6.8. After addition of glycerol (15% w/v final), aliquots of this purified laccase (around 900 U mL^{-1} and 3 mg total protein mL^{-1}) were stored at -20°C .

2.3. Allylamine plasma polymerization

Plasma polymerization of allylamine was performed in a home-made low pressure inductively excited radio frequency tubular quartz plasma reactor system (5 cm diameter, 40 cm length, base pressure of 0.03 mbar). The schematics of plasma deposition set-up and technical details of the process have been provided in our earlier work [39,40]. Briefly, prior to each experimental run, the reactor was scrubbed and cleaned with detergent, organic solvents and dried using compressed air. The plasma reactor system was reassembled and cleaned further with 20 W argon plasma discharge at 0.5 mbar pressure for 30 min. The power was generated by a Dressler Cesar RF generator which was delivered through the L-C matching network. The substrate is placed 9.0 cm below the coil. The plasma reactor was connected to the single stage rotary pump (Pfeiffer vacuum) via a chemical filter trap (Edwards high vacuum, Britain). The base pressure and operating pressure were 0.03 mbar and 0.5 mbar respectively. The partial pressure of monomer feed was controlled by flow rate of carrier gas (i.e. argon), which was regulated and measured by electronic mass flow controllers (MKS instruments).

2.4. Characterization of plasma allylamine coating

2.4.1. Surface wettability/stability

To test the stability of the PPAA coatings, the surface water contact angle (WCA) was measured on allylamine plasma coated silicon wafer before and after soaking in water for 30 min and quick air drying. The sessile drop contact angle values were measured by a video capture apparatus (Digidrop GBX-3S system, France). For each measurement, 6 μL deionized water droplets was dispensed onto the sample surface. The reported water contact angle values correspond to the average of three measurements, performed on different parts of the samples.

2.4.2. PPAA coating thickness

To determine the PPAA coating thickness, the silicon wafer was partially masked using a tape prior to plasma deposition. The mask

was peeled off to obtain a sharp edge of plasma allylamine coating and the PPAA thickness was determined using the atomic force microscopy (AFM) in tapping mode.

2.4.3. Fourier transform infrared (FT-IR) spectroscopy

Silicon wafers were used as deposition substrates for FT-IR analysis employing a Fourier transform infrared spectrometer (Bruker-Tensor 27 FT-IR spectrophotometer) in the transmission mode. The FT-IR spectra were recorded with the resolution of 4 cm^{-1} and averaged with 64 scans.

2.4.4. X-ray photoelectron spectroscopy (XPS)

The analysis of the samples was carried out in a VG Microlab 310-F electron spectrometer at base pressures, in the preparation and analysis chambers, of 2×10^{-8} and 1×10^{-8} Pa, respectively. The photoelectrons were excited with an X-ray source using $\text{MgK}\alpha$ ($h\nu = 1253.6\text{ eV}$) and the pass energy of the analyzer was 20 eV yielding a resolution of 1.1 eV. The C1s, N1s & O1s peaks were recorded along with 50–1000 eV survey scans. The intensities of the peaks were determined as the integrated peak areas assuming the background to be linear.

2.5. Laccase immobilization on the electrodes

The laccase was physically adsorbed and covalently immobilized on PPAA coated rough carbon electrodes. For covalent immobilization and in order to activate the carboxylic groups of the laccase, a mixture EDC/NHS was used. 50 μL of laccase solution ($0.72\text{ }\mu\text{g }\mu\text{L}^{-1}$) prepared in 10 mM phosphate buffer, (pH 7.0) was mixed with 22.5 μL of a 20 mM NHS solution (in 10 mM phosphate buffer, pH 7.0) and 27.5 μL of a 40 mM EDC solution (in 10 mM phosphate buffer, pH 7.0). The activation was allowed for 20 min. Then covalent immobilization of the enzyme was allowed to carry on during 2 h at ambient temperature. Finally, the modified electrode was soaked 6 times in a phosphate buffer (10 mM, pH 7.0) for 30 min with stirring to remove loosely bound protein until no laccase activity was detected in the washing solution. The same procedure in the absence of EDC/NHS was performed to immobilize laccase by physical adsorption. For all the experiments, tests were carried on physisorbed laccase electrodes (2 samples) and covalently immobilized laccase electrodes (3 samples) and repeated at least three times.

2.6. Laccase electrode performance determination

2.6.1. Determination of the immobilized laccase activity

The amount and activity of the immobilized laccase were spectrophotometrically determined at 420 nm with ABTS as the substrate. The electrode was soaked into 2 mL of citrate phosphate buffer (25 mM, pH = 3.0) containing 1 mM ABTS, at 30 °C. The formation of ABTS radicals was recorded by measuring the optical density (OD) at 420 nm during 1 min using a Varian Cary Spectrophotometer. The concentration of ABTS radical in the solution, c (M), was calculated using the Beer Lambert relationship: $\text{OD} = \epsilon l c$ where OD is the measured absorbance (no unit), $\epsilon_{420\text{ nm}}$ (the extinction coefficient measured at 420 nm) = $36,000\text{ M cm}^{-1}$ and the absorption path length $l = 1\text{ cm}$. The immobilized laccase activity Ac (U Laccase), was then determined as follows: $\text{Ac} = 5.56 \cdot 10^{-2} \Delta(\text{OD}) / \Delta t$ with $\Delta(\text{OD})/\Delta t$: being the slope of absorbance as a function of time (min). This corresponds to the immobilized laccase activity defined as the number of μmol of ABTS radical transformed in 1 min. One unit of activity (U) is the amount of enzyme that catalyzed the formation of 1 μmol of ABTS radical/min.

2.6.2. Electrochemical measurements

Cyclic voltammetry and amperometric measurements were carried out with a Princeton Applied Research Model 263A Potentiostat/

Galvanostat in an electrochemical cell using three electrodes: the carbon working electrode, a saturated calomel electrode (SCE) that was used as a reference and a platinum wire as the counter electrode. The three electrode system was placed in 30 mL of 50 mM acetate buffer, pH 4.2, containing 100 mM NaClO_4 .

In order to study the effect of the PPAA deposition duration on the current output of the laccase immobilized biosensor, CV was recorded in the presence of 1 mmol/L (500 mg/L) of ABTS [9] at room temperature. For all these measurements, the solutions were deaerated for 15 min with pure N_2 gas which was kept flowing over the solution during the electrochemical measurements in the absence of oxygen. The potential was cycled between 0.9 and -0.3 V (vs SCE) at 10 mV/s. The solution was then aerated for 10 min and cyclic voltammetry response in the presence of dioxygen was recorded in the same conditions. All the tests were conducted at room temperature. The current output values were determined at a potential of 0 V (vs. SCE) after deduction of the current recorded at the same potential under N_2 gas.

The analytical characterization of the covalently immobilized laccase PPAA carbon biosensors (30 min) for two substrates (ABTS and DMP) was performed by batch amperometry at fixed potential ($E_{\text{ABTS}} = 0\text{ V}$ and $E_{\text{DMP}} = -0.1\text{ V}$) in the same acetate buffer solution (pH 4.2, room temperature).

The laccase activity and electrochemical measurements were carried out on physically adsorbed laccase electrodes (2 samples) and covalently immobilized laccase electrodes (3 samples) and the measurements were reproduced at least three times.

3. Results and discussions

In order to select the optimal plasma deposition parameters, the allylamine flow rate was first optimized. The effect of the plasma power on the stability and the amine retention of the allylamine deposited coating were also studied. In a second step, the immobilization of the laccase on the plasma polymerized allylamine (PPAA) coating on rough carbon surfaces deposited under different deposition time was investigated by measuring the laccase activity and current output. Third, the manufactured allylamine/carbon biosensor sensitivity was evaluated in presence of ABTS and DMP.

3.1. Plasma deposition optimisation

3.1.1. Allylamine flow rate

Argon was used as a carrier gas to carry along the monomer into the reactor, taking advantage of the high vapor pressure of allylamine. Moreover, addition of argon to the monomer has been reported to enhance both plasma stability and allylamine hydrophilicity [29,41]. In fact the addition of argon in the discharge leads to an increase of the electron density due to ionization and this has a consequence on the plasma stability and also the fragmentation of the precursor, and therefore on the properties of the coatings. As shown in Fig. 1A, the allylamine flow rate depends on both monomer temperature and argon flow rate. The allylamine flow rate was slightly higher when the monomer temperature increased from $-50\text{ }^\circ\text{C}$ to $1\text{ }^\circ\text{C}$, while it increased significantly at room temperature ($20\text{ }^\circ\text{C}$) and high argon flow rates due to the fact that the monomer vapor pressure increased when the temperature became higher. The carrier gas flow rate also influenced remarkably the monomer flow rate. At room temperature, the latter increased from 8.4 sccm to 47.2 sccm (standard cubic centimeter per minute) when the argon flow rate increased from 1 to 5 sccm and a two fold increase in argon flow led to an increase of the allylamine vapor flow rate to 64.3 sccm. In order to ensure a significant allylamine flow rate, an argon flow rate of 5 sccm and a temperature of $20\text{ }^\circ\text{C}$ (room temperature) were fixed in the following experiments.

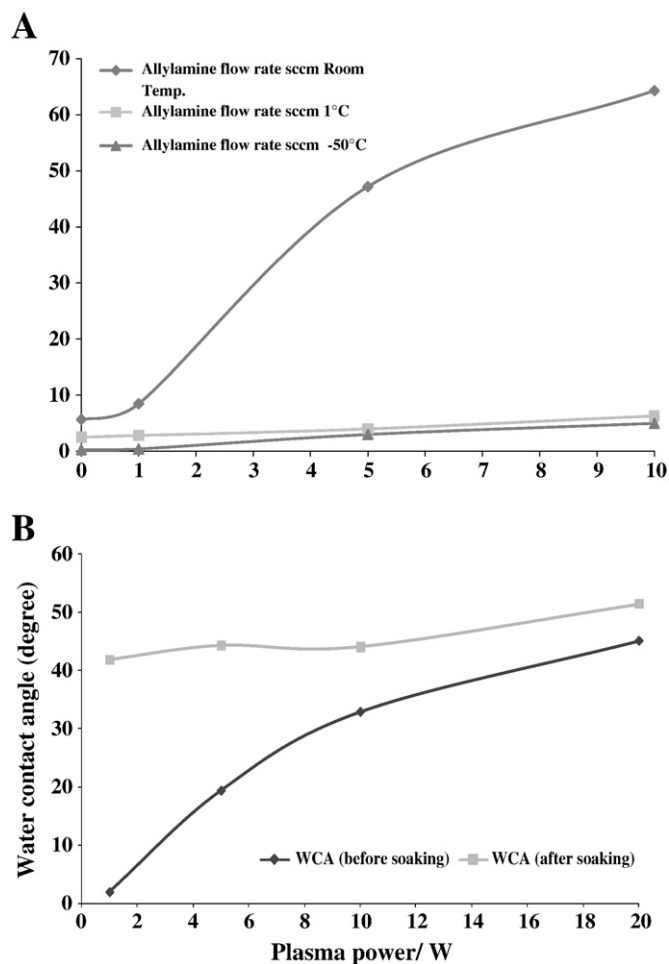


Fig. 1. (A) Allylamine flow rate at different precursor temperatures. (B) Effect of the plasma power on the water contact angle (error = ± 3) of plasma polymerized allylamine coatings before and after soaking with water for 30 min. All the coatings were deposited at 47 sccm monomer flow rate/room temperature during 20 min.

3.1.2. Plasma power-PPAA stability

The resistance to water of PPAA coatings deposited on silicon wafer was investigated in order to check the stability of the plasma polymerized allylamine coating which is essential for potent biosensor applications in biological media. The influence of the plasma power on the coating chemistry and stability was evaluated by measuring the WCA before and after soaking in water for 30 min. The PPAA coatings were deposited at a monomer flow rate of 47 sccm (Ar flow rate = 5 sccm) during 20 min and at a plasma power varying from 1 to 20 W. The results summarized in Fig. 1B demonstrate that, after plasma treatment and before soaking in water, the higher was the applied plasma power, the more hydrophobic was the deposited PPAA coating on silicon. This is probably due to high fragmentation of monomers and consequently lower retention of amine functions, and higher crosslinking of the deposited coatings at high plasma power, as already reported in the literature [19,21,27,29,37]. The stability tests also showed that, for all the coatings, the WCA after soaking increased indicating a possible degradation of the PPAA coating. However, this WCA increase became minimal (around 10°) when the coating was deposited at a plasma power superior than 10 W. When the coatings were deposited at a plasma power of 1 W, after soaking, the allylamine surface WCA increased from 2 to 41°. While, at higher plasma power (20 W), this WCA variation was not significant (5°). Thus it is clearly shown that the coatings prepared at plasma powers below 10 W were less stable than those deposited at higher power. Further increase of the plasma power may give stable coatings but a lower retention of amine functionality [19,37]. On the basis of the above experimental results, a plasma power of 10 W and allylamine flow rate of 47 sccm were chosen as plasma deposition parameters in the following experiments. Indeed, these conditions exhibited the best compromise between water stability, as shown by low WCA increase after rinsing and moderate WCA value, as an indicator of the amine retention on the surface.

3.1.3. Allylamine deposition duration — PPAA chemistry

To investigate the effect of the deposition duration on the PPAA coating thickness, wettability and chemistry, the allylamine was deposited on silicon wafer and rough spectrographic carbon surfaces for 3, 10, 20 and 30 min.

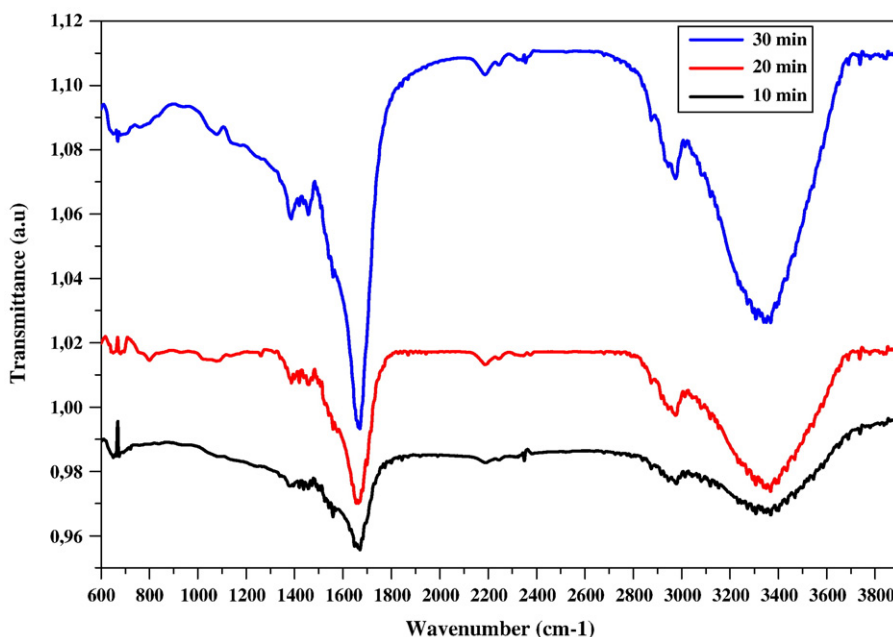


Fig. 2. FTIR spectra of PPAA deposited on silicon wafer at different deposition durations (P = 10 W, allylamine flow rate = 47 sccm).

FTIR spectra of the PPAA coatings deposited on silicon substrate are shown in Fig. 2. As expected, vibrational absorption bands were found in the range $3100\text{--}3650\text{ cm}^{-1}$ for the NH stretching mode and the

deformation mode of primary amine, secondary amine or imine. Multiple absorption peaks located around 2900 cm^{-1} can be assigned to the stretching mode of aliphatic C–H groups. The band around 1660 cm^{-1}

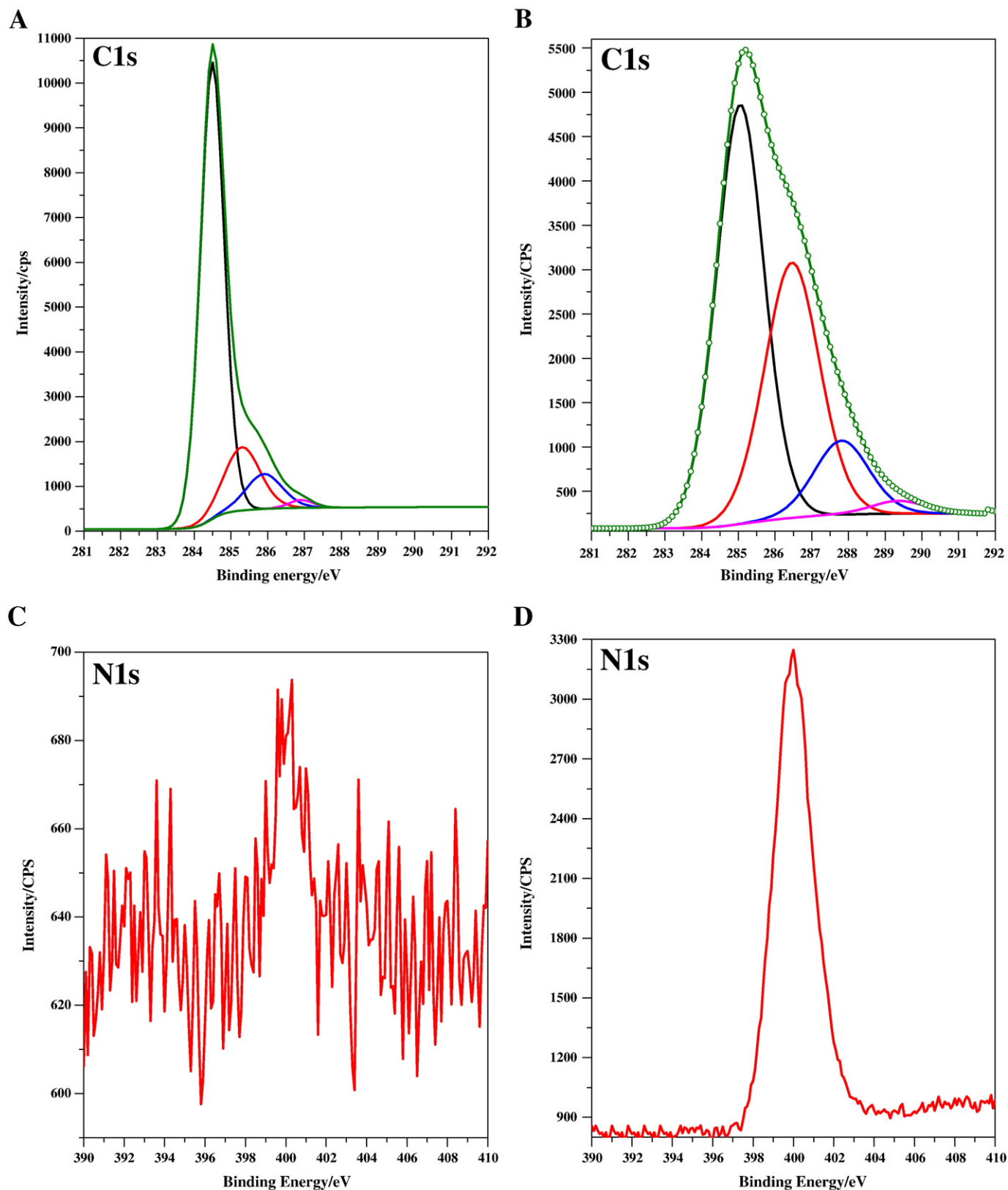


Fig. 3. Comparison between the XPS C1s and N1s spectra of non-plasma treated carbon (A, C) and the allylamine coated carbon ($t = 20\text{ min}$, $P = 10\text{ W}$, allylamine flow rate = 47 sccm)(B, D).

can be mainly assigned to the primary amine group bending and the imine stretching modes or azine functionalities ($C=N-N=C$). The C–H bending in methylene ($-CH_2-$, 1460 cm^{-1}), in vinyl groups ($-CH=CH_2$; 1430 cm^{-1}) is also present in the poly(allylamine) spectra. The spectrum features thus indicate that the monomer underwent, following a complex mechanism, fragmentation, reorganization and cross-linking of the excited monomer units assisted by the electrons and the reactive species in the plasma [28,42]. One can also notice that the longer is the plasma deposition duration, the more intense are the FTIR peaks confirming also the PPAA thickness increases. Indeed the intensity of the peak centered at around 1660 cm^{-1} increases linearly with the plasma deposition duration.

The polymer deposition rate was determined on partially masked silicon wafers by measuring the PPAA thickness using atomic force microscopy (AFM) operating in the tapping mode. A plasma deposition for 10 min generated a PPAA film thickness around 100 nm corresponding to a mean deposition rate of 10 nm min^{-1} .

XPS measurements were performed on non-treated carbon and allylamine plasma coated carbon (20 min) to confirm the previous conclusions. A peak-fitted spectrum for the C1s core level is shown in Fig. 3A and B for graphite and PPAA coated graphite respectively. The C1s binding energy for graphite is well known to be $284.4 \pm 0.2\text{ eV}$, as determined from XPS [43] and synchrotron radiation photoemission measurements [44] of single crystal graphite (Fig. 3A). The spectrum of the allylamine coated carbon (Fig. 3B) can be satisfactorily fitted by a combination of four distinct peaks: the peak at 285 eV corresponds to C–C and C–H moieties, the peak at 286.4 eV to C–O and/or C–N functional groups and the peak at 287.9 eV to C=O and/or N–C=O groups and the peaks at 289.4 eV to COOR or COOH. One can notice that the graphite peak at 284.4 was not detected on the allylamine coated sample, showing clearly that the films were homogeneous and covered completely the graphite sample. Results of elemental composition measurements carried on plasma allylamine coated carbon are shown in Table 1. The allylamine plasma deposition led to a significant increase of the surface nitrogen content from 0.76% (untreated carbon) to 16% when treated during 10 min (Table 1). The N1s peak was centered at 399.6 eV demonstrating that mostly amine groups were present on the surface after allylamine plasma treatment (Fig. 3C and D). The N/C ratio was around 0.20 which proved a good retention rate (60%) compared to the theoretical ratio of the poly-allylamine (0.33). As compared to the existing literature; we have prepared an allylamine coating with better or comparable amine retention at lower powers [28,35,41,45,46].

The XPS analysis revealed also an increase of the oxygen content from 5.24% to 7.67% when allylamine was plasma deposited on carbon surface. The oxygen is probably incorporated in the allylamine film from residual oxygen in the plasma chamber and/or by oxidation after exposure to air and led to the formation of some carboxylic groups, as shown in Fig. 3B.

Surface wettability was also measured on PPAA coated rough carbon. As illustrated in Table 1, the longer is the allylamine deposition duration, the more hydrophilic is the surface. The WCA dropped from 133° to 43.8° when allylamine was deposited for 3 min and reached 14° for

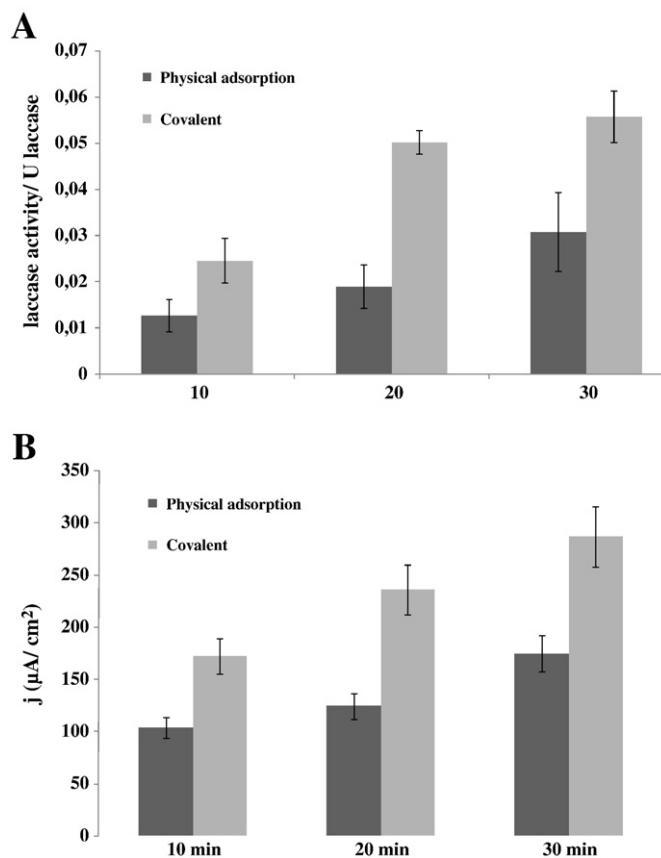


Fig. 4. Effect of the PPAA deposition duration on (A) the ABTS laccase activity and (B) the current density output for physically adsorbed and covalently immobilized laccase electrodes ($P = 10\text{ W}$, allylamine flow rate = 47 sccm).

PPAA coatings after 30 min. This behavior shows clearly that, on such PPAA coated rough carbon surfaces ($R_a = 2500\text{ nm}$), the longer is the plasma allylamine deposition, the better is the coverage of the rough carbon surface. The deposition of allylamine plasma for 30 min generates only 300 nm thick allylamine coating, covering partially the carbon surface the roughness of which was measured to be around 2500 nm.

3.2. Laccase coated electrode performance

3.2.1. Immobilized laccase activity

Laccase amount loaded by physical adsorption and covalent immobilization on the PPAA coated carbon was evaluated by measuring the laccase activity. As shown in Fig. 4A, for each plasma treatment duration, laccase activity was around two fold higher for covalently immobilized laccase compared to the physisorbed ones. For both immobilization methods, the longer was the allylamine deposition duration, the higher was the laccase activity. The highest laccase activity, 0.056 U, was measured on the electrode coated with allylamine

Table 1
Surface wettability, chemistry (XPS data) and the laccase coverage rate of PPAA deposited coatings on rough carbon graphite.

Plasma deposition duration (min)	θ (degree)	XPS				Covalently immobilized laccase/nm ²
		C1s (%)	O1s (%)	N1s (%)	N/C	
0	133 (± 3)	94	5.24	0.76	0.008	–
3	43.8 (± 3)	77.63	8.29	14.08	0.18	–
10	26.7 (± 3)	76.31	7.67	16.02	0.20	0.020
20	20 (± 3)	76.48	7.52	16	0.20	0.042
30	14 (± 3)	–	–	–	–	0.047

θ : Water contact angle.

during 30 min, followed by the covalent immobilization of the laccase. The active enzyme loading could thus be related to the higher density of amine groups available on the surface, as shown by the FTIR measurements on silicon wafers.

The same tendency was also observed for physically adsorbed laccase. The highest adsorbed laccase activity on carbon treated during 30 min (0.03 U laccase) is three fold higher than on carbon treated during 10 min. This could be attributed firstly to an enhanced adsorption of protein on the hydrophilic surfaces and secondly to a favorable electrostatic interaction between the negatively charged laccase (acidic isoelectric point) and the positively charged amine surface groups.

Assuming that the measured activity was representative of the amount of immobilized laccase and knowing that the molecular surface of one laccase molecule ($S_L = 35 \cdot 10^{-14} \text{ cm}^2$), as deduced from dimensions of laccase from crystallographic data [38], the total surface occupied by the enzyme on the carbon could be deduced. This surface coverage can be calculated according to the following relationship: $\Gamma = 100(N_L S_L) / S_E$ with Γ : surface coverage rate (%), N_L : number of laccase molecules immobilized on the surface, S_L : surface of one laccase molecule (cm^2) and S_E : electrode surface area (0.384 cm^2). Knowing that the laccase molecular mass is around 60 kDa, N_L was calculated as follows: $N_L = (Ac N_A) / (As M_L)$ with N_A : Avogadro constant ($6022 \cdot 10^{23} \text{ mol}^{-1}$), As : laccase specific activity (300 U/mg), Ac : laccase activity (U laccase) and M_L : laccase molar mass (610^7 mg/mol). When laccase was covalently immobilized on the electrode surface, a coverage rate of 74% ($0.02 \text{ laccase} \cdot \text{nm}^{-2}$) was calculated for the surface treated during 10 min. It reaches 152% ($0.042 \text{ laccase} \cdot \text{nm}^{-2}$) when the electrode was treated during 20 min (Table 1). For the physisorbed laccase, the electrode surface coverage rate doesn't exceed 38% when treated with allylamine for 10 min and it increases to 93% when the surface was treated during 30 min. This high coverage rates observed for covalently biofunctionalised carbon coated for more than 20 min could be explained by the fact that the calculation was done based on the apparent electrode surface, neglecting its roughness (2500 nm). On the other hand, the laccase coverage superior to one enzyme monolayer could result from laccase intramolecular crosslinking during the covalent binding procedure.

3.2.2. Bioelectrode O_2 reduction/sensitivity

The efficiency of the biosensor prepared by physical adsorption and covalent attachment of laccase was tested with two laccase substrates: a phenolic substrate (DMP) and a non-phenolic one (ABTS). Cyclic voltammograms (CVs) of ABTS at concentrations of 1 mM and scan rate of 10 mV/s, in absence and presence of laccase from *T. versicolor*, are shown in Fig. 5A. The CV in the absence of laccase shows clearly the ABTS oxidation at a potential of 0.472 V/Ag/AgCl as already reported by Bourbonnais et al. [47]. In the presence of laccase however, the CV signal is higher demonstrating that the biocatalyzed reduction of ABTS by the enzyme takes place. No current was measured, however, on both adsorbed and covalently immobilized laccase electrodes in the absence of ABTS (Fig. 5B). This could be probably due to the presence of the PPAA resistant layer avoiding a direct electron transfer between the enzyme and the carbon electrode surface. In the presence of 1 mM ABTS substrate, as mediator, however, a current was detected. The current output measured for covalently immobilized laccase biosensor was twofold higher than that for physically adsorbed laccase, as expected from activity measurements (Figs. 4B and 5B and C). For biosensors coated with allylamine during 20 min, the current density was $124 \pm 25 \mu\text{A cm}^{-2}$ when laccase was adsorbed. It reached $235 \pm 25 \mu\text{A cm}^{-2}$ when the enzyme was covalently immobilized. In addition, as for the laccase ABTS activity, the current output was correlated to the allylamine plasma deposition duration. For physically adsorbed laccase electrode, the current density increased from $103 \pm 25 \mu\text{A cm}^{-2}$ to $174 \pm 25 \mu\text{A cm}^{-2}$ when the PPAA deposition increased from 10 min to 30 min. Similar behavior was observed for

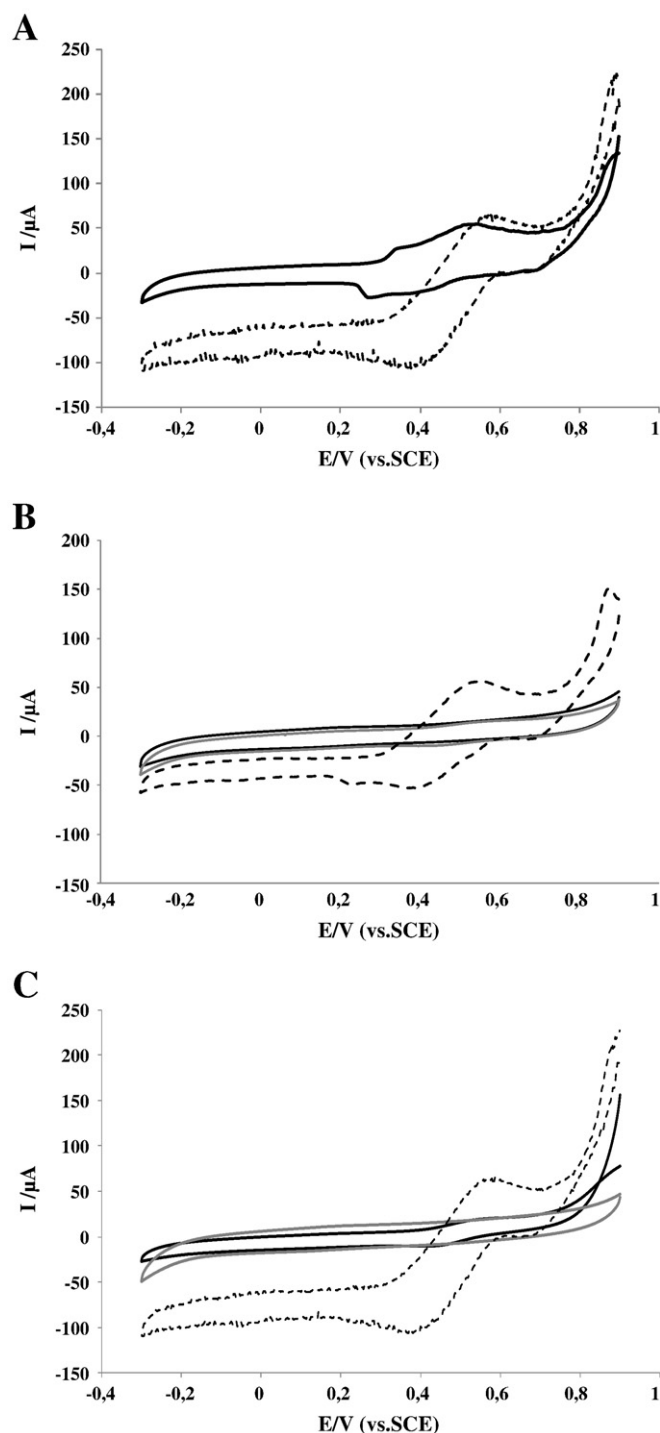


Fig. 5. (A) Cyclic voltammograms of PPAA functionalized carbon electrodes (20 min) in the presence of ABTS under O_2 without laccase (solid dark line) and with laccase (dashed dark line). Experimental conditions: 50 mM acetate buffer/100 mM NaClO_4 , pH 4.2, 10 mV/s, room temperature. (B) Cyclic voltammograms of laccase physically adsorbed on PPAA functionalized carbon electrodes (20 min): without ABTS: physisorbed laccase under N_2 (solid dark line) and under O_2 (solid grey line) and with ABTS: under O_2 (dashed dark line). Experimental conditions: 50 mM acetate buffer/100 mM NaClO_4 , pH 4.2, 10 mV/s, room temperature. (C) Cyclic voltammograms of laccase covalently immobilized on PPAA functionalized carbon electrodes (20 min): without ABTS: physisorbed laccase under N_2 (solid dark line) and under O_2 (solid grey line) and with ABTS: under O_2 (dashed dark line). Experimental conditions: 50 mM acetate buffer/100 mM NaClO_4 , pH 4.2, 10 mV/s, room temperature.

covalently immobilized laccase electrodes. The measured current density attains more than $280 \pm 25 \mu\text{A cm}^{-2}$ when the carbon electrode was coated with allylamine for 30 min (Fig. 4B).

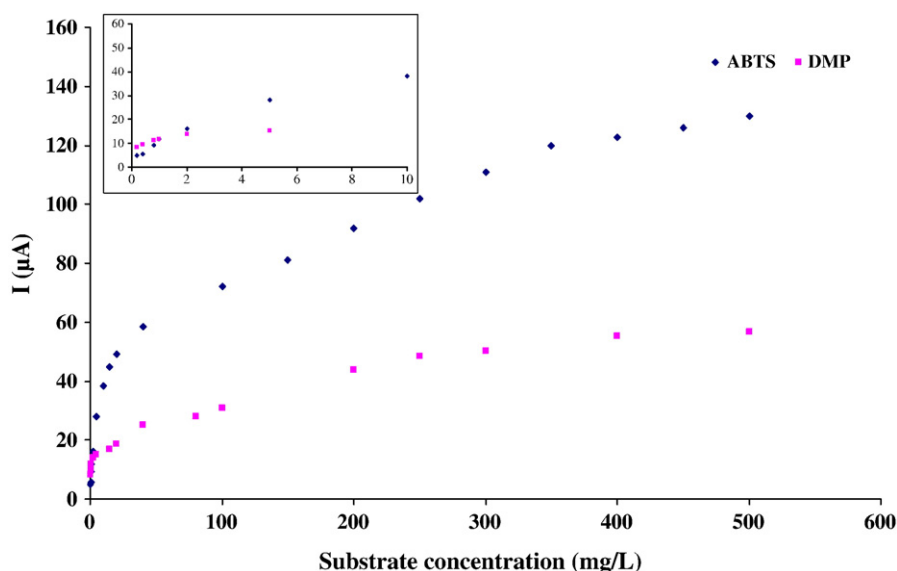


Fig. 6. Catalytic current responses of covalently immobilized laccase PPAA electrodes (30 min) obtained by chronoamperometry as function of ABTS and DMP concentrations. Inset: detail of linear range of the biosensor for both substrates. Experimental conditions: 50 mM acetate buffer/100 mM NaClO₄, pH 4.2, room temperature.

The sensitivity of the biosensor towards a phenolic substrate (DMP) and a non-phenolic one (ABTS) was evaluated for a covalently immobilized laccase PPAA carbon biosensor by amperometric detection. The variation of the measured current as a function of the concentration of the substrate is reported in Fig. 6. The high sensitivity to ABTS and DMP, related to the slope value, was found to be $4.8 \mu\text{A mg}^{-1} \text{ L}$ and $2.7 \mu\text{A mg}^{-1} \text{ L}$ respectively at ABTS concentration lower than 10 mg/L. For comparison, for covalently immobilized laccase on carbon fibers, the best sensitivity to catechol, another phenolic mediator, was reported to be $0.145 \mu\text{A mg}^{-1} \text{ L}$ [48]. Recently, for laccase covalently immobilized on multiwalled carbon nanotube (MWCNT) electrodes, Tortolini et al. [9] reported a sensitivity to both ABTS and catechol of $0.14 \mu\text{A mg}^{-1} \text{ L}$. Hussein et al. [49] obtained a current signal of around 1.8 mA/cm^2 for laccase physically adsorbed on bucky paper based-electrode in the presence of ABTS (0.02 M) corresponding to a sensitivity of around $4.8 \mu\text{A mg}^{-1} \text{ L}$ ($90 \text{ nA cm}^{-2} \mu\text{mol}^{-1} \text{ L}$). The high sensitivity of the PPAA carbon electrodes could be explained by both the high laccase loading mainly by covalent immobilization and the good diffusion properties of the PPAA immobilizing layer partially covering the carbon electrodes.

In addition to sensitivity, the stability of the biosensor is an important parameter. The electrodes prepared in this work retained laccase activity for more than 6 months (data not shown). Freire et al. [48], also reported a good catechol laccase activity, with covalently immobilized enzyme on carbon fiber electrodes, for over 2 months. Such a long-term stability of the PPAA coated carbon electrodes could be due to (i) the stability of the PPAA coatings (ii) the hydrophilicity of these coating leading to a strong adsorption of the enzyme and (iii) covalent immobilization of the laccase.

4. Conclusions

A simple and rapid method was used to functionalize carbon electrode in order to immobilize laccase for biosensor application. Amine functions have been successfully generated on carbon surfaces using allylamine as precursor under mild plasma conditions (low plasma power – short duration) leading to the deposition of a stable PPAA exhibiting good retention of amine and other nitrogen containing groups (N/C ratio > 0.18). The wettability measurements of PPAA deposited on rough carbon surface demonstrated that the surface coverage depended on the plasma deposition duration. The longer is the PPAA deposition, the higher is the nitrogen content of the coating

and the N/C ratio. The XPS analysis showed that mainly amine groups were found on the carbon surface after allylamine plasma polymerisation. Laccase activity and amperometric measurements showed a higher efficiency of the covalent immobilization of laccase compared to physical adsorption. Sensitivities of around $4.8 \mu\text{A mg}^{-1} \text{ L}$ for ABTS concentration ranging from 0.2 to 5 mg/L and up to $14.7 \mu\text{A mg}^{-1} \text{ L}$ for ABTS concentration ranging from 0.2 to 1 mg/L were attained. In the case of DMP, $2.7 \mu\text{A mg}^{-1} \text{ L}$ for concentrations ranging from 0.4 to 2 mg/L were measured. The laccase immobilization on PPAA coated electrode surfaces showed an excellent stability and the laccase activity and high sensitivity was maintained for more than 6 months. From this work we have highlighted the great potentiality of PPAA coatings as carbon functionalization method for subsequent laccase immobilization for the development of biosensor for analytical applications including in living organisms, due to the biocompatibility of PPAA coating. Furthermore, these electrodes can be applied and expanded for manufacturing bioelectronic devices such as implantable biofuel cells.

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