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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 μA mg^{-1} L and 2.7 μA mg^{-1} L were attained for ABTS and DMP respectively. An excellent stability of this laccase biosensor was observed for over 6 months.

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′-azinobis-(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 (Ra) is around 2500 nm (±200). Polished silicon wafers (100) (Ra = 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 ($CH_2{=}CHCH_2NH_2$, 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′-azinobis(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-xylidine (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 (Sepharose 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 homemade 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