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Oxidation of laccase for improved cathode biofuel cell performances

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ABSTRACT

Graphite rods were modified by substituted aryldiazonium salts allowing subsequent laccase immobilisation and direct electron transfer at the cathode. Two covalent enzyme immobilisation methods were performed with carboxy and amino substituted grafted groups, either via the formation of an amide bond or a Schiff base between the glycosidic groups of the enzyme and the amino groups on the electrode surface, respectively. Laccase adsorption efficiency was consistently compared to the covalent attachment method on the same carbon surface, showing that the latter method led to a higher immobilisation yield when the electrode surface was functionalised with carboxylic groups, as shown from both laccase activity measurement towards an organic reducing substrate, ABTS, and quantitative XPS analysis. Both analytical methods led to similar laccase surface coverage estimations. From activity measurements, when laccase was covalently immobilised on the electrode functionalised with carboxylic groups, the surface coverage was found to be 43 ± 2% whereas it was only 10 ± 3% when laccase was adsorbed. Biocatalysed dioxygen reduction current was also higher in the case of covalent immobilisation. For the first time, oxidised laccase performances were compared to unmodified laccase, showing significant improved efficiency when using oxidised laccase: the current obtained with oxidised laccase was 141 ± 37 μA cm^{-2} compared to 28 ± 6 μA cm^{-2} for unmodified laccase after covalent immobilisation of the enzyme on a graphite electrode functionalised with carboxylic groups.

1. Introduction

Due to the prospect of starvation of traditional fuel sources, alternative sources of sustainable electrical energy, including biofuel cells (BFC), have become a strategic research field [1–5]. Enzymatic biofuel cells are designed to generate electric current from bioelectrocatalysed chemical redox transformations. The oxidation of fuel, typically hydrogen or an organic compound such as glucose takes place at the anode while the cathode is responsible for the reduction of an oxidant such as dioxygen. These redox reactions are biocatalysed by different enzymes such as glucose oxidase or dehydrogenase at the anode and bilirubin oxidase [6], laccase or tyrosinase at the cathode and can occur directly at the electrodes or need the use of redox mediators that act as electron shuttles between the enzyme and the electrode.

Biomolecule immobilisation is a crucial process in many biotechnological applications, including the biofuel cell field. The immobilisation method should not only guarantee that the enzyme is retained on the support, but also should ensure a close proximity between the enzyme active site and the electrode surface in order to obtain an efficient electron transfer. Moreover, the immobilisation of the enzyme on the surface should preserve its activity, i.e. should not affect the 3D conformation of active sites. Over the past decade, the bulk of the research in enzymatic fuel cells has been directed towards enzyme/electrode integration methods that alleviate the power density limitations resulting from low electron transfer rate from the enzyme active site to the electrode [3]. Together with enhanced long-term operational stability, this point is a key challenge in the engineering of efficient electrochemical devices such as biofuel cells or biosensors [7–12].

A simple immobilisation method is physical adsorption. This technique, based on weak interactions (e.g. hydrogen bonds, electrostatic interactions and Van der Waals forces) usually does not induce significant enzyme structure modification that would affect its activity, especially when the surface is hydrophilic but again enzyme desorption cannot be excluded. Recently, Cinquin et al. used an original mechanical confinement of enzymes inside the electrodes by compressing some graphite [13] or CNT [14], the biocatalyst and eventually redox mediator

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mixture. Such a biofuel cell design using naphtoquinone-mediated oxidation of glucose by GOX and direct electron transfer (DET) using laccase at the cathode exhibited a power output of 1.54 mW cm^{-2} and current densities of 4.47 mA cm^{-2}, the highest reported to date, to the best of our knowledge [15]. The device was successfully implanted in a rat abdominal cavity and achieved in vivo enough current densities that an external capacitor could be charged to generate enough power to act as a sole power source for a light-emitting diode [16,17]. Interestingly, at the same period, Pankratov et al. also described a hybrid self charging electrochemical biocapacitor including an enzymatic fuel cell [18,19]. However, as Reuillard and coworkers [20] suggested in their conclusion, optimisation of the laccase wiring at the biocathode is still to be improved. Indeed, comparison between the highest currents obtained by DET with non-orientated laccase compressed with CNTs in 3D structures and covalently immobilised enzyme shows that in the first case, a large amount of laccase, typically a few to 15 mg [20] is needed whereas about four orders of magnitude lower amounts have been used to generate a power density only ten fold lower [21] or one hundred fold lower [22] on a similar electrode area after laccase covalent grafting. One reason for the higher currents obtained with the enzyme covalent grafting method could result from the amino acid composition of laccases from *Trametes versicolor* that contain 45 basic residues distributed all over the tridimensional structure but only 5 lysine side chains among 499 total amino acids [23]. Enzyme grafting via its lysine groups thus could lead to a better orientation control of the enzyme than via its carboxylic groups.

However, the covalent immobilisation method requires the presence of functional groups on the electrode surface to allow a covalent bonding with reactive groups on the protein, e.g. mostly carboxylic and amino groups. Among the methods developed to covalently bind chemical groups to carbon electrodes, a protocol initially proposed by Allongue et al. [24] has emerged. It involves the grafting of functionalised aryl groups via the electrochemical reduction of aryldiazonium salts bearing a wide range of functional groups. This easy-to-use method results in highly stable layers [25,26] and can be applied for microscale devices [27,28]. In recent studies [29,26], gold, graphite and glassy-carbon (GC) electrodes were functionalised using aryldiazonium salts bearing carboxylic acid groups, with subsequent grafting of GOx on the modified electrode. The modified electrodes retained much of their activity after six weeks, while control electrodes prepared by depositing the crosslinker and GOx directly onto the GC had lost all activity within only one week [29]. The diazonium electroreduction method also allowed the functionalisation of graphite carbon with amino groups. In the case of laccase, this aniline derivative mimics a potential enzyme reducing substrate, thus allowing molecular recognition and specific interactions to control the laccase orientation on the surface, which is a key element for the fabrication of bioelectrodes with high electron transfer efficiency [22,30]. Moreover, the functionalisation method by diazonium reduction allows a direct electron transfer between the electrode and the enzyme, as shown for glucose oxidase [29] and laccase [21]. Diazonium electrografting on carbon thus appears as a versatile functionalisation method for subsequent enzyme immobilisation and DET. Because different functional groups can be grafted on the stable phenyl layer, it provides a useful scaffold for the consistent study of the effect of enzyme orientation on the electrode surface.

In this work, functionalisation of graphite carbon by aryldiazonium salt electrografting has been performed to immobilise fungal laccase from *T. versicolor* on graphite rods. Both adsorption and covalent grafting were performed on functionalised surfaces according to the same protocol, thus allowing accurate comparison between these two immobilisation methods in terms of enzyme loading and electron transfer efficiency. In addition, the influence of the enzyme orientation induced by covalent grafting either via its carboxylic or amine residues has been studied on electrodes functionalised using similar methods, i.e. electrodeposition of diazonium salts. As an alternative grafting method to the formation of a peptidic bond, laccase was also cross-linked via its oxidised oligosaccharides. This method indeed was reported to lead to efficient wired laccase [31] and glucose oxidase [32] electrodes. However, the interest of using oxidised laccase as a biocatalyst in a biofuel cell without grafting it via its glycosidic groups has not been demonstrated yet, to the best of our knowledge. This issue will be addressed by comparing immobilisation of both unmodified and oxidised laccases on electrodes functionalised with carboxylic groups. All functionalised electrodes have been characterised by XPS, before and after enzyme immobilisation. The comparison between the immobilisation procedures was performed by measuring both the enzymatic activity of the bioelectrodes and their catalytic efficiency for dioxygen reduction.

2. Materials and methods

2.1. Reagents

4-Aminobenzoic acid, 4-nitrobenzenediazonium tetrafluoroborate, tetrabutylammonium tetrafluoroborate, 2,2′-azobis(3-ethylbenzothiazole-6-sulfonic acid) (ABTS), toluidine blue-O (TBO) and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich. 2-1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) was from Fluka. Buffer solutions were prepared with disodium hydrogenophosphate (Acros Organics), sodium dihydrogenophosphate, sodium acetate and acetic acid (Prolabo) in deionized water (Milli-Q grade, Millipore). Sodium perchlorate (Sigma-Aldrich) was used as an electrolyte.

2.1.1. Diazotization of 4-aminobenzoic acid

4 mL of tetrafluoroboric acid was cooled in an ice bath to 5 °C before adding 4×10^{-3} mol of 4-aminobenzoic acid. Following the addition, the temperature of the solution was maintained at 5 °C for 20 min. A yellow precipitate formed during the slow addition of 4.5×10^{-3} mol of sodium nitrite. The diazonium salt was suction filtered and washed with cold ether. The solid was then dissolved in less than 5 mL methanol and crystallized by ether addition.

2.2. Enzyme

Laccase was produced from *T. versicolor* (ATCC 32745) as previously described [33]. Purified laccase (around 900 U mL^{-1} and 3 mg total protein mL^{-1}) was stored at −20 °C in 50 mM phosphate buffer at pH 6.8, where the stability of the enzyme is maximal in the presence of glycerol (15% *v*/*v* final).

2.3. Electrochemical functionalisation of the carbon electrode

2.3.1. Electrode materials

7 mm diameter spectrographic Carbon–Graphite rods (Mersen, France) were used. Prior to surface modification the carbon electrodes were polished with SiC paper (Buehler, Germany) with grit size of 80, cleaned with Milli-Q water and dried by filtered compressed air. The roughness (Ra) of the resulting carbon surface was approximately 2500 (±300) nm. The geometric area of the carbon surface in contact with the electrolyte was 0.38 cm^2.

2.3.2. Functionalisation of the electrode surface and surface characterisation

2.3.2.1. Electrochemical modification. Carbon surfaces were functionalised by a diazonium cation electrochemical reduction procedure developed by Allongue and co-workers [24,34] and leading to the covalent attachment of aromatic groups onto the carbon surface.

a. Functionalisation with amino groups. In the first step, 4-nitrobenzene diazonium tetrafluoroborate (2 or 5 mM) electrochemical reduction was performed in anhydrous acetonitrile at the electrode surface. Then cyclic voltammetry was performed from 0.8 to −0.2 V/SCE at