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A reduced graphene oxide based biosensor for high-sensitive detection of phenols in water samples

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ABSTRACT

Laccase (Lac) from *Rhus vernificer*a was covalently immobilized onto 1-aminopyrene (1-AP) functionalized reduced graphene oxides (rGOs)-modified glassy carbon electrode by encapsulation with chitosan. The biosensor was characterized with respect to the optimum pH and polarized potentials for the determination of phenols, and its response time, sensitivity, linear range, detection limit, and stability. Hydroquinone and catechol were selected as the analytes and detected based on the direct electron transfer behavior of Lac and its enzymatic oxidation of analyte. The sensitivities of the electrode were 14.16, 15.79 μA mM^{-1} with linear ranges of 3–2000, 15–700 μM for hydroquinone and catechol, respectively. The detection limits (S/N = 3) were 2 and 7 μM for hydroquinone and catechol, respectively with a fast response time ($t_{95\%}$) of less than 5 s. Moreover, the immobilized Lac showed high affinity to hydroquinone and catechol with K_m^{app} values of 5 and 0.3 mM, respectively. As a consequence, the biosensor demonstrated suitable stability (*ca.* 7 days; over 300 determinations) and good repeatability with a relative standard deviation of 3.96%. The recovery study of hydroquinone in real water samples gave values from 82.7% ± 10% to 105.9% ± 8%, confirming its application potential in the measurement of phenols in real samples.

1. Introduction

Phenols are common byproducts of large-scale production and use of man-made organics such as drugs, dyes, antioxidants, paper pulp and pesticides and cause ecologically undesirable effects [1]. Most phenols exhibit different toxicities, and even some chlorophenols and nitrophenols are known to possess carcinogenic and immunosuppressive properties. As a consequence, both the US Environmental Protection Agency (EPA) and the European Union (EU) have included some phenols in their lists of priority pollutants. The maximum amount of phenols in wastewater allowed by the European Community is lower than 1 ppm [2]. In general, phenolic compounds are subjected to chromatographic separation before detection, however, the separation often requires pre-concentration, and is time-consuming [3]. In addition, these traditional methods, although accurate and with low detection limits, require expensive and sophisticated instrumentation, which prohibit on-line and real-time monitoring.

Biosensors are an attractive alternative to conventional analytical methods, such as gas/liquid chromatography, due to their fast response, high selectivity, cost-effectiveness, simplicity of operation and manufacturing [2,4]. In addition to the advantages mentioned above, enzyme electrode is available for the continuous measurement *in vivo*, which makes it preferred in some certain circumstances, such as direct monitoring of neurotransmitters in living organisms, compared with other biosensors [5].

Laccase (Lac, EC 1.10.3.2, p-benzenediol: oxygen oxidoreductase) belongs to a family of multicopper oxidases that include one type-1 (T1) copper ion and three additional copper atoms including one type-2 (T2) and two type-3 (T3 and T3′) copper ions which form a trinuclear copper cluster [6]. Lac is capable of catalyzing the oxidation of a range of inorganic and aromatic compounds (particularly phenols) at the T1 copper site with the concomitant reduction of molecular oxygen to water at the T2/T3 cluster [7–9]. Consequently, many electrodes combined with Lac have been developed for detection of phenolic compounds [3,4,10,11]. Recently, great effort has been made to develop new mediator-free (or reagentless) enzyme electrodes based on direct electron transfer (DET) by immobilizing enzymes on conducting substratum [4,12–15]. However, the redox center in biomolecules is usually embedded deeply into the large three dimensional structures of enzyme molecules [14,16]. As far as we known, the direct electron transfer of Lac is only limited on the gold and few carbon electrodes due to the complex structures of its redox centers and the unfavorable orientations of Lac at electrodes [17,18]. Therefore, immobilizing enzymes efficiently on the electrode

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surface and promotion of the electron transfer processes between them are still the most challenging tasks in the construction of biosensors.

Graphene is a star nanomaterial due to its large accessible surface area, electrical conductivity, and capacity for immobilizing enzymes in the development of high performance electrochemical biosensors [19]. In comparison with the carbon nanotubes (CNTs), graphene exhibits potential advantages of low cost, high surface area, excellent conductivity, ease of processing and safety [20]. Until now, numerous strategies have been successfully employed for immobilizing enzymes efficiently on graphene and its derivatives as depicted by [21,22]. Among them, the reduced graphene oxides (rGOs) is confirmed more suitable for biosensors because its electrical conductivity is around 8 orders of magnitude larger than those of GO film [23]. Moreover, the effective deoxygenating process would benefit the subsequent modification and immobilization of biological macromolecules. Despite these subtle properties, the potential applications of rGOs have not been fully explored yet.

1-Aminopyrene (1-AP) is a bifunctional molecule with a pyrenyl group and an amino functional group. The pyrenyl group of 1-AP, being highly aromatic in nature, can interact strongly with the graphene *via* π-stacking. Recently, 1-AP functionalized CNTs were used successfully as the support to immobilize Laccase (Lac) for oxygen reduction with the assistance of the amino functional groups [24]. The amino functional group can be used to immobilize enzyme by a typical glutaraldehyde (GA) cross-linking reaction. By functionalization of CNTs with 1-AP, lots of amino functional groups can be introduced uniformly on the CNT surface and used to immobilize enzymes to construct biofuel cells [24]. Biopolymer chitosan, a copolymer of N-acetyl-D-glucosamine and D-glucosamine, is a polysaccharide derived by deacetylation of chitin. It is hydrophilic, biocompatible and biodegradable, with low toxicity, and shows the significant properties as the support for enzyme immobilization in the construction of biosensors [11,25]. Motivated by the lack of reports on the fabrication of non-covalent immobilization of Lac on rGOs and its electrochemical applications, we selected Lac as the model due to its important applications in biosensors and biofuel cells [3,10,11]. By functionalizing rGOs with 1-AP to form the AP-rGOs composite, the enzyme was immobilized on the AP-rGOs by the glutaraldehyde (GA) cross-linking. The composite of Lac/AP-rGOs was coated on the glassy carbon electrode (GCE) by embedded with chitosan (Chit). After the whole procedure, the DET properties of Lac immobilized on AP-rGOs (Lac/AP-rGOs) and its electrocatalytic response to phenols in real water samples and the related influence factors was evaluated and optimized.

2. Materials and methods

2.1. Reagents and instruments

Lac from *Rhus vernificera* (10 U/mg) was purchased from Sigma–Aldrich. 1-AP was purchased from Alfa Aesar. Graphene oxide (GO) were purchased from XFNANO Materials Tech Co. Glutaraldehyde (GA) and other reagents were analytical grade. A series of HAC–NaAC buffer solutions in which the pH values varied from 3.5 to 5.5 were prepared. Chit was purchased from Sinopharm Chemical Reagent Co. All stock solutions were stored at 4 °C.

All electrochemical experiments were carried out on CHI-660 electrochemical working station (CH Instruments Inc, USA), with modified GCE (3 mm in diameter) as working electrode, platinum wire as counter electrode and saturated calomel electrode (SCE) as reference electrode at room temperature of about 25 °C.

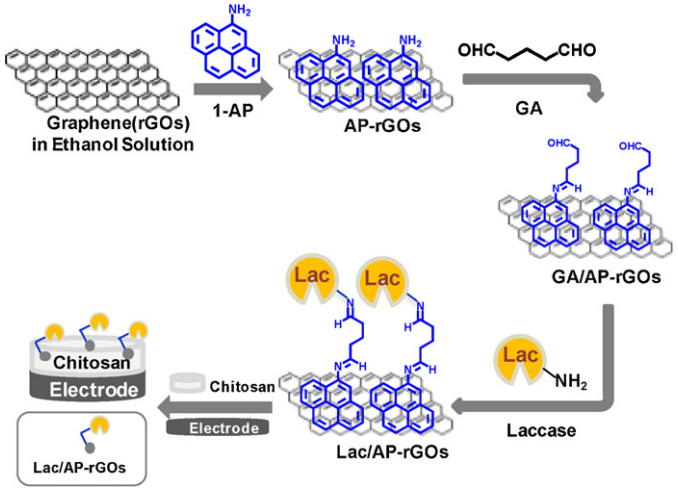

Fig. 1. Schematic diagram for the immobilization of Lac on 1-AP non-covalent functionalized rGOs and its capsulated on GCE by chitosan.

The X-ray photoelectron spectroscopic (XPS) were obtained using the Thermo Scientific Escalab 250xi.

2.2. Preparation and electrochemical characterization of Lac/AP-rGOs/Chit/GCE

The GCE was polished with 0.05 μm alumina slurry. After successive sonication in ethanol and deionized water for several minutes, the electrode was dried under a high-purity nitrogen stream.

Fig. 1 illustrates the schematic of the preparation of the Lac/AP-rGOs/Chit/GCE biosensor. First of all, GO was reduced by the hydrohalic acid method [26] except that the GO was ultrasonically dispersed in alcohol prior to its reduction. And then the rGOs and 1-AP with the optimized ratio of 1/8 were dissolved in ethanol and mixed under ultrasonication for 2 h. The mixture was shaken for 10 h and then stored at room temperature (20 ± 2 °C) overnight. After filtration and washing with ethanol several times, the resulting deposit was dried at 70 °C for 12 h to obtain AP-rGOs.

In the procedure of enzyme immobilization, GA aqueous solution (5 wt%) was mixed with 200 μL of 2 mg/mL AP-rGOs suspension dispersed in HAC–NaAC buffer solution (pH 4.5) to obtain a homogenous suspension. After that, 200 μL of 1 mg/mL Lac solution dissolved in HAC–NaAC buffer solution (pH 4.5) was added and the mixture was shaken for 30 min to obtain the Lac/AP-rGOs suspension. Subsequently, 200 μL of chitosan (0.5 wt%) was added to the suspension to form Lac/AP-rGOs/Chit. Lac/AP-rGOs/Chit stock solution (12 μL) was dripped on the freshly pretreated GCE surface and dried at 4 °C overnight. Before each measurement, the modified electrode was washed with 0.1 M HAC–NaAC buffer solution (pH 4.5) and stored at 4 °C.

3. Results and discussion

3.1. Characterization of Lac/AP-rGOs composites

XPS was used to characterize the Lac/AP-rGOs and AP-rGOs by comparison as shown in Fig. 2. The C1s spectrum of the Lac/AP-rGOs reveals that it consists of two main components originating from the carbon–oxygen double bond (C=O) at 287.96 eV and carbon–oxygen single bond (C—O, C—R) at 284.66 eV. Compared with the C1s spectrum of the AP-rGOs, the C=O bond exists mainly due to the successful covalent immobilization of Lac. Moreover, The Cu spectrum of the Lac/AP-rGOs further confirms it although the Cu peaks aren't very high-resolution (Fig. 3), which maybe because