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A glucose biosensor based on direct attachment of *in situ* generated nile blue diazonium cations to the electrode surface

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ABSTRACT

We describe here the covalent modification of glassy carbon electrode with nile blue (NB), a redox dye, by electrochemical reduction of its corresponding diazonium salt, which is generated *in situ* from the reaction between the aromatic amino phenyl group of NB and sodium nitrite. NB is attached directly to the electrode surface without any cross linking agent or complex matrices. This modification method is very rapid, simple, in one step and provides a very stable grafted NB film. Cyclic voltammetry and electrochemical impedance spectroscopy were used to trace the reaction. The resulting NB films have a very stable and reversible electrochemical response and exhibit excellent electrocatalytic behavior toward hydrogen peroxide and oxygen reduction. The amperometric detection of hydrogen peroxide is performed at −0.15 V vs. Ag/AgCl. This strong catalytic effect for reduction of hydrogen peroxide and oxygen exhibited a biocompatible platform for development of glucose biosensors. Therefore, a glucose biosensor is developed using glucose oxidase by simple casting method based on decreasing of cathodic peak current of oxygen. This biosensor has been successfully applied to determination of glucose in human plasma sample. The great stability and reusability, excellent electrochemical reversibility, technically simple and possibility of preparation at short period of time make this method suitable for low-cost bioelectronical devices.

1. Introduction

Recently, the electrochemical reductive adsorption of aryl diazonium salts has been receiving increasing attention [1]. This immobilization method is based on the electrochemical generation of aryl radicals by reduction of the aryldiazonium salts which leads to strong bonding between the substrate and the aryl functional group [2,3]. The aryldiazonium salts can be reduced onto a variety of substrates such as carbon surfaces like glassy carbon, carbon nanotubes, graphite and diamond [4–6], metals [7,8], semiconductors [9–13], indium tin oxide and organic surfaces [14,15]. This direct introduction of functional groups leads to robust thin films which have thermal stability [16] and electrochemical stability over a wide potential range [17,18]. Large varieties of substituent aryl groups can be grafted to the electrode surface and impart useful properties to the modified electrodes [5].

Nile blue is a phenoxazine dye with highly promising properties as a redox mediator. Redox mediators can reduce the oxidation overpotential and improve the electron transfer rate [19]. This compound has been used as mediator for the electrocatalytic oxidation of hydrogen peroxide [20–23], NADH [24–28], nitrite [29] and l-cysteine [30] and also for the electrocatalytic reduction of hemoglobin [31,32] and oxygen [33]. NB can be immobilized on the electrode surface to shuttle electrons between the analyte and the electrode. Usually, NB is adsorbed [34,35], supported on the carbon nanotubes [21–23,36] or electropolymerized [37–39] on the electrode surface. But these modified electrodes suffer from low stability and mediator leaking as NB can easily diffuse from electrode surface to the bulk solution. Covalent attachment can overcome this disadvantage. In this respect a well known and widely used approach for the covalent attachment of NB is obtained by formation of self-assembled monolayers (SAMs) of thiols on gold surface [40]. The popularity of SAMs arises from their ease of preparation, the well defined monolayer that results and the possibility of introducing a vast number of functional groups at the monolayer surfaces [41]. However the limited potential window and the poor long-term stability of the SAMs offset this popularity [1]. To solve these limitations of the SAMs, the use of the reductive adsorption of aryl diazonium salts on the electrode surfaces has been explored. Despite the mentioned advantages of this method, there are only a few papers in which the electrochemical reduction of diazonium salts is used for the covalent modification of the electrode surface by nile blue. In our previous work, we have functionalized the glassy carbon (GC) electrode with 4-nitrophenyl group by electrochemical reduction of 4-nitrobenzenediazonium

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salt. After the nitro group reduced to amino group, nile blue attached to amine using glutaraldehyde linker [20]. In spite of the good electrocatalytic activity of this modified electrode toward hydrogen peroxide, this modification strategy is long and time consuming and also the mediator is not in direct contact with the electrode surface. As NB is an aromatic amine, it is possible to synthesize its diazonium salt and bond it to the electrode surface directly. So we decided here to propose a strategy for the easily and rapidly preparation of NB diazonium salt. In this strategy, immobilization of NB on GC electrode is carried out by the electrochemical reduction of its *in situ* generated diazonium salt. *In situ* generation of diazonium salt is simple and quick, in one step and offers the distinct advantage of working in aqueous media and 0 °C. The significance of this modified electrode is that NB has been immobilized on the electrode surface directly by covalent bonds and without any cross linking agent or complex matrices. The attached NB molecules help to achieve rapid electron transfer between hydrogen peroxide and oxygen with the electrode surface. So NB modified GC electrode was applied for the detection and stable monitoring of hydrogen peroxide. In addition, a glucose biosensor was developed by electrostatic adsorption of negatively charged glucose oxidase (GOx) enzyme [42] on positively charged NB molecules based on the decreasing of cathodic peak current of oxygen. Electrochemical performance of this biosensor is investigated by amperometric measurements of glucose. Finally the proposed biosensor was applied for glucose determination in human plasma sample.

2. Experimental

2.1. Reagents

Glucose oxidase (EC 1.1.3.4. Type II: from *Aspergillus niger*) was purchased from Sigma. Nile blue was obtained from Merck. All other chemicals were of analytical grade from Merck, Fluka and Aldrich and used without further purification. All aqueous solutions were prepared with 18 MΩ water using a Millipore-MilliQ purifier (Millipore Inc.). The phosphate buffer solutions (0.1 M) were prepared from NaH_2PO_4, Na_2HPO_4 and 0.1 M KCl. HCl and NaOH solutions were used for pH adjustment. For hydrogen peroxide detection, the solutions were deaerated by bubbling high purity argon gas (99.99%) through them prior to the experiments.

2.2. Apparatus

All electrochemical measurements including cyclic voltammetry, amperometry and electrochemical impedance spectroscopy were performed with a computer-controlled potentiostat, Autolab electrochemical analyzer model PGSTAT30 (Eco Chemie, Utrecht, The Netherlands) and a standard three electrode cell consisting of a chemically modified or unmodified GC working electrode, a platinum wire auxiliary electrode and an Ag/AgCl (3 M KCl) reference electrode.

2.3. Electrode preparation

Prior to electrode modification, the glassy carbon electrode was polished with 1 μm, 0.3 μm and 0.05 μm Al_2O_3 slurry and then ultrasonically cleaned in ethanol and distilled water, successively. For *in situ* generation of NB diazonium salt, NB was dissolved in 0.5 M HCl. Ice-cold sodium nitrite aqueous solution was added into this mixture drop by drop. Final concentrations of NB and $NaNO_2$ were 1 mM and 2 mM, respectively. After the mixture was stirred for 1 h at 0 °C, the polished GC electrode was immersed into this solution derivatized by cyclic voltammetry scanning from 0 to -1.0 V at 100 mV s^{-1} scan rate for 5 scans. The resultant modified GC electrode was rinsed with phosphate buffer solution several times and sonicated for 60 s to remove the loosely adsorbed NB molecules. The NB modified electrode was then utilized for the fabrication of GOx/NB modified electrode by drop casting 10 μl of 3 mg ml^{-1} GOx solution on NB modified electrode and then dried at room temperature.

3. Results and discussion

3.1. Electrochemical reduction of NB diazonium salt onto glassy carbon electrode

The strategy used to modify the surface of the electrode with NB is shown in Scheme 1. The grafting possibility of NB molecules on GC electrode was investigated using the electrochemical reduction of *in situ* generated diazonium salt. Fig. 1 shows the cyclic voltammograms (CVs) recorded under *in situ* conditions using a scan rate of 100 mV s^{-1}. The first scan shows a broad irreversible wave at $E_p = -0.45$ V, indicating that an irreversible reaction is associated with the reduction of diazonium function and this is the loss of dinitrogen and formation of radicals. In further scans, the wave disappears nearly completely and this is because of the covalent attachment of very reactive radicals to the GC surface and formation of an organic layer blocking further reduction of diazonium salt [7]. After washing the modified electrode with buffer solution and thorough 60 s ultrasonic treatment, the resulting voltammogram in phosphate buffer (pH 7.0) shows a reversible well-defined peak (Fig. 2). As can be seen, the electrode surface is covered with an electroactive film that shows anodic and cathodic peaks at −0.08 V and −0.15 V, respectively. The peaks can be attributed to the redox process of attached NB. When no potential is applied to the GC electrode immersed in the diazonium salt solution, no redox peak is observed in phosphate buffer, indicating that the spontaneous grafting of the diazonium is negligible.

3.2. Cyclic voltammetry and electrochemical impedance spectroscopy analysis

The surface of the NB modified GC electrode was probed using cyclic voltammetry and electrochemical impedance spectroscopy (EIS). The CVs of 100 μM $[Ru(NH_3)_6]^{3+/2+}$ as positively charged redox probe were recorded in pH 2.0 and pH 10.0 phosphate buffer solution on bare and NB modified GC electrode. The results in Fig. 3 shows that in pH 2.0, after immobilization of NB on the electrode surface, the peak current (I_p) of probe is decreased significantly. This behavior can be attributed to the electrostatic repulsion between protonated and positively charged NB molecules on the electrode surface and $[Ru(NH_3)_6]^{3+/2+}$ redox probe in solution. But in pH 10.0, the NB molecules on the electrode surface have no charge, so the redox peak of probe is similar for bare and NB modified electrode.

EIS is a powerful and informative technique for probing the interface features of the modified electrodes. In the current work, the EIS was applied for the recognition of NB modified GC electrode. The measurements were performed in the blank phosphate buffer solution at pH 7.0; the results are presented in Fig. 4. The plot includes a vertical line for the bare GC electrode, which is an indicative of a capacitive effect, suggesting no faradic reaction at the electrode interface, implying the behavior of an ideally polarized interface. But for the NB modified electrode that shows almost a deformed semicircle, related to the faradic reaction at the interface of this electrode which is because of the attached NB molecules on the surface.