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A single-layer structured microbial sensor for fast detection of biochemical oxygen demand

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ABSTRACT

In this paper, a novel biochemical oxygen demand (BOD) sensor based on a single layer of immobilized microorganisms on electrode for fast detection is investigated. Instead of the previous two-layer structure of oxygen permselective membrane and microorganism membrane, a single layer structure using electrodes as the support for microorganism immobilization has been studied. Amino functional groups on the surface of microorganisms have been bonded with carboxyl modified electrodes. Without extra supports for immobilization, mass transfer resistances are reduced, which facilitates fast current response. *Bacillus subtilis* cells with high biodegradation ability are obtained after 12 h cultivation. The immobilization process has been investigated by CV and EIS. *B. subtilis* cultivation time, response time and linear range have been optimized. The proposed sensor can reach equilibrium in 5 min which has the advantage for real-time BOD determination in the future. The linear range is 5–30 mg/l with correlation coefficient of 0.978 and limit of detection of 1.65 mg/l.

1. Introduction

Biochemical oxygen demand (BOD) is an international regulatory index for defining organic water pollution and indicating water environment quality [1,2]. The standardized method for BOD measurement is the 5-day method (BOD_5), which obtains BOD results by measuring the oxygen consumption of microorganisms' biodegradation in five days under carefully controlled conditions. However, this method requires complicated procedures, accurate controls and most importantly five days, which make automatic and real-time BOD monitoring unavailable. Karube and Matsunaga [3] developed the first microbial sensor for fast BOD detection in 15 min. Since then, researches into BOD microbial sensors for fast measurement have attracted extensive attention.

A BOD microbial biosensor is an integrated device containing a biorecognition element coupled with a transducer. Microorganisms used as biorecognition element require low selectivity and high biooxidation activity over a wide range of organics. Various strains have been studied including *Trichosporon cutaneum* [4,5], *Bacillus subtilis* [6], *Pseudomonas putida* [7,8], *Pseudomonas fluorescens* [9–11], *Escherichia coli* [12,13], *Saccharomyces cerevisiae* [14–16], activated sludge [17,18], BODseed [19] etc. Apart from selection of microorganisms, the immobilization method plays an important role in biosensors' stability and reproducibility. Compared with suspended microorganisms, immobilized microorganisms provide improved stability, repeatable utilization, protection against environmental stresses and toxicity, and no need for separation from samples [20–23]. A commonly used method is the encapsulation of microorganisms between gas permeable membrane and cellulose membrane [24]. Another widely studied method is entrapment which creates a cell immobilization matrix [5,19,25–27]. However, these methods usually bring in large mass transfer resistances as cellulose membranes and sel-gel have been used. Thus, a proper method for microorganism immobilization and BOD detection needs to be studied. As for the transducer, many dissolved oxygen detections employ electrochemical methods [19,20,28] for simple operation, high sensitivity and selectivity, and extension of application after modification. However, the oxygen permselective membrane (most commonly Nafion membrane) covered on the electrode needs to be replaced in a period of time to avoid blockage and ensure sensor's validity, which brings loss of reliability. Taking

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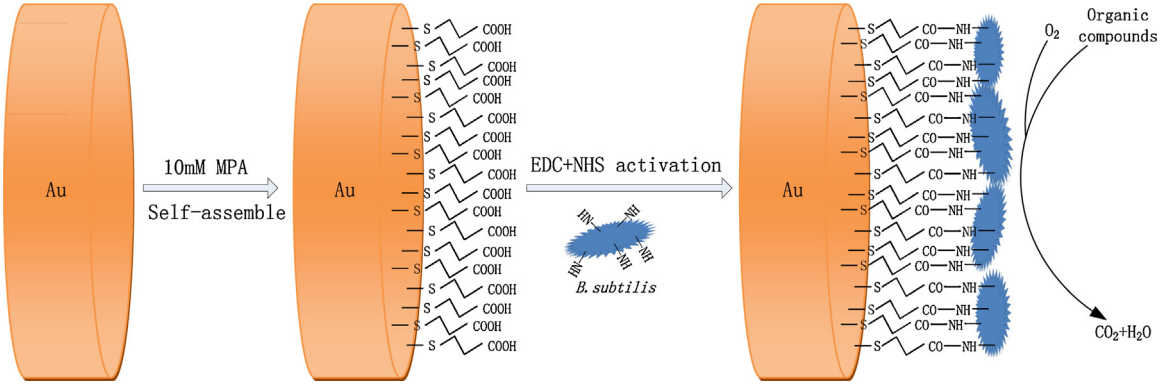

Scheme 1. The procedure of *B. subtilis*' immobilization on gold electrode.

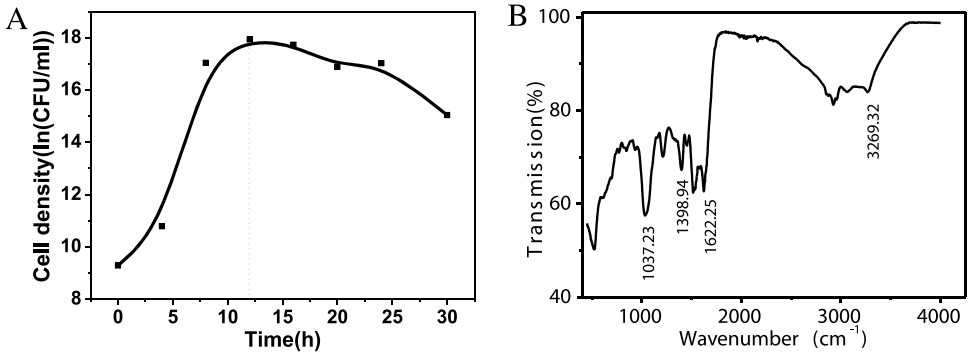

Fig. 1. The growth curve (A) and infrared spectrum (B) of *B. subtilis*.

whole microbial sensor into consideration, two-layer structure of oxygen permselective membrane and microorganism membrane increases mass transfer resistances, which can be simplified.

Self-assembled monolayers (SAMs) have been widely studied for their applications in biosensors and biomolecular electronics [29]. By designing the functional groups in the alkyl chain and at the chain terminal, SAMs can form highly ordered and oriented interfaces, which are promising in the integration of biomaterials with solid-state devices. Besides, the biomimetic and biocompatible nature of SAMs facilitates their developments in biochemical sensing [30]. The advantages of self-assembly immobilization also include rapid response and easy regeneration [31]. Different types of biomaterials [32–36] have been chemically bonded with the electrodes based on SAMs. However, immobilization of microorganisms on electrodes based on SAMs has seldom been studied to our knowledge. Actually, as many literatures [37,38] have reported, there are many functional groups including carboxyl, amino and hydroxyl on the surface of microorganisms, which can be utilized to covalently bond with the well-defined terminal groups of SAMs. Thus, firm immobilization of microorganisms on gold electrode can be formed. In addition, *B. subtilis* was selected as the biorecognition element due to its good biooxidation ability to many kinds of organics and tolerance to negative environmental factors.

In this paper, *B. subtilis* was chosen to be the recognition element. 3-mercaptopropionic acid was self-assembled on gold electrodes, leaving carboxyl functional groups exposed for further immobilization. The amino groups on *B. subtilis* surface were covalently bonded with activated self-assembled carboxyl groups for firm connection. No toxic reagents reacted with bacteria and no extra cellulose membranes were brought in. Thus, the activity of microorganisms was retained and diffusion was facilitated. Optimal cultivation time, response time and linear range were studied. Replacing two-layer structure with directly immobilizing microorganisms on the surface of electrodes obviously simplified sensor's structure, shortened diffusion path of reagents and reduced diffusion resistance, which led to less response time.

2. Methods

2.1. Reagents

Cells of the strain *B. subtilis* were obtained from the Institute of Microbiology, Chinese Academy of Sciences, and stored at 4 °C. 3-mercaptopropionic acid (MPA, $HOOCCH_2CH_2SH$), dichloroethane (EDC), *N*-hydroxysuccinimide (NHS) and fluorescein diacetate (FDA) were purchased from Sigma & Aldrich. MPA solution of 10 mM was dissolved in ethanol (99.7%). Ferricyanide solution (2 mM) was prepared with 0.1 M KCl solution. Phosphate buffer solutions (PBS, 5 mM, pH 7.0) was prepared with 5 mM KH_2PO_4 and 5 mM Na_2HPO_4. Dissolved oxygen (DO) solutions were prepared by dissolving Na_2SO_3 in PBS. The BOD standard solution (150 mg/l glucose and 150 mg/l glutamic acid, GGA) was prepared. GGA solutions of different concentrations were prepared by appropriate dilutions. Deionized water was used throughout the experiment.

2.2. Apparatus

Electrochemical experiments were performed with the Gamry Reference 600 electrochemical measurement system (Gamry Instruments Co., Ltd., USA). Measurements were carried out with a three-electrode cell consisting of a gold-disk (d = 1 mm) working electrode, a Pt-disk (d = 3 mm) counter electrode and a commercial saturated Ag/AgCl (commercial reference electrode, CRE). DO concentration was measured by commercial DO meter (Eutech CyberScan DO110 Dissolved Oxygen Meter, Vernon Hills, IL, USA) at 25 °C. The optical microscopy images were carried out at