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Short communication

A novel method to directionally stabilize enzymes together with redox mediators by electrodeposition

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ABSTRACT

This paper depicts a novel method to directionally stabilize enzymes together with redox mediators by electrodeposition. Chitosan was used as a stabilizing matrix. By electrochemical removal of local H^+, chitosan close to working electrode became locally insoluble, and enzymes and redox mediators in chitosan were stabilized. The microelectrode on home-made microelectrode array (MEA) served as the working electrode. Three model enzymes––horseradish peroxidase (HRP), glucose oxidase (GOD), and glutamate oxidase (GlOD)––were used to fabricate different biosensors, and the redox mediator model was a poly(vinylpyridine) complex of $Os(bpy)_2Cl$ and a diepoxide (PVP-Os). Biosensors fabricated by the method exhibited very high performance. For HRP biosensor fabricated by this method, the sensitivity was 5.274 nA μM^{-1} mm^{-2}, with linear detection range (LDR) of 2–220 μM and limit of detection (LOD) of 1 μM ($S/N=3$); for GOD biosensor, the sensitivity was 2.65 nA μM^{-1} mm^{-2}, with LDR of 4–500 μM and LOD of 2 μM ($S/N=3$); for GlOD biosensor, the sensitivity was 0.33 nA μM^{-1} mm^{-2}, with LDR of 4–500 μM and LOD of 2 μM ($S/N=3$). Since this method is very simple and especially suitable for directionally introducing enzymes and redox mediators onto microelectrode without contaminating other sites in the same microenvironment, it could be used for fabricating in vivo or in vitro 2nd generation biosensors in μm-scale, especially in neuroscience.

1. Introduction

There has been substantial progress in the past decade in electrochemical biosensors of biomolecules (especially enzymes; Li et al., 2009). With development of the industry, biosensor has involved 3 generations (Wang, 2008): (1st) classical Clark biosensor which is based on O_2 as mediator to oxidize reduced enzyme (Clark and Lyons, 1962); (2nd) mediator biosensor which is made by replacing mediator from O_2 to other redox compounds (Cregg and Heller, 1991); and (3rd) direct electron-transfer biosensor which conducts electron directly from enzyme to electrode without mediators (Xiao et al., 2003). Among the three, the 2nd generation of biosensor is applied most widely, because of its lack of need for O_2, lower working potential to reduce interference, much faster electron transfer so as to give larger LDR and higher sensitivity (Wang, 2008).

Immobilization of enzyme is one of the most difficult tasks when making a biosensor. Many methods have been developed to conveniently and effectively immobilize enzyme onto electrode. An extensive literature on enzyme immobilization techniques applied to biosensors has been reviewed (Sassolas et al., 2012; Chaniotakis 2004; Guilbault, 1984). Most common are cross-linking of the enzyme with an inert protein such as BSA using glutaraldehyde, simple adsorption of enzyme to electrode surfaces or covalent binding of enzyme to insoluble carriers such as nylon or glass. Enzymes immobilized in these ways often show improved stability relative to enzyme in solution. Another immobilization technique involves bulk modification of an electrode material where enzyme is mixed with a material such as carbon paste, which serves as both the enzyme immobilization matrix and the electroactive surface (Gorton, 1995). In spite of these techniques, the most widespread method for immobilizing enzymes and mediators is directly-dropcoating or screen printing. In laboratories, the dropcoating method is utilized more frequently, while in industry production, screen printing shows more advantages, such as being suitable for mass production, easy to produce graphically, and more economical (Wang et al., 2012).

Even though the methods used to introduce enzyme onto the surfaces of the electrodes have been highly developed, there are some issues that require further investigation and improvement. In recent years, along with the need for specialized biosensors, biosensors have evolved to be smaller and smaller. For example, biosensors are widely applied to detect chemical signals generated

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from minute tissues or even cells (Song et al., 2012). The working electrodes of such biosensors must be very tiny, close to ten or tens of μm. In such small ranges, dropcoating and screen printing are not suitable for immobilizing enzyme: for dropcoating, it is hard to generate an enzyme mixture drop less than 10^{-13} L to drop-coat one electrode of the MEA; for screen printing, the electrode of interest can be hardly distinguished and modified separately. Ammam et al. used a method called alternating current electrophoretic deposition to directly immobilize GOD and lactic oxidase by immersing working electrode in high concentration enzyme and applying alternating potential (Ammam and Fransaer, 2009, 2010;). Since there were no redox mediators, the biosensors they obtained were 1st generation ones.

Chitosan (CS), a polysaccharide-based gel, has very interesting properties. CS with abundant amino groups exhibits good biocompatibility (Liu et al., 2005) and excellent film-forming ability originating from its protonation and solubility in slightly acidic solution and from insolubility in solution with pH over its pKa (6.3; Pillai et al., 2009). Therefore, it is a very suitable matrix for immobilizing bioactive molecules and constructing biosensors. Biosensors utilizing CS as matrix were developed to detect glucose (Shi and Ma, 2010; Ren et al., 2012), NH^{4+} (Azmi et al., 2009), phenol (Wang et al., 2002), choline (Sassolas et al., 2009), ethanol (Lee and Tsai, 2009), lactate (Cui et al., 2007; Tsai et al. 2007), and cholesterol (Tsai et al., 2008). Meanwhile, because of its low fluidness, CS can become locally insoluble once pH in the very site is over its pKa (Pang and Zhitomirsky, 2005). Du et al. developed a simple method to fabricate a CS–gold nanoparticles film by electrodeposition and applied the film to sense glucose (Du et al., 2007). Herein, this paper depicts a novel method to directionally stabilize enzymes together with redox mediators by increasing the local pH of soluble CS close to electrodes. This method was developed to effectively fabricate 2nd generation biosensors. Especially for directionally immobilizing enzymes and redox mediators onto microelectrode without contaminating other sites in the same microenvironment, the method was rather useful. Three model enzymes—GOD, HRP, and GlOD—were involved to fabricate different biosensors, and the redox mediator was PVP-Os. Amperometric measurement results show that biosensors fabricated by the method exhibit very high performance, both in sensitivity and LOD. The performance of the obtained biosensors with respect to LDR and response time is presented and discussed.

2. Experimental

2.1. Materials

Poly(4-vinylpyridine), $Os(bpy)_2Cl_2$, 2-bromoethylamine hydrobromide, and CS were purchased from Sigma-Aldrich Co., Ltd. Ethylene glycol and DMF were obtained from Beijing Chemical Reagents Company (Beijing, China). Other chemicals were of analytic grade and were used as received unless stated otherwise. Water was purified through a Michem ultrapure water apparatus (Michem, Chengdu, China, resistivity > 18 MΩ). The phosphate buffer saline (PBS, 0.1 mM Na_2HPO_4–NaH_2PO_4–KCl, pH 7.4) was prepared from a PBS tablet (Sigma).

The reference electrode that was used for entrapment of PVP-Os and enzyme was a typical KCl saturated Ag|AgCl electrode, and the Ag wire was inserted in a glass capillary with pulled terminal.

2.2. Apparatus

Stereomicroscope image was taken with a Leika M205C stereomicroscope (Leika, Germany). All electrochemical measurements were performed on a CH Instruments 660A electrochemical Workstation (CHI-660A, CH Instruments, Texas, USA).

2.3. Preparation of PVP-Os

The PVP-Os was prepared according to Cregg and Heller (1991)). Briefly, 0.494 g of $Os(bpy)_2Cl_2$ and 0.430 g of poly(4-vinylpyridine) (4.09 mequiv) were heated in nitrogen under reflux in 18 mL of ethylene glycol for 2 h. After the solution was cooled to room temperature, 30 mL of DMF and 1.5 g of 2-bromoethylamine hydrobromide were added, and the solution was stirred overnight. Acetone was added to obtain the precipitate PVP-Os, which was dried and stored in a dark glass bottle, and could be stable for more than 2 years.

2.4. Entrapment of enzyme and PVP-Os

In a typical experiment, HRP (5 μL, 200 U/mL) and PVP-Os (1 μL, 50 mg/mL) were added to 25 μL of CS solution (Grabar et al., 1995) 1.0 wt%) in microtubes separately, and mixed by a micropipettor for 1 min to give a dark brown and slightly viscous mixture. Then 5 μL of H_2O_2 (3 wt%) was added into the mixture and mixed up. 5 μL of the obtained mixture was drop-coated onto the homemade microelectrode array (MEA; Song et al., 2012), and the reference electrode bound with a Pt wire as auxiliary electrode was hanged on the top of the MEA with both terminals immersed in the mixture (Scheme S1). Together with one electrode of the MEA as work electrode, a typical three-electrode system was formed. A working potential of −0.35 V was applied to the system for a duration of 100 s. A flush of pure water was used to quickly remove the remaining mixture, and then the MEA was dried with flow of air. The electrodeposited MEA (Ed-MEA) was stored on the top of PBS (pH 7.4) with saturated humidity at 4 °C for at least 24 h before use. Changing the enzyme from HRP to others, such as GOD and GlOD, would yield biosensors for detecting glucose and glutamate, respectively.

For comparison, the same MEA was modified with similar concentration of HRP (200 U/mL), PVP-Os and CS by drop-coating 1 μL of the mixture onto the MEA without electrodeposition. After drying with slow flow of air at room temperature, the drop-coated MEA (Dc-MEA) was also stored on the top of PBS with saturated humidity at 4 °C for at least 24 h before use.

2.5. Electrochemical recording

Electrochemical measurements were performed on a CHI-660A connected to a PC. A traditional three-electrode system with a platinum wire as the counter electrode, Ag|AgCl (saturated KCl) as the reference electrode and a modified Pt microelectrode of the modified MEA as the working electrode was used. Once the background current reached a steady-state value, different concentrations of H_2O_2 or other reagents were injected into the PBS and response currents were recorded with time at a constant applied potential. All experiments were conducted at room temperature.

3. Results and discussion

3.1. Electrodeposition process

A typical electrodeposited HRP/PVP-Os@CS membrane is shown in Fig. 1A, in which the electrodeposition time is 100 s. The role of H_2O_2 in the mixture of enzyme, CS and PVP-Os is to assist soluble CS become locally insoluble, so as to immobilize enzyme and PVP-Os onto MEA. When the potential of working