



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.

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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 O₂ as mediator to oxidize reduced enzyme (Clark and Lyons, 1962); (2nd) mediator biosensor which is made by replacing mediator from O₂ 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 O₂, 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), $\text{Os}(\text{bpy})_2\text{Cl}_2$, 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 $> 18 \text{ M}\Omega$). 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 $\text{Os}(\text{bpy})_2\text{Cl}_2$ 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

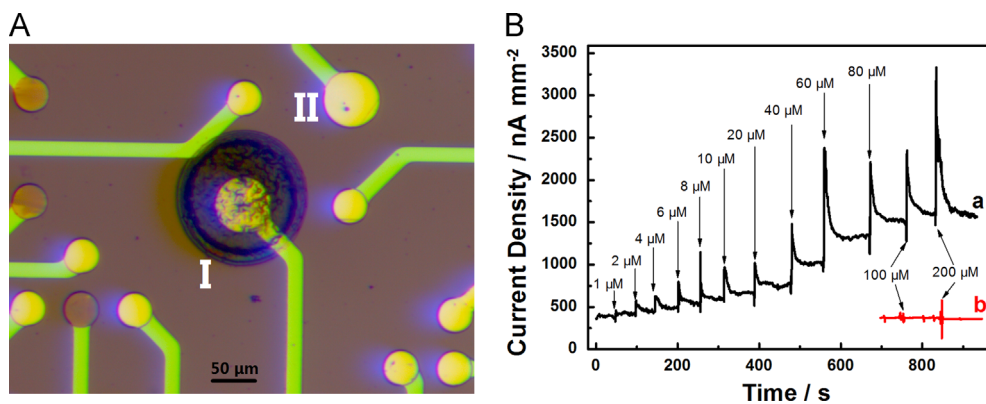


Fig. 1. (A) Stereomicroscope image of the Ed-MEA. (B) Current–time responses to successive injections of different concentrations of H₂O₂ in static PBS: the electrode site I (a, black) and II (b, red) in (A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

electrode is low enough to provide enough electrons, the following electrochemical reactions occur:



The reactions can happen easily, and the needed applied potential can be as high as -0.3 V. Without H₂O₂, in which case the reaction is as Eq. (2) shows, H⁺ cannot be removed unless the applied potential is less than -0.8 V. At such lower potential, the Os²⁺ in PVP-Os may not be stable. What is more, the product of Eq. (2) is gas, and the resulting membrane would be full of minute bubbles. Therefore, the introduction of H₂O₂ is more effective.

The selection of CS as the matrix for entrapment of PVP-Os and enzyme is attributed to the outstanding properties of CS, which is a linear polysaccharide with many amino groups that exhibits good biocompatibility (Liu, et al., 2010). Its ability to form films is excellent due to its protonation and solubility in slightly acidic solution, and its stability arising from insolubility in solution with pH > pKa (6.3). The membrane formed due to the film generating ability of CS cannot be easily removed by water-rinsing, but can easily dissolve in acidic condition. This is because CS in the membrane would be reprotonated in acids, thus becoming dissolvable. Meanwhile, since the pH of the electrodeposition mixture was much less than 6.3, the newly-prepared membrane might re-dissolve in such an acidic condition. As a result, when preparing, the excess mixture should be removed as quickly as possible by a flush of deionized water to guarantee an appropriate membrane.

Because of the presence of the amino group in the CS, the membrane shows an excellent hydrophilicity, which enhances the matter exchange between the membrane and the solution. Meanwhile, the good attachment of the CS on the electrode makes the electron transfer between the membrane and the electrode more efficient. The thickness and the area of the membrane could be controlled by the electrodeposition time. In a word, the larger the time of electrodeposition procedure, the thicker and larger the membrane. However, the thickness and the electron transfer efficiency of the membrane are inversely related. Thicker membrane means more loaded enzyme that would increase the Faraday current, but also means lower electron transfer efficiency because electrons cannot get through the membrane easily.

As described in the Experimental section, the electrodeposition process is based on the deprotonization of CS. As a result, whether the electrodeposition can succeed or not is principally determined by (1) the pH of the mixture, (2) the concentration of the H₂O₂, and (3) the applied potential for electrodeposition, all of which might not be considered independently. The lower the pH value of the mixture, the higher the concentration of proton in the mixture,

and the harder the electrodeposition of the mixture; the higher the concentration of the H₂O₂ in the mixture, the higher the consumption of protons in the CS, and the easier the electrodeposition; the higher the applied potential for electrodeposition, the quicker the removal of protons in the solution. However, there are some limits for the decisive factors. It is hard to obtain transparent mixture when pH of used CS is higher than 6.0; higher concentration of H₂O₂ means higher oxidation ability, which would damage enzymes and PVP-Os; electrodeposition at lower potential would make the membrane full of bubbles through Eq. (2), while higher potential would not be sufficient to make the mixture insoluble. After a series of experiments (Fig. S1), an applied potential of -0.35 V, a final H₂O₂ concentration of 80 mM, and a mixture pH of 5.5 were selected.

The enzyme-immobilization method could be easily applied to different kinds of electrodes, including μm-scale microelectrodes, such as MEA on a glass substrate (Fig. 1A) or silicon plate, as well as the normal millimeter-scale electrodes, such as Pt disk and column electrodes.

3.2. Biosensing performance of HRP/PVP-Os@CS biosensor

To evaluate the advantages of the PVP-Os and enzyme modified MEA, the current–time response of Ed-MEA to successive injections of different concentrations of H₂O₂ was obtained. For electrode I in Fig. 1A, as shown in Fig. 1B-a, the injections could invoke quick and dramatic responses of the current, and it took a relatively long time (~ 20 s) for the responses to be stable. This may be because the injections were performed by adding small amounts of high concentrations of H₂O₂ into bulk PBS. Once the solution was added, a rather dramatic current response was reached. Along with the diffusion of the solute, the current response tended to be stable. Therefore, more exactly speaking, Fig. 1B-a actually exhibits the diffusion process of H₂O₂. The response time of the modified biosensor for H₂O₂ would be much less than 20 s. What is more, the experiments have been performed at a fixed potential of 0 V (vs. Ag/AgCl), which means that many electroactive compounds cannot become interferents when using the biosensor to detect H₂O₂. For a comparison, a bare MEA electrode and an MEA electrode modified with only CS and HRP by the same electrodeposition method showed no remarkable response to the equivalent concentration of H₂O₂, at the potential of 0 V. Therefore, the employment of PVP-Os in the membrane played a very important role as a mediator. Comparatively, when changing the working electrode to electrode II, even the increase of H₂O₂ concentration as high as 200 μM cannot invoke a discernable response (Fig. 1B-b). Therefore, this method can be used to

selectively modify the electrode of interest in MEA without contaminating other sites.

Also, for comparison, the Dc-MEA was used to perform the same experiment as described above. As shown in Fig. 2, the results of the experiment, the sensitivity ($5.274 \text{ nA } \mu\text{M}^{-1} \text{ mm}^{-2}$) of the Ed-MEA (curve a) was 2.3 times more than that ($2.291 \text{ nA } \mu\text{M}^{-1} \text{ mm}^{-2}$) of the Dc-MEA (curve b). These results prove that the Ed-MEA can enhance the current response. This might result from the difference of ingredient between the two coatings. In the process of electrodeposition, the mixture close to the electrode became insoluble because of the consumption of H^+ , and the congealed part of the mixture attached tightly to the electrode. After removal of the surplus mixture, the remaining part had a relatively high pH value, say, a low H^+ concentration. On the other hand, the membrane on the electrode modified with Dc-MEA did not experience such H^+ -diminishing process; as a result, the maximum part of H^+ that made the membrane soluble still remained in the membrane. The nuance between the two membranes resulted in significant difference in the response when sensing H_2O_2 . When the Dc-MEA was immersed in PBS, the membrane showed a significant expansion under the stereomicroscope, leading to the increase of thickness of the membrane. As described above, the thickness would lower the electron transfer efficiency. On the other side, the membrane of Ed-MEA did not expand after immersed in PBS; in other words, with the same amount of enzymes, the membrane of Ed-MEA would be much thinner than that of Dc-MEA. Expansion of the membrane caused by immersing Dc-MEA in PBS buffer may give a plausible proof.

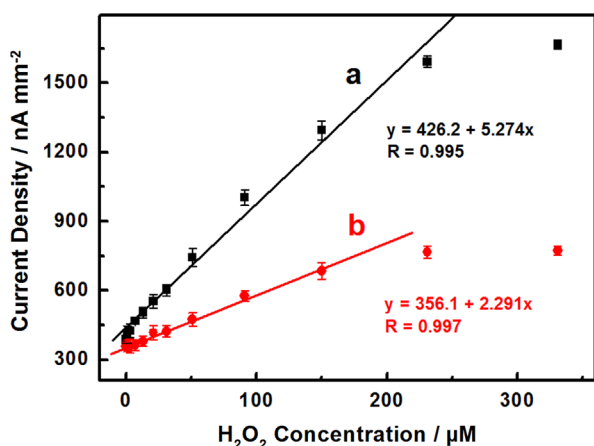


Fig. 2. Calibration curve of Ed-MEA modified with HRP (a) and Dc-MEA modified with HRP (b). All electrochemical measurements were performed in 0.1 mM phosphate buffer, pH 7.4.

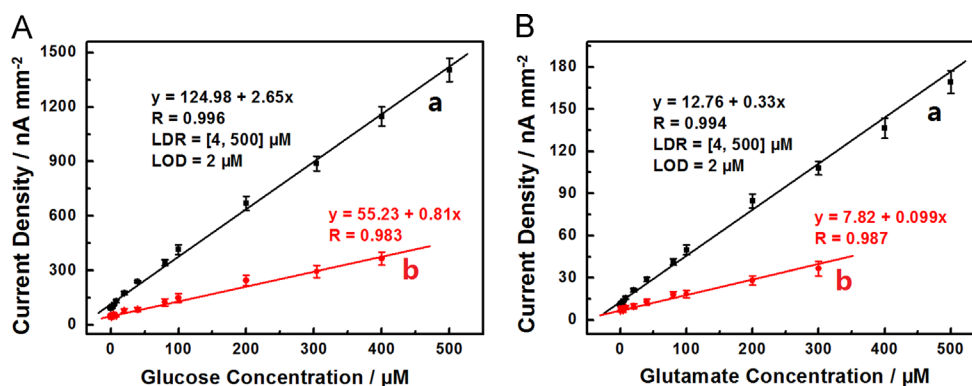


Fig. 3. Calibration curve of Ed-MEA modified with GOD (A-a) and with GIOD (B-a), and calibration curve of Dc-MEA modified with GOD (A-b) and with GIOD (B-b). Data were calibrated from 5 replicates.

3.3. Biosensing performance of GOD/PVP-Os@CS and GIOD/PVP-Os@CS biosensor

If the HRP in the membrane is changed into other enzymes, such as GOD and GIOD, novel glucose and glutamate biosensor would be fabricated. Fig. 3A-a and B-a respectively shows the glucose and glutamate biosensors modified by the same method. The role of PVP-Os in the glucose and glutamate biosensors is different from the one in the H_2O_2 biosensor. It is well known that the by-products of both GOD catalyzing glucose and GIOD catalyzing glutamate are H_2O_2 , and both of the two reactions need O_2 to oxidize the GOD and GIOD in reductive state to oxidative state. When PVP-Os involves in the reaction, the Os^{3+} in PVP-Os can play the same role as that of O_2 to oxidize the reduced GOD, and Os^{3+} is reduced to Os^{2+} . To regenerate the oxidative Os^{3+} , a relatively high potential must be applied to the sensor. As a result, the work potential for the glucose sensor must be high enough to oxidize the Os^{2+} to Os^{3+} , which is fixed at +0.35 V (vs. Ag/AgCl). The glucose and glutamate biosensors prepared by the Dc method show similar comparisons with the proposed Ed glucose and glutamate biosensors respectively. The performances of Dc method biosensors are shown as the curves of Fig. 3A-b and 3B-b, showing lower sensitivity, narrower LDR and lower LOD compared with Ed method biosensors.

3.4. Real samples detection and reproducibility

To examine the possible use of the proposed electrode in a practical application, experiments were studied in samples of milk, human serum, and SD-rats' Cerebrospinal Fluid (CSF) for determination of H_2O_2 , glucose and glutamate respectively. The direct detection results and spiked results are shown in Supplementary materials, Table S1–S3. Both H_2O_2 and glucose biosensors give exact recovery (within $100 \pm 4\%$) and good R.S.D. ($< 4.0\%$). Glutamate biosensor shows a little higher recovery and R.S.D., which may result from the relatively low glutamate concentration in CSF samples. However, the results of the three experiments show that the three biosensors can be applied for real samples detection.

The biosensors made by this method also showed good reproducibility and high stability. For example, for three independently prepared HRP biosensors, the relative standard deviation was less than 5% for the response of $50 \mu\text{M}$ H_2O_2 . The current response of biosensor retained is about 84% of its original response after a month.

4. Conclusion

A novel method to directionally stabilize enzymes together with redox mediators has been developed. The method can be utilized to

fabricate 2nd generation biosensors. Biosensors fabricated by the method exhibited very high performance. This method is very suitable for directionally immobilizing enzymes and redox mediators onto very small electrode without contaminating other sites in the same microenvironment, showing great potential for in vivo or in vitro detection of different chemical signals in very small scale, especially in neuroscience.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.07.062>.

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