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# Preparation of Porous Titania Sol−Gel Matrix for Immobilization of Horseradish Peroxidase by a Vapor Deposition Method

Jiuhong Yu and Huangxian Ju*

*Department of Chemistry, The State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, People's Republic of China*

**A new and facile vapor deposition method has been developed for the preparation of sol−gel matrix. This method was used to form a titania sol−gel thin film and to immobilize horseradish peroxidase (HRP) on a glassy carbon electrode surface for the production of an amperometric hydrogen peroxide biosensor. This process prevented the cracking of conventional sol−gel-derived glasses. The morphologies of both titania sol−gel and the enzyme membranes were characterized using scanning electron microscopy and proved to be chemically clean, porous, and homogeneous and to have a very narrow particle size distribution. The sol−gel-derived titania-modified electrode retained the enzyme bioactivity and provided for long-term stability of the enzyme in storage. In the presence of catechol as a mediator, the sensor exhibited a rapid electrocatalytic response (less than 5 s), a linear calibration range from 0.08 to 0.56 mM with a detection limit of 1.5 $\mu$M and a high sensitivity (61.5 $\mu$A mM$^{-1}$) for monitoring of $H_2O_2$. Effects of pH and operating potential were also explored for optimum analytical performance by using the amperometric method. The apparent Michaelis−Menten constant of the encapsulated HRP was 1.89 ± 0.21 mM.**

Most biological macromolecules are highly efficient at recognizing specific molecules or catalyzing reactions in aqueous biological media. Efforts are being made to utilize these reagents for the preparation of biosensors by immobilizing them in alternative matrixes. These matrixes must preserve the native stabilities and reactivities of biological macromolecules for sensing.[1] Recently sol−gel-derived materials have emerged as attractive matrixes well suitable for immobilization of biomolecules. Silica glasses, the main kind of these materials, obtained by a sol−gel process can retain the function of those biomolecules to a large extent.[2−6] They are usually prepared under ambient conditions and exhibit tunable porosity, high thermal stability, chemical inertness, and negligible swelling in aqueous and nonaqueous solutions. For example, Carturan and co-workers presented a method to immobilize plant cells by exposing the cells to gas-phase silica sol−gel procursors.[7] These materials are also particularly useful for immobilization of enzymes to fabricate electrochemical biosensors.[8−12] Numerous studies of enzyme encapsulation indicate that the enzyme can be firmly trapped within a silica gel or glass matrix and the pores in the matrix permit substrates to access the enzyme.[13,14] However, the silica sol−gel matrixes have some notable drawbacks, including fragility; hydrolysis at high acidity, and complicated procedures, which result in the loss of enzyme stability[2] and limit their applications in electrochemical biosensors.[15] On the other hand, nonuniformity of the pores in silica sol−gel matrixes causes cracks and fractures in dry monolithic sensors upon immersion in water.[15,16] To solve this problem, some researchers used surface-active drying control chemical additives such as Triton-X and quaternary ammonium compounds[17] or copolymers[8] to prevent the drying fractures of the sol−gel film.[17] As is well known, surfactants are detrimental to the enzyme and, thus, limit the sensitivity of such biosensors.[8]

Some non-silica sol−gel materials have been developed to immobilize enzymes for the construction of biosensors[18,19] and to

* Corresponding author. Fax: +86(25)3317761. Tel: +86(25)3593593. E-mail: hxju@jlonline.com.

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synthesize new catalysts for the functional devices.[20−24] Deng et al. proved that alumina sol−gel was a suitable matrix to improve the immobilization of tyrosinase for detection of trace phenols.[18,19] Titania is another kind of non-silica material easily obtained from the sol−gel process.[20−22] It is of great interest for potential applications in photovoltaic cells,[23] electrochemical photolysis of water, and semiconductors.[24] For analytical purposes, the porous titania microspheres have been synthesized as HPLC packing material.[20] Yamada et al.[21] studied the photocurrent response of porphyrin−titania−fullerene assemblies by using self-assembly and surface sol−gel techniques. A sol−gel $Pt/TiO_2$ catalyst has been prepared for catalytic reduction of NO.[22] The sol−gel process has also been used to synthesize nanocrystalline $TiO_2$ for preparation of charge-transfer sensitizers[25] and new catalysts[26] and for adsorption of proteins[27] because of the biocompatibility of $TiO_2$.[28] All these procedures need a calcination step at more than 300 °C. Thus, compared to the silica sol−gel technique, no titania sol−gel material obtained has been used for the direct immobilization of enzymes during the gelation process. An improved method recently reported is to mix titanium(IV) tetrabutoxide ethanol solution with tetraethoxysilane ethanol solution at very high acidity to prepare a silica−titania surface for immobilization of porphyrin−cobalt(II).[29] A coating of titania/hydroxyapatite can be obtained by adding hydroxyapatite−ethanol solution to a mixture of titanium isopropoxide and nitric acid.[30] Obviously, these processes use extremely acidic conditions to form titania sol−gel, which are also unsuitable for enzyme immobilization, particularly for enzymes sensitive to acid.

In this work, we report the first attempt to construct a titania sol−gel thin film through a novel method of vapor deposition in a biocompatible neutral medium. The sol−gel film formed has been used to immobilize horseradish peroxidase for preparation of an electrochemical sensor. Horseradish peroxidase is inexpensive and stable and has been extensively used for the preparation of $H_2O_2$ sensors based on silica sol−gel materials.[31−36] The vapor deposition method simplifies the preparation process of titania sol−gel containing immobilized enzyme and also avoids the side effects caused by acid catalysts and organic cosolvents needed for previous sol−gel processes. The resulting sensor shows rapid electrocatalytic response and high sensitivity for monitoring of $H_2O_2$, providing a promising platform for the development of biosensors, affinity supports, and immobilized enzyme reactors.

## EXPERIMENTAL SECTION

**Reagents.** Horseradish peroxidase (HRP, EC 1.11.1.7, RZ > 3.0, A > 250 units/mg) was purchased from Sigma Chemical. Titanium isopropoxide ($Ti(i\text{-}PrO)_4$) was obtained from Aldrich and catechol from Shanghai Chemical Reagent Co. (Shanghai, China). All other chemicals were of analytical grade and were used without further purification. All solutions were made up with twice-distilled water.

**Apparatus.** Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems) with a conventional three-electrode system with the enzyme electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference against which all potentials were measured. Cyclic voltammetric measurements were done in an unstirred electrochemical cell at 25 ± 0.2 °C. Amperometric experiments were carried out by applying a potential step of −100 mV to a stirred cell at 25 ± 0.2 °C. Aliquots of a standard solution of $H_2O_2$ were successively added to the solution. Current−time data were recorded after a steady-state current had been achieved. All experimental solutions were deaerated by highly pure nitrogen for 10 min, and a nitrogen atmosphere was kept over the solutions during measurements.

Scanning electron micrographs (SEMs) of titania sol−gel membranes and HRP/sol−gel membranes were obtained with a Hitachi X-650 scanning electron microscope (Hitachi Lid., Tokyo, Japan) at an acceleration voltage of 20 kV.

**Preparation of Enzyme Electrode.** Glassy carbon electrodes (diameter of 4 mm) were prepared by subsequent polishing with 1.0-, 0.3-, and 0.05-$\mu$m alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. The electrodes were then successively sonicated in 1:1 nitric acid, acetone, and doubly distilled water and then allowed to dry at room temperature.

For preparation of a horseradish peroxidase electrode, a HRP solution was first obtained by dissolving 5 mg of HRP in 1 mL of 0.02 M pH 7.0 phosphate buffer solution (PBS). A 10-$\mu$L aliquot of HRP solution was dropped onto the surface of a pretreated glassy carbon electrode. The electrode was then suspended vertically above titanium isopropoxide in a sealed flask kept at a constant temperature of 25 °C for 6 h. This resulted in absorption of saturate titanium isopropoxide vapor at 25 °C by the enzyme solution and slow formation of a titania sol−gel membrane through hydrolysis of titanium isopropoxide on the surface trapping the HRP in the membrane. Prior to electrochemical experiments, the electrode was rinsed thoroughly with doubly distilled water and kept in PBS at 4 °C.

## RESULTS AND DISCUSSION

**Cyclic Voltammetric Behavior of Enzyme Electrode.** Figure 1 shows the cyclic voltammograms of the sensor in 0.02 M phosphate buffer solution (pH 7.0). In the absence of catechol and $H_2O_2$, no response of the enzyme electrode is observed. The


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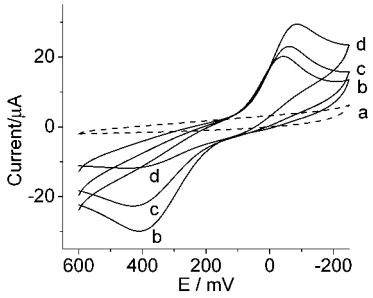


Figure 1. Cyclic voltammograms of a HRP enzyme electrode in (a) 0.02 M pH 7.0 PBS, (b) 0.02 M pH 7.0 PBS containing 1.0 mM catechol, (c) 0.02 M pH 7.0 PBS containing 1.0 mM catechol and 0.3 mM $H_2O_2$, and (d) 0.02 M pH 7.0 PBS containing 1.0 mM catechol and 1.0 mM $H_2O_2$. Scan rate, 100 mV $s^{-1}$.

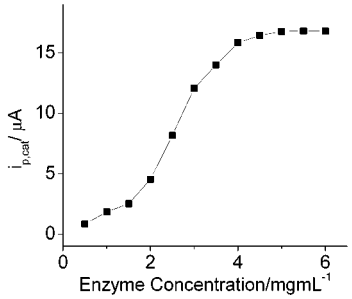


Figure 2. Effect of enzyme concentration for the preparation of the enzyme electrode on the voltammetric response of 1.0 mM. $H_2O_2$ in 0.02 M pH 7.0 PBS containing 1.0 mM catechol. Scan rate, 100 mV $s^{-1}$.

electrode displays a low background current. When 1.0 mM catechol is added to PBS, the cyclic voltammogram shows a couple of oxidation and reduction peaks for catechol (Figure 1b). With increasing scan rate, the peak-to-peak separation increases and the peak current is proportional to the square root of the scan rate, indicating a typical quasi-reversible behavior and no adsorption of the oxidized and reduced forms of catechol on the enzyme electrode surface. Upon addition of $H_2O_2$ to the solution, the reduction peak current increases dramatically and the oxidation peak current decreases dramatically (Figure 1c and d), indicating efficient electrocatalytic reduction of catechol to $H_2O_2$ at the enzyme electrode.

**Optimization of Enzyme Electrode Preparation.** The performance of the enzyme electrode mainly depends on two aspects: temperature and the amount of enzyme dropped on the electrode surface. Titanium isopropoxide is much more reactive with water than tetraethyl orthosilicate. Thus, it is difficult to prepare a titania sol−gel by direct hydrolysis of titanium isopropoxide due to the quick formation of the precipitate of titanium dioxide when contacting water. The vapor deposition method greatly decreases the hydrolysis rate, resulting in the formation of titania sol−gel without $TiO_2$ precipitation. Meanwhile, the temperature affects directly the vapor pressure of titanium isopropoxide, thus controlling the hydrolysis rate.

Experiments show that an enzyme electrode with maximum response is formed at a preparation temperature of 25 °C. Higher temperatures result in high vapor pressures of titanium isopropoxide and thus a rapid hydrolysis rate. At preparation temperatures higher than 25 °C, some $TiO_2$ powder formation is observed on the electrode surface. Thus, the enzyme cannot be effectively immobilized on the electrode surface. At lower temperatures, the rate of vapor deposition is slower than that of water volatilization, resulting in a poor yield of the hydrolysis product, titania sol−gel, which limits the amount of enzyme immobilized on the surface.

The amount of enzyme immobilized on the electrode surface is another important parameter. Figure 2 shows the effect of the concentration of enzyme solution dropped on the electrode surface on the voltammetric response of the enzyme electrode. With increasing enzyme concentration, the catalytic peak current increases and then tends toward a constant value. At enzyme concentrations greater than 5 mg $mL^{-1}$, the current reaches a maximum value, indicating a saturation of enzyme in the titania sol−gel thin film. Thus, a 5 mg $mL^{-1}$ HRP solution was used for preparation of the enzyme biosensor.

**Morphologies of Titania Sol−Gel Film and Enzyme Electrode.** The response of an enzyme electrode is related to its physical morphology. Thus, the surface morphology of the titania sol−gel matrix is an important factor affecting its performance. Figure 3 shows the morphologies of titania sol−gel (Ti/GCE) and enzyme electrodes (HRP/Ti/GCE) characterized by SEM. The SEM of the Ti/GCE membrane displays a chemically clean three-dimensional uniform porous structure. The aggregates of the titania sol−gel matrix on the electrode surface produced a very narrow particle size distribution (Figure 3a). This uniform open structure provids a significant increase of effective electrode surface for enzyme loading and a good preparation reproducibility of the enzyme electrode. When HRP is immobilized in the titania sol−gel matrix, the uniform open structure is retained and bright particles of HRP are observed (Figure 3b). The aggregates of the trapped enzyme molecules are distributed regularly and show an islandlike structure. This structure facilitates substrate access to the enzyme and results in a good amperometric enzyme electrode response.

**Effect of pH on the Response of HRP/Ti/GCE.** The effect of pH on the response of the $H_2O_2$ sensor depends on two factors: the activity of HRP and the peak potentials of the electrode reaction. Figure 4 shows the relationship between the catalytic peak current for HRP and catechol to $H_2O_2$ reduction and solution pH. Obviously, the optimal pH range is between 6.4 and 7.0 with the maximum peak current at pH 7.0. Similar results were also observed for soluble HRP,[37] indicating that the titania sol−gel matrix does not alter the optimal pH value for catalytic behavior of peroxidase. Plots of cathodic and anodic peak potentials versus pH yield two straight lines with slopes of $-43 \pm 2$ and $45 \pm 4$ mV $pH^{-1}$ in the pH range of 2.6−8.6, respectively. The changes of peak potentials result from the participation of $H^+$ in the electrode reaction for catechol. Considering a two-electron-transfer process for catechol, the number of $H^+$ participating in the electrode process is 2.

**Amperometric Response of $H_2O_2$ Sensor.** At an applied potential of around 75 mV, the reduction of $H_2O_2$ is already observed. Upon decreasing the applied potential from 75 to −150 mV, the steady-state current increases due to the increased driving force for the fast reduction of catechol at the lower potentials.

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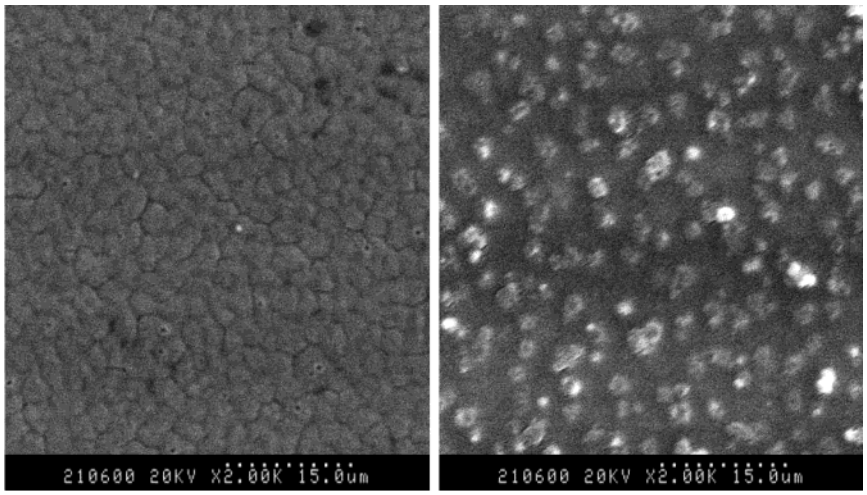


(a) (b)

Figure 3. Scanning electron micrographs of the glassy carbon electrodes coated with (a) titania sol−gel film and (b) titania sol−gel film doped with HRP.

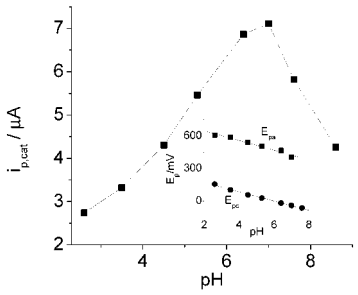


Figure 4. Effect of pH of detection solution on the catalytic peak current for the sensor in 0.02 M PBS containing 1.0 mM catechol and 0.5 mM $H_2O_2$. Inset: plots of peak potentials vs pH. Scan rate, 100 mV $s^{-1}$.

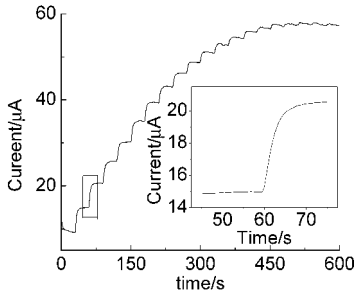


Figure 5. Typical current−time response curve for the sensor upon successive additions of 0.08 mM $H_2O_2$ in 0.02 M pH 7.0 PBS containing 1.0 mM catechol at an applied potential of −150 mV. Inset shows a magnification of the second addition of $H_2O_2$.

The reduced form of catechol reduces further the oxidized form of HRP to form reduced HRP, which catalyzes the reduction of $H_2O_2$.[38] The response approaches a plateau value at −150 mV, so we select this value as the working potential for amperometric determinations.

Figure 5 illustrates a typical current−time plot for the sensor upon successive step changes of $H_2O_2$ concentration. Upon addition of an aliquot of hydrogen peroxide to the buffer solution, the reduction current increases steeply to reach a stable value. The enzyme electrode achieves 95% of the steady-state-current in 5 s. The response rate is much faster than that reported for HRP in a silica sol−gel.[39]

**Calibration Curve of Sensor.** With increasing $H_2O_2$ concentration, the amperometric response of the enzyme electrode increases. The calibration range of $H_2O_2$ is 0.08−1.4 mM. The linear response range of the sensor to $H_2O_2$ concentration is from 0.08 to 0.56 mM with a correlation coefficient of 0.9979 ($n = 7$) and a detection limit of 1.5 $\mu$M at a signal-to-noise ratio of 3. In the linear range, the sensor has a high sensitivity of 61.5 $\mu$A $mM^{-1}$. Both fast response and high sensitivity are attributed to the uniform porous structure of the titania sol−gel matrix, which yields a very low mass transport barrier and results in a rapid diffusion of substrate from bulk solution to enzyme.

At higher $H_2O_2$ concentrations, the response shows a shape of that follows a typical Michaelis−Menten process. The apparent Michaelis−Menten constant ($K_M^{app}$) is calculated to be 1.89 ± 0.21 mM according to the Lineweaver−Burk equation.[40] The value of $K_M^{app}$ is less than that of the native system in buffer solution. The decrease is in agreement with that reported previously[39] and results from the use of catechol (hexacyanoferrate(II) in ref 39) as an electron mediator, which accelerates electron transfer between substrate and the active center of enzyme.

**Reproducibility and Stability of the $H_2O_2$ Sensor.** The reproducibility of the current response for the enzyme electrode was examined at a $H_2O_2$ concentration of 0.2 mM. The relative standard deviation is 1.6% for eight successive assays. The fabrication reproducibility of six sensors, made at the same electrode independently, shows an acceptable reproducibility with a relative standard deviation of 5.2% for the current determined at 0.2 mM $H_2O_2$.

When the enzyme electrode was not in use, it was stored in PBS at 4 °C. No obvious decrease in the response to $H_2O_2$ is observed after a two-month storage. After a 90-day storage period, the sensor retains 94% of its initial current response. The biosensor

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loses its sensitivity more rapidly if stored in air. This is attributed to the fast deactivation of HRP. Thus, titania sol−gel composite film is very efficient for retaining the activity of HRP and preventing it from leaking out of the film. This indicates that titania sol−gel prepared by a vapor deposition sol−gel method, a mild process, provids a biocompatible microenvironment around the enzyme to stabilize its biological activity to a large extent.[2] On the other hand, large quantities of hydroxyl groups in the sol−gel hybrid material can form strong hydrogen bonds. These hydrogen bonds and the intermolecular interactions between enzyme and specific sites of titania sol−gel prevent the enzyme from leaking out of the thin film.

## CONCLUSIONS

The preparation of titania sol−gel by the direct hydrolysis of titanium isopropoxide or other related compounds under ambient conditions is very difficult due to the rapid formation of a precipitate of titanium dioxide with the presence of water. This work develops a new vapor deposition method for the preparation of a titania sol−gel matrix and the immobilization of an enzyme in this matrix. The uniform porous structure of the titania sol−gel matrix results in a very low mass transport barrier, a high catalytic activity, and a fast response rate of the immobilized enzyme and provides a biocompatible microenvironment around the enzyme. This film is very efficient for retaining the enzyme activity and preventing its leakage out of the film. The biocompatibility of the titania sol−gel material means that this immobilization matrix can not only be used for HRP but also can be extended to other enzymes and other bioactive molecules, thus providing a promising platform for the development of biosensors.

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