ANALYTICAL SCIENCES SEPTEMBER 2006, VOL. 22

Notes

Application of the Nanogold-4,4′-bis(methanethiol)biphenyl Modified Gold Electrode to the Determination of Tyrosinase–Catechol Reaction Kinetics in Acetonitrile

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The reactivity of tyrosinase adsorbed on nanogold bound with 4,4′-bis(methanethiol)biphenyl monolayer self-assembled on a gold disk with catechol in a dipolar aprotic solvent, acetonitrile (AN), was studied by cyclic voltammetric and amperometric methods. Tyrosinase exhibited characteristics of a Michaelis–Menten kinetic mechanism. The tyrosinase attached to the nanogold continued to react with substrates in AN even when the water content was lower than 0.01 w/w%. The apparent Michaelis–Menten constant K_m of tyrosinase for catechol is 5.5 ± 0.4 mM (n = 5).

(Received May 31, 2006; Accepted July 10, 2006)

Introduction

Many studies on the use of enzymatic activity in nonaqueous solutions have been carried out after Dastoli and Price's report of their investigation of the catalytic activity of xanthine oxidase suspended in several organic media.[1] Updike and Hicks published the results of their experiment involving an enzyme electrode with cross-linked polyacrylamide (PAA) membrane as matrix for use in aqueous solutions.[2] Enzymes have been found to have numerous applications as practical and specific catalysts in chemical and pharmaceutical syntheses and as recognition elements in biosensors. The discovery that they are catalytically active in organic solvents containing little or no water has expanded their repertoire of use still further.[3,4] Some of the benefits in utilizing enzymes in nonaqueous solvents over the use of conventional aqueous reaction media include the high solubility of many hydrophobic substrates in such solvents, the suppression of various side reactions promoted by water, altered enzymatic selectivity and the ability to control it with the solvent, and the comparative simplicity of immobilization procedures due to the insolubility of enzymes in organic solvents. Many studies have been conducted in order to gain an understanding of so-called solvent effects due to organic solvent properties. Factors such as solvent hydrophobicity,[5] solvent polarity, water content,[6] and substrate hydrophobicity[7] can affect the enzymatic reaction. It remains important to continue the search for new enzymatic reaction systems because of the use which might be made of such systems in both pure and applied research.

Many enzyme-based electrochemical biosensors have been described for use in both aqueous and nonaqueous solutions. A tyrosinase-immobilized polyacrylamide membrane platinum electrode has been prepared and the electrochemical behavior of *o*-hydroxybenzene derivatives in acetonitrile[8] and dimethylacetamide[9] has been studied. Because of unique properties of nanoparticles, recent investigations have focused on the development of enzyme-modified nanoparticle-based electrochemical biosensors.[10–12] The ability of nanoparticles to stabilize enzymes, and to improve the efficiency of immobilized enzymes is extremely useful when preparing biosensors. Moreover, other characteristics of nanoparticles, such as their high surface-to-volume ratio, their high surface energy, and their ability to decrease the distance between enzyme and metal particles, may facilitate electron transfer between redox sites and the electrode surfaces. In our laboratory, a gold nanoparticle self-assembled electrode has been constructed by using 4,4′-bis(methanethiol)biphenyl (MTP), rigid rod dithiols, as a binder between a gold disk and gold nanoparticles. The effect of gold nanoparticles on the interaction of $Co(phen)_3^{3+}$ with DNA, and the characteristics of the electrode in a nonaqueous solution were described in a recent report.[13] We have also devised a method of preparing density-controlled gold nano-particle self-assembled monolayer (SAM) interfaces by using both MTP and 1-octadecanethiol and have investigated their electrochemical behavior.[14] The MTP forms assemblies in which one thiol group binds to the surface, while the other thiol moiety projects upward at the exposed surface of the SAM. There was no indication of in-looped structures.[15] So, the electrode based MTP is expected to apply to various biosensing situations. In the paper, as an application of the MTP based electrode, the construction of such a tyrosinase-modified nanogold-based gold electrode and the voltammetric properties of the electrode to catechol in very low water content-acetonitrile (AN) solution are described.

Experimental

Materials

Tetraethylammonium perchlorate (Et_4NClO_4, code 33016-32), $AgNO_3$, KOH, molecular sieves (4A 1/16), and CaH_2 were purchased from Nacalai Tesque, Inc. Hydrogen tetrachloroaurate(III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$), $C_6H_5Na_3O_7 \cdot 2H_2O$, P_2O_5, H_2NCSNH_2, H_2SO_4, H_2O_2, and AN were Wako products.

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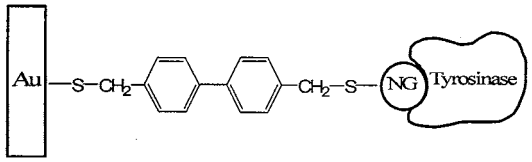
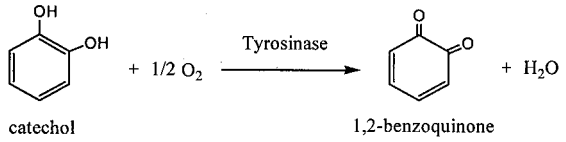


Scheme 1 Tyrosinase, NG-MTP-Au electrode.



Scheme 2 Enzymatic oxidation of catechol.

4,4′-Bis(chloromethyl)-1,1′-biphenyl ($ClCH_2C_6H_4C_6H_4CH_2Cl$) was purchased from Aldrich and tyrosinase (T-3824) was purchased from Sigma. Et_4NClO_4 was dried at 65°C for 3 h under a high vacuum with P_2O_5. AN from Wako was purified by the same method as that reported in the literature.[16] 4,4′-Bis(methanethiol)biphenyl (MTP) was prepared using the following procedure: 2.3 g of thiourea was added to 20 mL of 95% ethanol, and the solution was stirred at reflux until it became clear (about 15 min). Then, 2.5 g of 4,4′-bis(chloromethyl)-1,1′-biphenyl was added to the solution. A white substance formed in the solution after several minutes. After filtering, the white substance was stirred again with 9.6 g of KOH in 20 mL H_2O at reflux for 9 h. The solution turned light yellow. After being acidified with diluent H_2SO_4 to pH 7 – 8, the product was obtained by filtering. The crude compound was recrystallized in CH_2Cl_2 twice. m.p.: 146.8 – 148.5. NMR ($CDCl_3$ 500 M): δ 1.714 – 1.733 (t, 2H), 3.700 – 3.719 (d, 4H), 7.317 – 7.321 (d, 4H), 7.439 – 7.444 (d, 4H). IR (cm^{-1}) ν: 2549.9 (-SH). Colloidal gold sols with average particle diameters of 6 nm were prepared as previously reported.[13]

Preparation of tyrosinase-modified electrodes

Bulk gold disk electrodes were prepared by using polycrystalline gold rod (diameter 2 mm) in Teflon tubes. The electrodes were carefully polished first with emery paper (No. 2500), and then with 0.05 μm alumina slurry on microcloth pads. After removal of the trace alumina from the surface by rinsing with water, the electrodes were ultrasonicated for 10 min in fresh piranha solution (H_2SO_4:H_2O_2 = 3:1). The electrodes were then sonicated in water and anhydrous ethanol. The tyrosinase/nano-gold modified gold disk electrode was prepared as follows; The electrodes were firstly modified by incubation in 50 μm MTP anhydrous ethanol solution for 6 h. The modified electrodes (MTP-Au electrode) were washed twice by ethanol and subsequently by distilled water prior to adsorption of gold nano-particles. The electrodes were then immersed in gold colloid solution for 12 h to produce the nano-gold particle-modified electrode (NG-MTP-Au electrode). After being washed with water, each electrode was modified with tyrosinase by transfering a droplet of 20 μL tyrosinase solution, containing 0.2 mg (450 units) tyrosinase, onto the surface of the electrode followed by air-drying overnight at 4°C. Thus, tyrosinase-modified electrode (tyrosinase, NG-MTP-Au electrode) was obtained (Scheme 1).

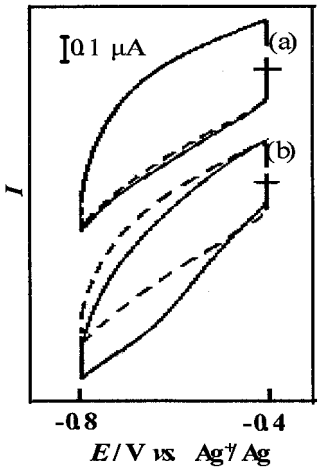


Fig. 1 Cyclic voltammograms of catechol in 50 mM Et_4NClO_4–AN solution at (a) Au and (b) the tyrosinase, NG-MTP-Au electrode. Dotted and solid lines were for blank and 30 mM sample solutions, respectively. Scan rate was 0.100 V/s.

Electrochemical measurements

Cyclic voltammetric and amperometric measurements were carried out using a potentiostat/galvanostat combined with an arbitrary function generator (HOKUTO DENKO) and an X-Y recorder at 35 ± 0.1°C. A conventional three-electrode system was used. The working electrode was a tyrosinase-modified nano-gold electrode, the reference electrode was a Ag/Ag^+ (10 mM) electrode, and the counter electrode was a platinum wire. The solutions were stirred by a stirrer at a stated speed when amperometric experiments were carried out, while the solutions were stirred for 4 min and then the stirrer was held still for 1 min before cyclic voltammetric experiments were performed.

Results and Discussion

Cyclic voltammetric measurements

Cyclic voltammetric measurements of the tyrosinase, NG-MTP-Au, the NG-MTP-Au, the tyrosinase/Au and bare gold electrodes were carried out for the substrates of catechol in 50 mM Et_4NClO_4–AN solution. In AN solution a hydrophilic tyrosinase adsorbed on nano-gold particles is stable enough. The typical cyclic voltammograms of 30 mM catechol in AN solution are shown in Fig. 1. The voltammogram of the tyrosinase, NG-MTP-Au electrode showed a reduction peak at –0.63 V *vs.* Ag/Ag^+. The reduction potential was almost the same as that obtained at the tyrosinase immobilized polyacrylamide (PAA) electrode in AN.[8] The 1,2-benzoquinone was produced from the enzymatic oxidation of catechol with dissolved oxygen at the tyrosinase modified electrode (Scheme 2) and it was reduced electrically at the nano-gold surface, and the electron transfer occurred through MTP to give a reduction current on cyclic voltammograms.

A clear cathodic peak at about –0.6 V *vs.* Ag/Ag^+ had been obtained at the PAA-tyrosinase electrode because the enzymatic oxidation product, 1,2-benzoquinone, was accumulated in a PAA membrane matrix while the electrode was kept at rest potential for 5 min before applying the potential for cyclic voltammogram recording.[8] In the absence of substrates, both the tyrosinase, NG-MTP-Au and the NG-MTP-Au electrode gave no detectable signal. And after the substrate addition into the cell, no response was also observed either at the bare gold

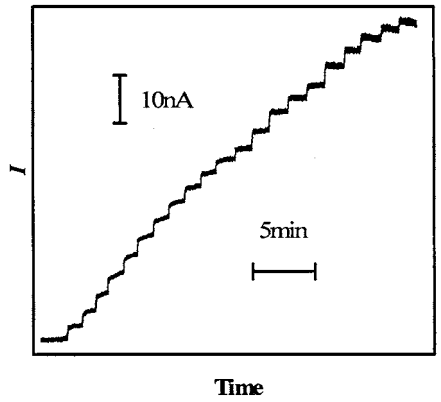


Fig. 2 Typical current–time response curve of the tyrosinase, NG-MTP-Au electrode on stepwise additions of catechol in 50 mM Et_4NClO_4–AN solution at –0.70 V *vs*. Ag/Ag^+.

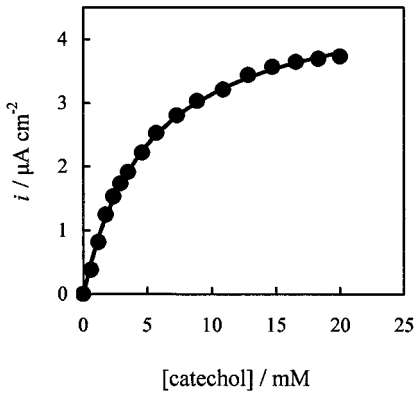


Fig. 3 Calibration plot of the tyrosinase, NG-MTP-Au electrode for catechol in 50 mM Et_4NClO_4–AN solution at –0.70 V *vs*. Ag/Ag^+.

electrode (Fig. 1(a)) or at the NG-MTP-Au electrode. It could be now firmly recognized from the results obtained above that the current increase at about –0.6 V *vs*. Ag/Ag^+ should be due to the reduction of the enzymatic oxidation product of catechol.

Amperometric results

A typical current–time plot for the tyrosinase, NG-MTP-Au electrode on stepwise addition of catechol from 0.60 mM to 22 mM at –0.70 V *vs*. Ag/Ag^+ is shown in Fig. 2. When catechol was added into the cell, the reduction current rose steeply to reach a stable value. On the other hand, when a tyrosinase was attached directly at a bare gold electrode in place of the tyrosinase, NG-MTP-Au electrode, noisy and not reproducible currents were observed amperometrically when catechol was added into the cell. This may be because tyrosinase attached on a gold-nano particle holds a three dimensional conformation and is present at low density, different from its presence on a gold disk surface.

Figure 3 shows the calibration plot of the tyrosinase, NG-MTP-Au electrode for catechol in 50 mM Et_4NClO_4–AN solution at –0.70 V *vs*. Ag/Ag^+. The response of the electrode exhibits characteristics of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant, K_m, of tyrosinase on the tyrosinase, NG-MTP-Au electrode to substrate can be calculated according to the Lineweaver–Burk equation,[9,17-19]

$$i^{-1} = i_{max}^{-1} K_m [S]^{-1} + i_{max}^{-1},$$

where [S] is the concentration of substrate.

Figure 4 shows the Lineweaver–Burk plot for the response of the tyrosinase, NG-MTP-Au electrode to catechol in AN. The apparent constant, K_m, for the reaction of tyrosinase with catechol at the tyrosinase, NG-MTP-Au electrode was calculated to be 5.5 ± 0.4 mM (n = 5), which is similar to that from 3 to 4 mM for the same enzyme in aqueous solution.[20] It is obvious that tyrosinase remains active to the substrates dissolved in the dipolar aprotic solvent AN. Furthermore, we deduced that tyrosinase attached to nanogold surface works as a redox enzyme.

Enzymes have been traditionally considered to work in aqueous systems. When an enzyme is placed in an organic solution and still remains active, it is considered that the enzyme activity is related to the amount of water bound to the enzyme.[21-23] The water bound to the enzyme in organic solvent

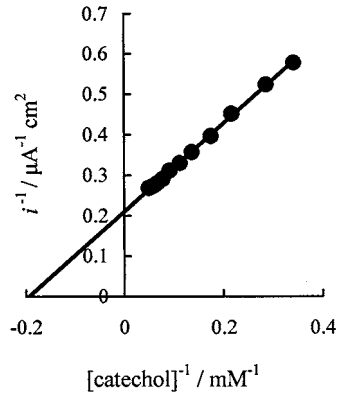


Fig. 4 Lineweaver–Burk plot for the response of the tyrosinase, NG-MTP-Au electrode to catechol in AN.

may not form a layer covering the enzyme. It may be just a few clusters around charged and polar regions on the enzyme surface, which could be regarded as the regions through which the enzymatic reaction proceeds.[23] So, even in organic solvent of lower water content, enzymes still exhibit activity. In this study, the fact that tyrosinase remained active may also be because tyrosinase itself is a so-called membrane protein,[24] so that it is naturally accustomed to a nonaqueous environment. Generally, an enzymatic reaction may occur at the hydrophobic site of enzymes and substrates react with enzymes after releasing their solvated solvent molecules and reaching the reaction site. Therefore, the main factors concerning the reaction rates may be independent of so-called solvent effects. We concluded that the MTP based electrode can be applied to monitor both the enzyme activity in very low water-content aprotic solvents and the enzymatic reaction rate.

The present paper has displayed that an entirely stable biosensor could be constructed by direct adsorption of hydrophilic enzyme on nano-gold. Such a sensor shows one way for getting reaction kinetics of substrates dissolved in dipolar aprotic solvents. Further applications will be expected to get basic data concerning the invention of medicines, for example.

Conclusions

The tyrosinase, NG-MTP-Au electrode exhibits characteristics

of Michaelis-Menten kinetic mechanism in a dipolar aprotic solvent. Tyrosinase immobilized on gold nano-particles conjugated to a rigid rod dithiol remains its activity to catechol in AN even when the water content is lower than 0.01 w/w%. The apparent Michaelis-Menten constant, K_m, of tyrosinase on the electrode for catechol in AN is similar to that for the enzyme in aqueous solution.

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