Journal of Electroanalytical Chemistry 804 (2017) 128–132

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Putrescine oxidase/peroxidase-co-immobilized and mediator-less mesoporous microelectrode for diffusion-controlled steady-state amperometric detection of putrescine

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ARTICLE INFO

Keywords:
Direct electron transfer
Microdisk electrode
Peroxidase
Putrescine oxidase
Putrescine biosensor

ABSTRACT

A mediator-less amperometric biosensor for putrescine detection was proposed to obtain a (pseudo) steady-state catalytic current. Putrescine oxidase (PuOD) and peroxidase (POD) were co-immobilized with glutaraldehyde on a Ketjen Black (KB)-based mesoporous electrode. A POD-catalyzed direct electron transfer-type reduction wave of H_2O_2 generated by the reaction of PuOD was observed at a PuOD/POD-immobilized and KB-modified rotating disk glassy carbon electrode with an onset potential of 0.60 V *vs.* Ag|AgCl. A PuOD/POD-immobilized and KB-modified microdisk electrode produced a spherical diffusion-controlled (pseudo) steady-state catalytic current under quiescent conditions in the presence of putrescine. The bienzyme mesoporous microelectrode exhibited a linear range from 17 μM to 500 μM with a sensitivity of 0.33 ± 0.01 mA mM^{-1} cm^{-2} and a lower detection limit of 5 μM (S/N > 3).

1. Introduction

Putrescine (1,4-diaminobutane), a small aliphatic diamine, is ubiquitous in a wide variety of living cells and plays important roles in many physiological processes, especially in cell growth [1–3]. Increase in putrescine is often associated with several diseases, for example malignant tumor [4,5]. In addition, putrescine is also usually found in spoiled foodstuffs due to the decarboxylation of amino acids by microorganisms [6,7].Therefore, the determination of putrescine concentration is very important in clinical, biological, and chemical samples, as well as food processing and fermentation. In pursuit of rapid, simple, and sensitive putrescine detection, a great number of methods have been developed [8–11], in which redox enzyme-based amperometric biosensors continue to be a topic of interest.

Putrescine oxidase (PuOD) is a flavoenzyme catalyzing the oxidation of putrescine and the concomitant reduction of O_2 to H_2O_2 [12]:

$$\text{putrescine} + O_2 + H_2O \rightarrow 4-\text{aminobutanal} + H_2O_2 + NH_3 \quad (1)$$

Several oxidases can utilize artificial electron acceptors (or mediators) (M_{Ox}) in place of O_2 (with their dehydrogenase activity), and second generation-type biosensors are often constructed based on mediated electron transfer- (MET-) type bioelectrocatalysis. In the case of PuOD, a similar MET-type electrode was attempted to be constructed based on the reactions shown in Eqs. (2) and (3) [13].

$$\text{putrescine} + 2M_{Ox} + H_2O \rightarrow 4-\text{aminobutanal} + 2M_{Red} + NH_3 \quad (2)$$

$$M_{Red} \rightarrow M_{Ox} + H^+ + e^- \text{ (at } E\text{>the formal potential of the mediator)} \quad (3)$$

However, PuOD preferentially and almost exclusively uses O_2 as an electron acceptor. Therefore, the second generation-type biosensor showed very low performance due to low dehydrogenase activity. Therefore, the amperometric methods reported to date for putrescine detection are almost all of the first generation type based on the direct oxidative detection of H_2O_2 [14–16]:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \text{ (at } E > 0.5 \text{ V)} \quad (4)$$

However, the oxidation of H_2O_2 requires relatively high operation potentials, which are always accompanied by the co-oxidation of other electroactive metabolites in physiological fluids; thus, the oxidative detection of H_2O_2 does not seem to be practical for *in vivo* analysis.

On the other hand, peroxidase (POD)-catalyzed reductive detection of H_2O_2 has often been coupled with several oxidase reactions to construct a variety of biosensors for the detection of biologically related compounds as the substrates of the oxidases [17–21]. POD catalyzes the reduction of H_2O_2 with several artificial mediators (M_{Red}), and the oxidized form of the mediator (M_{Ox}) is reductively detected at electrodes as MET-type bioelectrocatalysis.

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http://dx.doi.org/10.1016/j.jelechem.2017.09.056
Received 19 July 2017; Received in revised form 24 September 2017; Accepted 26 September 2017
Available online 28 September 2017
1572-6657/

$$H_2O_2 + 2M_{Red} \rightarrow 2H_2O + 2M_{Ox} \quad (5)$$

$$M_{Ox} + H^+ + e^- \rightarrow M_{Re} \text{ (at } E\text{<the formal potential of the mediator)} \quad (6)$$

Coupling of an oxidase reaction and the MET-type bioelectrocatalysis of H_2O_2 reduction frequently provides bienzyme-type sensors of high sensitivity. However, the system requires a suitable mediator, and more importantly, the coupling often causes a cross reaction in which the M_{Ox} can work as an electron acceptor for the oxidase reaction, leading to negative interference in the quantitative analysis [17].

Recently, our group has reported the direct electron transfer- (DET-) type bioelectrocatalysis of POD at mesoporous electrodes with an onset potential of *ca.* 0.7 V *vs.* Ag|AgCl [22]:

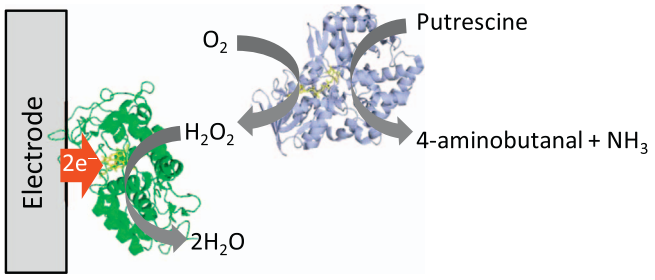
$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \text{ (at } E < \text{ the formal potential of POD)} \quad (7)$$

The mesoporous structure with a suitable pore size seems to be very useful and essential for rapid electron transfer between an enzyme and an electrode [22–25]. It can be considered that the DET-type POD-based bioelectrocatalytic system will be coupled with the PuOD reaction (Eq. (1)) for putrescine detection without any mediator (Scheme 1).

Another important issue to be solved for such biosensors is that the response is liable to be affected by time-dependent characteristics. Rotating disk electrodes or magnetic stirring is often used to minimize the influence of time-dependent mass transfer and to provide steady-state currents. Such methods always make the system so "fat" that they would not be convenient for *in vivo* detection.

On the other hand, it has been reported that microelectrodes [26] provide steady-state responses under quiescent conditions even in MET-type bioelectrocatalysis under suitable conditions [27–29]. In the case of DET-type biosensors, one may get (pseudo) steady-state diffusion-controlled responses with microdisk electrodes when bioelectrocatalysis proceeds at a very large rate constant. From this viewpoint, a useful strategy is to develop a mesoporous microelectrode for DET-type biosensors; the mesoporous structure suitable for a redox enzyme improves the DET-type bioelectrocatalysis of the enzyme and the microdisk electrode provides rapid (pseudo) steady-state amperometric responses proportional to the substrate concentration even in a quiescent electrolyte solution.

In this work, Kejten Black (KB) was utilized to construct a mesoporous carbon platform for co-immobilization of POD and PuOD. DET-type bioelectrocatalytic reduction of H_2O_2 by POD (Eq. (7)) and the PuOD reaction (Eq. (1)) were coupled to detect putrescine. In addition, a bienzyme mesoporous microsensor was constructed to obtain spherical diffusion-controlled (pseudo) steady-state currents under quiescent conditions (Scheme 2). The proposed bienzyme microelectrode was capable of putrescine determination under quiescent conditions with a lower detection limit of 10 μM (S/N > 3).



Scheme 1. Proposed cascade reactions at a PuOD/POD bienzyme electrode.

2. Experimental

2.1. Materials and reagents

Ketjen Black EC300J (KB) was kindly donated by Lion Co. (Japan). Poly (tetrafluoroethylene) fine powder (PTFE, 6-J) was obtained from DuPont Mitsui Fluorochemicals (Japan). Glutaraldehyde (20%), putrescine, and H_2O_2 were obtained from Wako Chemicals Co. (Osaka, Japan). Peroxidase from horseradish (POD, EC 1.11.1.7, 282 U mg^{-1}) was purchased from Toyobo Co. (Japan) and used without further purification. Putrescine oxidase from *Rhodococcus erythropolis* (PuOD, EC 1.4.3.10, 9 U mg^{-1}) was expressed from a plasmid pBAD*puo*$_{Rh}$ and purified as described previously [30]. All other chemicals used in this study were of analytical grade.

2.2. Preparation of bienzyme rotating disk electrode

KB-modified glassy carbon rotating disk electrodes (KB/GCE) were constructed as follows. KB (40 mg) and PTFE (10 mg) were distributed in 3.5 mL of 2-propanol and homogenized with an ultrasonic disruptor (Heat Systems GmbH & Co.) for 3 min in an ice bath to prepare a KB slurry. A 3 μL aliquot of the KB slurry was applied onto a GCE surface and dried at room temperature for 10 min. Then, a 20 μL aliquot of an enzyme/reagent mixture containing POD (usually 0.1 mg mL^{-1}), PuOD (2.5 mg mL^{-1}), and glutaraldehyde (5%) was dropped onto the KB/GCE surface and dried at 4 °C for 2 h. Glutaraldehyde was used here as a cross-linker to form a stable enzyme layer for DET-type bioelectrocatalysis. The prepared bienzyme electrode, referred to as PuOD/POD/KB/GCE, was washed with fresh buffer before electrochemical measurements.

2.3. Preparation of bienzyme mesoporous microelectrode

Scheme 2 shows the preparation process of a bienzyme mesoporous microelectrode. A polished gold microdisk electrode (AuMDE) was immersed in 4 mL of aqua regia (35% HCl: 70% HNO_3 = 3:1 v:v) for 50 min. The etched AuMDE was washed with and sonicated in distilled water. A 1 μL aliquot of the KB slurry was applied onto the top of the etched AuMDE and dried at room temperature. Any KB particles outside the etched microdisk portion were carefully removed. The KB-modified AuMDE is referred to as KB/AuMDE. Then, a 10 μL aliquot of the enzyme/reagent mixture containing POD, PuOD, and glutaraldehyde was dropped onto the KB/AuMDE surface and dried at 4 °C for 2 h. The bienzyme microporous microelectrode was washed with fresh buffer before electrochemical measurements and is referred to as PuOD/POD/KB/AuMDE. For a long-time storage, the PuOD/POD/KB/AuMDE was immersed in a fresh buffer and stored at 4 °C.

2.4. Electrochemical measurements

Cyclic voltammetry and chronoamperometry were performed using an electrochemical analyzer (ALS 701 E, ALS Co. Ltd., Japan) with either PuOD/POD/KB/GCE or PuOD/POD/KB/AuMDE as the working electrode, a Pt wire as the counter electrode, and an Ag|AgCl|KCl (sat.) electrode as the reference electrode. All potentials were referenced against this reference electrode.

3. Results and discussion

3.1. PuOD/POD modified KB rotating disk electrode

Fig. 1 shows typical rotating-disk cyclic voltammograms (CVs) obtained at the PuOD/POD/KB/GCE in the presence and absence of putrescine. A well-defined reduction wave was observed in the presence of putrescine, whereas no clear wave was observed in the absence of putrescine. Such a reduction wave was also not observed in the absence of