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A lactate electrochemical biosensor with a titanate nanotube as direct electron transfer promoter

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Abstract
Hydrogen titanate ($H_2Ti_3O_7$) nanotubes (TNTs) have been synthesized by a one-step hydrothermal processing. Lactate oxidase (LOx) enzyme has been immobilized on the three-dimensional porous TNT network to make an electrochemical biosensor for lactate detection. Cyclic voltammetry and amperometry tests reveal that the LOx enzyme, which is supported on TNTs, maintains their substrate-specific catalytic activity. The nanotubes offer the pathway for direct electron transfer between the electrode surface and the active redox centers of LOx, which enables the biosensor to operate at a low working potential and to avoid the influence of the presence of O_2 on the amperometric current response. The biosensor exhibits a sensitivity of 0.24 μA cm^{-2} mM^{-1}, a 90% response time of 5 s, and a linear response in the range from 0.5 to 14 mM and the redox center of enzyme obviates the need of redox mediators for electrochemical enzymatic sensors, which is attractive for the development of reagentless biosensors.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Lactate is a universal metabolite of nearly all living organisms, and a natural or an artificial component of food products. Selective and sensitive detection of lactate has significant implication in clinical diagnostics, sports medicine, and food processing [1]. The lactate level is an indicator of cell integrity, tissue hypoxia and drug toxicity [2, 3]. It is also useful in sports medicine, particularly in evaluation of the physical conditions of athletes [4]. In addition, L-lactate is an indicator of the fermentative process and the quality of alcoholic beverages and dairy products [5]. Determination of lactate by laboratory-based chromatographic or spectroscopic techniques is relatively expensive, time-consuming, complex to perform, and requires large sample volume. Therefore, great attention has been drawn to developing inexpensive portable devices, especially electrochemical biosensors, for rapid, real-time monitoring of the lactate level.

Lactate oxidase (LOx) enzyme, which is a flavoprotein with flavin adenine dinucleotide (FAD) as a prosthetic group, is commonly used in lactate electrochemical biosensors. It catalyzes selectively the conversion of lactate to pyruvate by the redox center ($FAD/FADH_2$), accompanying oxygen

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consumption and hydrogen peroxide production. Thus the lactate concentration could be determined by probing O_2 using a Clark electrode or by measuring the H_2O_2 oxidation. However, the fluctuation of O_2 concentration in the solution brings about the system complexity, and affects the detection limit. On the other hand, direct amperometric detection of H_2O_2 requires a relatively high oxidation potential at an unmodified electrode, which causes severe interference arising from easily electro-oxidizable substances such as ascorbic acid and uric acid existing inherently in biological fluids. In order to eliminate these problems, several strategies have been developed to reduce the applied potential for H_2O_2 oxidation. For example, bi-enzyme systems [6, 7] have been developed by co-immobilizing horseradish peroxidase (HRP) and LOx. In addition, artificial electron mediators [8, 9], redox catalysts [10] or polymers [11, 12] have been employed in the working electrode. While offering effective electron transfer channels between the LOx enzyme and the electrode, some of the above-mentioned procedures require expensive reagents. Some of the electron mediators, such as ferrocene derivatives and osmium complexes, are toxic for living forms if leakage occurs, which limits the *in vivo* applications of biosensors. In addition, the redox catalysts may catalyze the interfering reactions [13]. Furthermore, there always is a competition between an artificial mediator and oxygen owing to the difficulty in complete elimination of oxygen. Therefore, there has been an increasing interest in further improving the electrical coupling between the enzyme and the electrode. If direct electron transfer between the enzyme and the electrode can be realized, electrochemical biosensors will be operated in a potential window close to the redox potential of the enzyme itself, and thus will be less susceptible to interfering reactions. However, it is difficult to realize direct electrochemistry of enzymes on bare (unmodified) electrodes [14]. One of the reasons is that the active redox center of the enzyme is deeply embedded in an electrically insulate protein shell that is the majority part of enzymes. Thus the active redox sites are isolated from the electrode surface, preventing the direct electron transfer. Furthermore, proteins directly adsorbed on a bare (unmodified) electrode like carbon, platinum or gold tend to denature, leading to electrode fouling. The challenge could be overcome by employing roughened electrodes that can penetrate the insulting protein shell [15], thus permitting direct electron transfer. Alternate strategies are to immobilize enzymes with self-assembled monolayers or multilayers [16], or with appropriate nanosized particles [17–20]. To the best of our knowledge, direct electron transfer between the lactate oxidase and an electrode has not been reported.

Titanium oxides are environmentally benign, chemically stable in physiological fluids, and have good mechanical strength. The titanate surface contains a functional hydroxyl group, offering a hydrophilic surface. The hydrophilic surfaces are expected to provide an aqueous-like environment that facilitates the stabilization of the immobilized proteins [21]. It has been reported that adsorbed proteins on nanocrystalline TiO_2 surfaces have exhibited less denaturation [22]. In addition, titanate ($H_2Ti_3O_7$) nanotubes are n-type semiconductors [23] with a band gap of around 3.3 eV [24].

In the present work, we attempted to use titanate nanotubes (TNTs) as the direct electron transfer promoter to develop a reagentless electrochemical biosensor for lactate detection. Cyclic voltammetric and amperometric measurements were conducted to characterize the electrochemical behaviors of the enzyme/TNT modified electrodes and to explore the underlying electron transfer mechanism.

2. Experimental method

Chemicals

Nafion® (5% ethanol solution) was obtained from Alfa Aesar (Ward Hill, MA, USA). L(+)-lactic acid (98%), ascorbic acid (99%), uric acid (99%), and lactate oxidase (EC 1.1.3.2 from *Pediococcus* species) lyophilized powders containing 38 units mg^{-1} solid were purchased from Sigma (St Louis, MO, USA). All the reagents were analytical grade and used without further purification. 10 mM phosphate buffer solution (PBS) (pH 7.4) containing 2.7 mM potassium chloride and 137 mM sodium chloride was prepared by dissolving one tablet (Sigma) into 200 ml of deionized water. Stock LOx solution was prepared by dissolving 2.6 mg of the LOx lyophilized powder into 250 μl of 10 mM PBS with pH of 7.4. The aliquoted (10 μl) LOx solutions (4U/10 μl) were stored at −20 °C. All solutions were prepared with deionized water (resistance >18 MΩ cm). Gold-coated glass slides (25 mm × 75 mm) were obtained from EMF Corporation (Ithaca, NY, USA).

Titanate nanotube synthesis

The TNTs were synthesized by a one-step hydrothermal processing of TiO_2 particles in NaOH, as described in our previous work [25]. Briefly, 1.2 g TiO_2 particles were added to 10 M NaOH aqueous solution. After stirring for 1 h, the suspension was transferred to a preheated Teflon-lined stainless-steel autoclave and then held at 120 °C for 24 h. The products in the autoclave were subsequently collected and washed with 1 M HCl and deionized water until the pH was less than 7.

Lactate electrode fabrication

Gold-coated glass chips were immersed in piranha solution (70% concentrated H_2SO_4:30% H_2O_2) at 80 °C for 15 min (*Caution*: *Piranha solution reacts violently with organic chemicals*), and rinsed with deionized water. After being nitrogen-dried, the cleaned chip was covered by an adhesive tape with a hole (3 mm diameter) to confine an exposed area of 0.07 cm^2. Then 2 μl 0.3 wt% Nafion® in ethanol was dropped into the confined area and the sample was nitrogen-dried (denoted as Nafion®–Au). The TNTs were added into deionized water and treated with sonication to form a 5 g l^{-1} TNT suspension. This suspension was then mixed with 10 μl of the stock LOx solution at a volume ratio of 1:1. Subsequently 2 μl of such mixed suspension

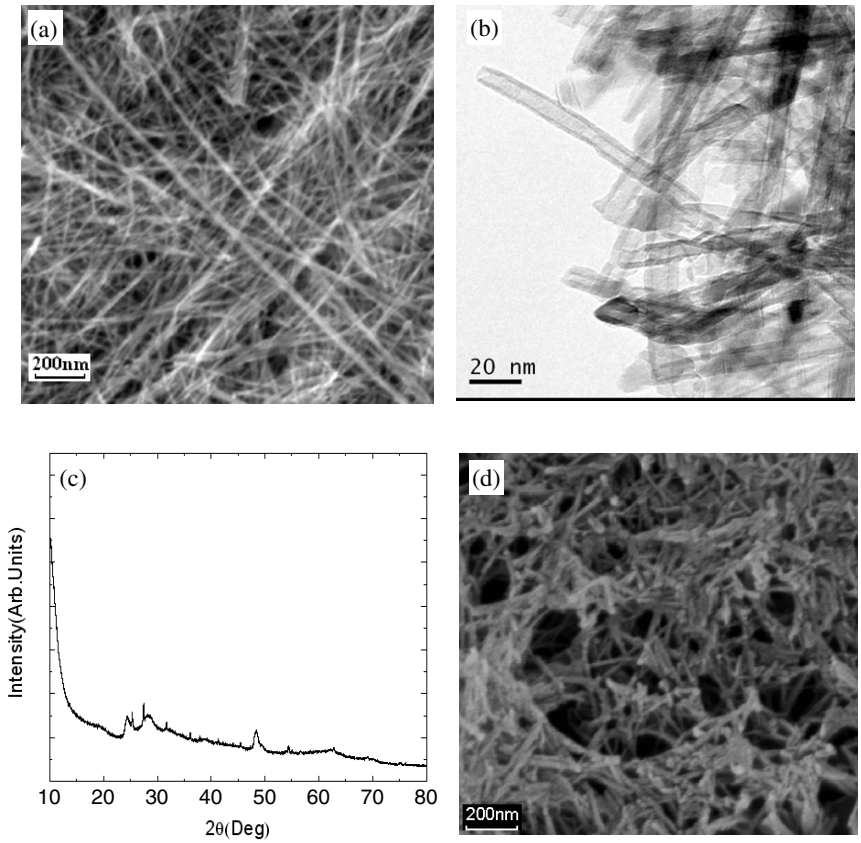

Figure 1. (a) SEM photograph of nanotubes; (b) TEM image of nanotubes; (c) XRD pattern of the TNTs; (d) SEM photograph of the Au–Nafion®–TNTs–LOx electrode.

was dropped onto the confined gold electrode surface, and then dried in N_2, followed by rinsing with deionized water. This resulted in the enzyme/TNT modified electrode denoted as Au–Nafion®–TNTs–LOx. For comparison, we also prepared the working electrode without TNTs (Au–Nafion®–LOx).

Apparatus

Cyclic voltammetric and amperometric measurements were performed with a conventional three-electrode system, where a platinum wire (1 mm in diameter) was used as the counter electrode, a KCl-saturated Ag/AgCl electrode as the reference electrode, and the modified gold chip as the working electrode. Electrochemical measurement was carried out with a Solartron SI 1287 electrochemical interface in 10 mM PBS (pH 7.4) as the background solution. The crystal structure of TNTs was determined with an X'Pert PW3040-Pro x-ray powder diffractometer (XRD) (Panalytical Inc.). The morphology of the TNTs was observed with a Hitachi S-4700 field-emission scanning electron microscope (FE-SEM) and Philips CM 20 transmission electron microscope (TEM) equipped with EDS (EDAX/4pi). The specific surface area of TNTs was measure by the BET (Brunauer, Emmett and Teller) method with a Micromeritics ASAP 2020 nitrogen adsorption surface area analyzer.

3. Results and discussion

3.1. Morphology and structure of nanotubes

Figure 1 shows the morphology and structure of the TNTs prepared by hydrothermal processing. It can be seen that the hollow titanate nanotubes with two open ends were around 17 nm in diameter and several hundred nanometers to several microns long. The BET surface area of TNTs was estimated to be 277 $m^2\ g^{-1}$. The XRD pattern in figure 1(c) was indexed to hydrogen titanate ($H_2Ti_3O_7$) [25]. Figure 1(d) reveals the architecture of the Au–Nafion®–TNTs–LOx electrode. The nanotubes formed a porous three-dimensional (3D) network as the enzyme support matrix. Negatively charged groups are usually present on the TNT surface at the working PBS solution, which probably favored the enzyme adsorption that results from the electrostatic interactions between the negatively charged groups on the TNT surface and the positively charged surface lysine and arginine residues of LOx. A large amount of enzyme can be loaded onto the electrode due to the large specific surface area of individual TNTs and the unique porous 3D network. In addition, this architecture provides a friendly micro-environment for immobilization of enzymes, which could retain the biocatalytic activity of enzymes. Also, the porous structure enhances the ability of the substrate molecules to access the enzymes. Furthermore, the cross-linked semiconducting TNTs offer electron transport channels throughout the whole electrode.

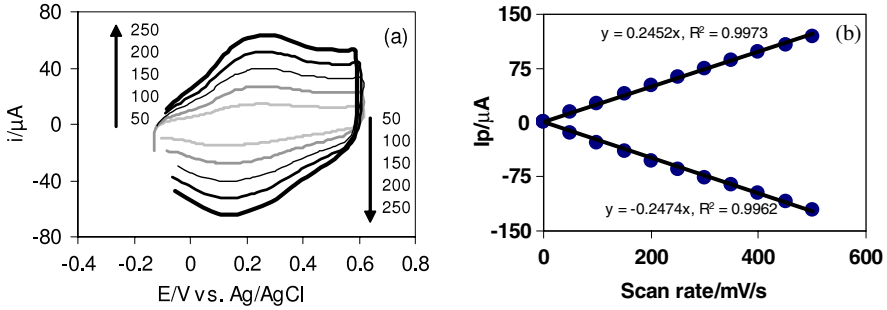

Figure 2. (a) Cyclic voltammograms obtained from the Au–Nafion®–TNTs–LOx electrode in the 10 mM PBS at different scan rates; (b) plots of peak currents versus scan rates; scan rates: 50–500 mV s^{-1}.

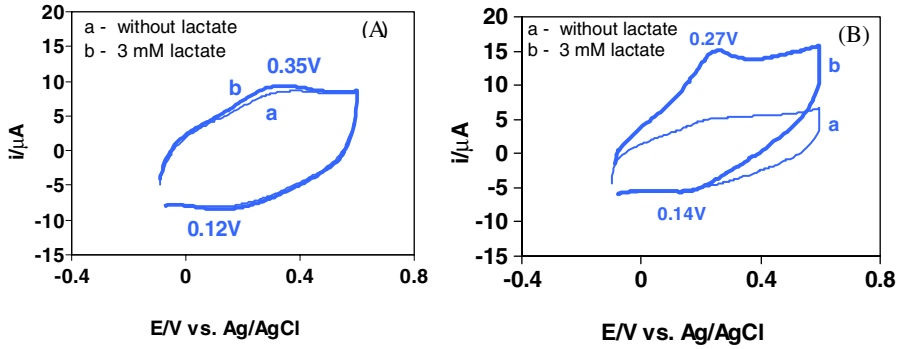

Figure 3. Cyclic voltammograms of the working electrodes in the 10 mM PBS at scan rate of 50 mV s^{-1}; (A) the Au–Nafion®–LOx electrode without TNTs; (B) the Au-Nafion®–TNTs–LOx electrode with TNTs.

3.2. Role of TNTs in electron transfer

Figure 2(a) shows the cyclic voltammograms (CVs) of the Au–Nafion®–TNTs–LOx electrode in the 10 mM PBS at different scan rates. The shape of both the cathodic and anodic waves was nearly symmetrical. The reduction and oxidation peaks exhibited almost equivalent height. The peak separation between the anodic and cathodic peaks was 130 mV. This suggests that the redox reaction was quasi-reversible. Figure 2(b) demonstrates that both the cathodic and anodic peak currents increased linearly with the scan rate from 50 to 500 mV s^{-1}, which indicated a surface-confined electrochemistry [26, 27]. According to the Laviron equation [28], the plot of the peak current versus scan rate can be expressed by

$$I_p = \frac{n^2F^2}{4RT} \cdot A\Gamma v, \qquad (1)$$

where I_p is the peak current, n is the number of electrons transferred, v is the scan rate, F is Faraday's constant, R is the gas constant and A is the electrode real area. From figure 2(b), the surface concentration (Γ) of LOx enzyme on the electrode was estimated to be 6.8×10^{-11} mol cm^{-2} (equivalent to 4.1×10^{13} cm^{-2}).

For the Au–Nafion®–LOx electrode without the nanotubes, the apparent anodic and cathodic peak potentials were located at 0.35 V and 0.12 V, respectively (figure 3(A)). The peak split was 230 mV. For the Au–Nafion®–TNTs–LOx electrode with the nanotubes, the apparent oxidation potential negatively shifted to around 0.27 V (figure 3(B)). The peak split was reduced to 130 mV. When 3 mM lactic acid was present in the PBS solution, a weak oxidation peak appeared in the CV of the Au–Nafion®–LOx electrode without TNTs. In contrast, the oxidation peak current of the electrode with TNTs was about 13 times higher than that of the electrode without TNTs. This suggested that the porous 3D TNT network provided a favorable micro-environment for the LOx enzyme to maintain biocatalytic activity. The nanotubes effectively shuttle the electrons from the active redox co-factor ($FAD/FADH_2$) of LOx to the gold electrode. From the saturated anodic current density of 7.56 μA cm^{-2}, and from the surface coverage (4.1×10^{13} cm^{-2}) of the LOx molecules on the electrode, the unimolecular electron transfer rate constant was estimated to be 0.57 s^{-1} according to the approach described previously [29]. The mechanism of direct electron transfer needs to be further studied. Based on the hypothesis by Guiseppi-Elie *et al* [30], an enzyme supported on a nanotube is analogous to a balloon sitting on a sharp needle. Individual nanotubes could penetrate into the protein shell of the flavoenzymes, and thus are physically contacted with or located within the electron tunneling distance of the prosthetic group FAD of the enzyme due to the small diameter of the nanotubes. This is one of the possible mechanisms of direct electron transfer.

Generally, for a LOx-based lactate biosensor, lactate is oxidized into pyruvate via dehydrogenation. After accepting two produced hydrogen atoms, the prosthetic group FAD of LOx becomes its reductive form of $FADH_2$, which needs to be re-oxidized to the original form of FAD in the presence of oxygen in a natural cycle. This reaction leads

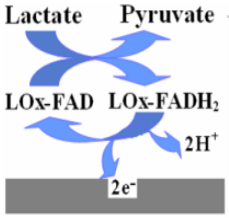

Figure 4. Principle of electro-enzymatic detection of lactate, illustrating the nanotube-promoted direct electron transfer between the LOx enzyme and the electrode.

to the formation of hydrogen peroxide (reaction (c)). The corresponding reaction pathway is described as follows:

$$\text{LOx-FAD} + \text{Lacate} \rightarrow \text{LOx-FADH}_2 + \text{Pyruvate} \quad \text{(a)}$$

$$\text{LOx-FADH}_2 \rightarrow \text{LOx-FAD} + 2\text{H}^+ + 2\text{e} \quad \text{(b)}$$

$$\text{LOx-FADH}_2 + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{LOx-FAD} \quad \text{(c)}$$

$$\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + 1/2\text{O}_2 + 2\text{e} \quad \text{(d)}.$$

It has been reported by Rubianes and Rivas [31] that the LOx enzyme was immobilized on a carbon nanotube paste electrode (CNTPE) prepared by dispersion of multi-wall carbon nanotubes (MWNT) in mineral oil. The carbon nanotubes catalyzed the oxidation and reduction of the hydrogen peroxide enzymatically generated, as shown by reactions (c) and (d). Therefore, the accelerated electron transfer reaction of hydrogen peroxide at the CNTPE allowed lactate determination at a low potential. In the present work, we have measured the CVs of the Au–Nafion® electrode and the Au–Nafion®–TNT electrode without LOx in the 40 mM PBS solution (pH 7.4) containing 10 mM hydrogen peroxide. Our results showed that TNTs had no electro-catalytic activity toward both the oxidation and the reduction of the hydrogen peroxide. In addition, when the testing solution was deaerated by N_2 bubbling, the electrochemical response of the biosensor did not change as compared to the aerated condition. This indicated that the influence of oxygen on the biosensor system was eliminated. Our results suggested that the role of TNTs in our TNT-based LOx biosensor was different from that of MWNTs reported by Rubianes and Rivas [31]. We propose that titanate nanotubes shuttle the direct electron transfer between the active redox sites of the LOx enzyme and the gold surface, as demonstrated in figure 4. This significantly reduces the redox potential. The catalytic process goes via only reactions (a) and (b). As a result, reactions (c) and (d) become negligible.

3.3. Amperometric response to lactic acid

Figure 5(a) demonstrates the amperometric response of the nanotube-based biosensor, which was obtained at the controlled voltage of 0.27 V (versus Ag|AgCl, 3 M KCl) after successive addition of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mM lactic acid into the 10 mM PBS solution every 50 s. It can be seen that the biosensor exhibited a fast and sensitive response to the change in lactic acid concentration. The biosensor achieved 90% of the steady current within 5 s. Figure 5(b) depicts a typical calibration curve obtained from the Au–Nafion®–TNTs–LOx electrode at an operating potential of 0.27 V (versus Ag|AgCl, 3 M KCl). Initially the current increased linearly with increase in the lactic acid concentration and then saturated at a high lactic acid concentration of 23 mM. The calibration curves exhibited an excellent linear relationship (correlation coefficient $R = 0.9928$) in the range from 0.5 to 14 mM with a sensitivity of 0.24 μA cm^{-2} mM^{-1}. The limit of detection (LOD) was determined to be 0.2 mM at the signal-to-noise ratio of 3. The relative standard deviation (RSD) of five successive measurements of 6 mM lactic acid was 2.47%. The wide linear response range (from 0.5 to 14 mM) is of great importance for the application of the developed biosensor in monitoring the physical condition of athletes or individuals who do exercise, because the lactate level measurement can help determine appropriate training intensities and help predict the endurance performance [32]. The physiological level of lactic acid is 0.8–1.5 mM in normal blood for most humans at rest conditions. Lactate concentration increases during exercise, and could reach the lactate threshold (LT) that is the level of exercise

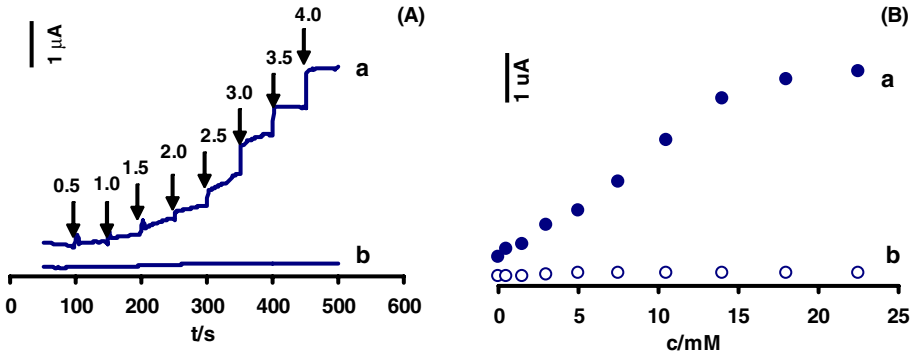

Figure 5. (A) Amperometric response to lactic acid of the Au–Nafion®–TNTs–LOx electrode with TNTs (a) and of the Au–Nafion®–LOx electrode without TNTs (b) by successively adding 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mM lactic acid into the 10 mM PBS solution every 50 s at the operating potential of 0.27 V versus Ag|AgCl; (B) calibration curve for the Au–Nafion®–TNTs–LOx electrode with TNTs (a) and for the Au–Nafion®–LOx electrode without TNTs (b), showing the oxidation current versus the lactate concentration.

intensity at the point of lactate accumulation and at the onset of the anaerobic performance. The LT is commonly set at level of 4 mM. High intensity training can even produce a lactate level as high as 15 mM in human blood.

4. Conclusions

In summary, the 3D titanate nanotube network not only provided a good micro-environment for the LOx enzyme to retain its bioactivity but also offered the pathway for direct electron transfer between the gold electrode surface and the active redox center of LOx. Effective direct electron transfer lowered the redox potential, and eliminated the influence of the presence of O_2 on the biosensor signal. The developed biosensor showed a fast response to lactic acid. The linear response ranged from 0.5 to 14 mM. The direct electron transfer between the electrode and the redox center of enzyme obviates the need of redox mediators for electrochemical enzymatic sensors, which is attractive for the development of reagentless biosensors.

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