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ORIGINAL PAPER

α-MoO_3 nanowire-based amperometric biosensor for L-lactate detection

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Abstract Large-scale orthorhombic single-crystalline molybdenum trioxide nanowires were synthesized using a facile one-pot hydrothermal method. Lactate oxidase enzyme was immobilized on the nanowires to produce a highly sensitive electrochemical biosensor for L-lactate detection. At an applied potential of 0.5 V, the sensor exhibited a high sensitivity of 0.87 μA/mM with a fast response to L-lactate (90% of response times within 10 s). A linear response was obtained over a concentration range from 0.5 to 8 mM with a detection limit of 0.15 mM (*S/N*=3). The developed biosensor showed excellent reproducibility and operational stability, as well as the ability to be stored long term.

Keywords Biosensor · L-lactate · MoO_3 nanowires · Direct electron transfer

Introduction

The development of biosensors based on enzyme electrodes for the detection of ions and molecules in many biological, clinical, and environmental monitoring applications is gaining wider attention [1–3]. Among various enzymatic sensors, L-lactate sensors are particularly important because of their potentially widespread application in areas such as clinical diagnostics, sports medicine, and food analysis [4–7]. The commonly used chromatographic or spectroscopic techniques for the detection of L-lactate are expensive, time-consuming, and require a large volume sample. Therefore, enormous efforts have been put into the development of inexpensive portable devices such as electrochemical biosensors for rapid and real-time monitoring of L-lactate [8, 9]. Electrochemical L-lactate biosensors have been fabricated by using either lactate oxidase (LOx) or lactate dehydrogenase (LDH) as the immobilized enzyme [8, 9]. However, biosensors based on immobilized LOx are preferable to those based on LDH since the natural cofactor flavin adenine dinucleotide (FAD) is already in the LOx content [10]. The FAD in LOx is preferred to nicotinamide adenine dinucleotide in LDH because of faster electron transfer and higher reaction rates between the FAD and biomacromolecules [11]. The immobilization of LOx on a sensing layer has been achieved by methods such as physical adsorption, chemical cross-linking, self-assembly, covalent bonding, and sol–gel immobilization [12–15]. Recently, the immobilization of LOx on electrodes through the use of transition metal oxide nanostructures has gained attention because of their diverse physical, chemical, and electrical properties [16, 17]. Among the different transition metal oxides, molybdenum trioxide (MoO_3) nanostructures possess the most desirable characteristics in terms of high specific surface area, optical transparency, chemical and photochemical stability, electrochemical activity, and ease of fabrication [18, 19]. Despite such interesting properties, the application of 1D MoO_3 nanostructures in amperometric biosensors has not yet been fully explored. Recently, glassy carbon electrodes modified with a molybdenum oxide layer were found to give a good response to the anodic oxidation of nitrite at relatively moderate positive potentials. The use of molybdenum oxide-modified electrodes as amperometric sensors for monitoring nitrite enhanced the sensitivity of detection compared to what had previously been achieved [17]. Herein, we propose a simple and sensitive biosensor using MoO_3 nanowires for L-

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lactate detection. We demonstrate its expanded linear range, high sensitivity, and excellent efficiency. These results suggest that MoO_3 nanowires are a promising matrix for the fabrication of electrochemical biosensors and thus can be widely applied to biomedical detection and environmental analysis.

Experimental section

MoO_2 powder, 5% Nafion solution, L(+)-lactic acid (98%), phosphate-buffered saline (PBS) tablets, and lactate oxidase (EC 1.1.3.2 from *Pediococcus* species, 38 units mg^{-1} solid) were purchased from Sigma Aldrich (St. Louis, MO, USA). All reagents were analytical grade and used without further purification. PBS solution (10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7) was prepared by dissolving one tablet of PBS into 200 ml of deionized water. The MoO_3 nanowires were synthesized by a hydrothermal method in which 3 mmol of MoO_2 powder was dissolved in HCl/H_2O_2 in a 1:5 *v/v* ratio and stirred magnetically for 30 min at pH 1–2. The final yellowish solution was transferred into a Teflon-lined stainless steel autoclave (25-ml capacity), which was kept at 180 °C for 12 h and cooled naturally to room temperature. The precipitates were filtered, washed several times with deionized water and ethanol, then dried on a hot plate at 100 °C for 12 h. This process resulted in the production of high-quality MoO_3 nanowires.

SiO_2 substrates coated with a 500-nm-thick gold layer were immersed in a piranha solution (70% H_2SO_4:30% H_2O_2) at 80 °C for 15 min, rinsed with deionized water, and dried with a highly pure nitrogen stream. After being nitrogen-dried, the cleaned chip was covered by an adhesive tape with a hole to create an exposed area of 0.07 cm^2. Uniform dispersion of MoO_3 nanowires was achieved by mixing 5 mg of MoO_3 nanowires in 2 mL of the ethanol and sonicating for 30 min. The LOx solution was prepared by dissolving 5.0 mg LOx in 1.0 mL of PBS solution. One hundred microliters of the MoO_3 nanowire-dispersed solution was mixed with 100 μL of the LOx solution. Twenty microliters of the mixture was dropped onto the surface of a gold-coated SiO_2 substrate electrode and allowed to dry in ambient conditions. Then, 10 μL of 0.5% Nafion solution was dropped onto the exposed area, and the electrode was nitrogen-dried. This process resulted in an enzyme-modified electrode we denote as Au–MoO_3–LOx–Nafion. For comparison, we also prepared a working electrode without MoO_3 nanowires (Au–LOx–Nafion). The prepared enzyme electrodes were washed numerous times with deionized water before use.

Results and discussion

The as-synthesized MoO_3 nanowires were examined by X-ray diffraction (XRD). Figure 1a shows the XRD pattern of the α-MoO_3 nanowires, which were in good agreement with the standard peaks for orthorhombic phase MoO_3 (JCPDS card 89-7112). Field-emission scanning electron microscopy (FE-SEM; JEOL JSM–7401F) analysis indicates that the length of the MoO_3 nanowires was about 5 μm with an average diameter of 110 nm (Fig. 1b). The activity of the functionalized LOx on the MoO_3 nanowires depends on the pH of the supporting electrolyte. Thus, the exact effect of the pH levels was investigated over the range of pH 5–8.

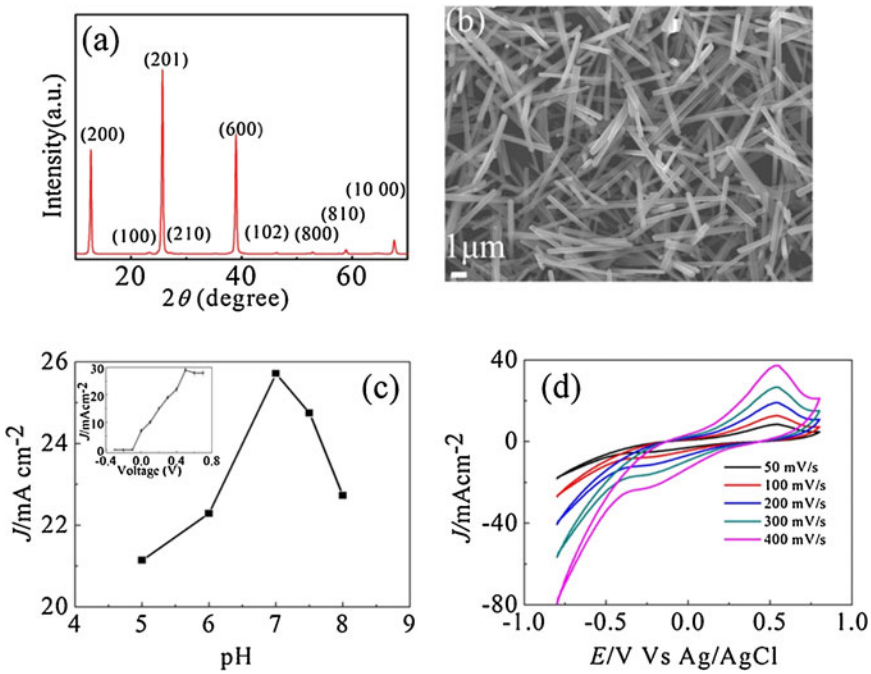

Fig. 1 **a** XRD pattern of MoO_3 nanowires synthesized at 180 °C, **b** FE-SEM image of MoO_3 nanowires, **c** effect of pH on the response of Au–MoO_3–LOx–Nafion electrode at 0.5 V in the presence of lactate (1 mM) and 0.05 M phosphate-buffered saline, and **d** CV obtained from the Au–MoO_3–LOx–Nafion electrode in 10 mM PBS at different scan rates

Figure 1c illustrates the effect of pH on the oxidation current density when the biosensor was subjected to 1 mM of L-lactate standards in PBS solutions at different pHs. The results indicate that the oxidation current density increases significantly with increasing pH, reaching a maximum at pH 7. Above pH 7, we observed a decrease in enzyme activity, which is in good agreement with results obtained for other previously reported electrochemical sensors [20–22].

Cyclic voltammetry (CH Instruments Inc., TX, USA) was used to investigate the possibility of direct electron transfer between LOx and the electrode surface. The influence of scan rate on the cyclic voltammetric (CV) performance for Au–MoO_3–LOx–Nafion in 10 mM PBS was investigated, and the results are shown in Fig. 1d. The well-defined and quasi-reversible redox peaks suggest favorable direct electron transfer between the electrode and redox centers of the LOx [23–25]. All other electrochemical measurements were carried out with a constant scan rate of 20 mV s^{-1}, and the L-lactate concentration in PBS was increased by an increment of 0.5 mM. The CV of Au–MoO_3–enzyme–Nafion with 2 mM L-lactate is shown in Fig. 2. An anodic peak was observed at approximately 0.50 V for a 2-mM L-lactate solution which is in the physiological range of L-lactate. For comparison, CV measurements of the Au–LOx–Nafion electrode without nanowires were performed, and apparent anodic peak potentials were found at 0.54 V (Fig. 2a). When 2 mM L-lactate was added to the PBS solution, a weak oxidation peak appeared in the CV of the Au–LOx–Nafion electrode without nanowires. In contrast, the oxidation peak current density of the electrode with MoO_3 nanowires was about 20 times higher than that of the electrode without nanowires (Fig. 2b). We also measured the current density response in the range of −0.3 to 0.5 V and found that current density increased rather slowly when the voltage was below 0.3 V. However, when voltage is higher than 0.3 V, the current density rises drastically and reaches maximum value at +0.5 V (inset of Fig. 1c). Based on the above observations, the applied voltage was set to be +0.5 V in all the other experiments which is consistent with that used in other literature [26–29]. In order to understand the role of LOx in the sensor, we also prepared Au–MoO_3–Lox–Nafion and Au–MoO_3–Nafion and measured the CVs of electrodes in a 2-mL PBS solution (pH 7) with and without lactate. The results indicate that MoO_3 has no electro-catalytic activity in either the oxidation or reduction of lactate without LOx, suggesting that the synthesized biosensor is mainly enzymatic and cannot be operated without the functionalization of LOx.

We also measured the CVs of an Au–Nafion electrode and Au–MoO_3–Nafion electrode without the LOx in a 2-mL PBS solution (pH 7) containing 10 mM H_2O_2. The results indicate that MoO_3 has no electro-catalytic activity in either the oxidation or reduction of H_2O_2. Nanowires have been shown to enhance direct electron transfer between active redox sites of the LOx enzyme and a gold surface. In addition, the electrochemical response of the Au–MoO_3–LOx–Nafion electrode in deaerated PBS did not show any change compared to aerated conditions. This suggests that the influence of oxygen on the biosensor system can be

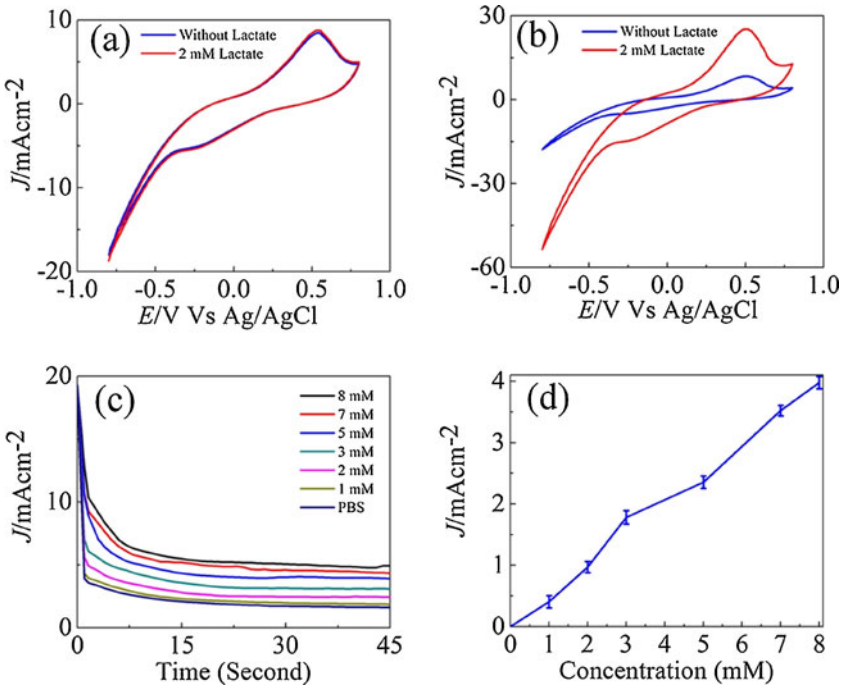

Fig. 2 Comparison of the CV responses of the **a** Au–LOx–Nafion electrode, **b** Au–MoO_3–LOx–Nafion electrode, in PBS and after the addition of 2 mM lactate, **c** amperometric response of the Au–MoO_3–LOx–Nafion electrode at 0.5 V upon the addition of lactic acid into the 2 mM PBS, and **d** calibration curve for the Au–MoO_3–LOx–Nafion electrode showing the oxidation current density versus lactate concentration

ruled out. This also confirms that the observed electrochemical change is due to the electron transfer from enzyme to electrode by enzymatic L-lactate oxidation [25, 29].

$$\mathrm{LOx-FAD+L-lactate \rightarrow LOx-FADH_2+pyruvate} \quad \text{(a)}$$

$$\mathrm{LOx-FADH_2 \rightarrow LOx-FAD+2H^+ + 2e} \quad \text{(b)}$$

$$\mathrm{LOx-FADH_2 + O_2 \rightarrow H_2O_2 + LOx-FAD} \quad \text{(c)}$$

In order to further confirm the mechanism of direct electron transfer, we calculated the unimolecular electron transfer rate constant with and without the presence of O_2. We found that the electron transfer through the electrode is much faster than electron transfer to O_2 and that the current is not affected by changes in the dissolved O_2 concentration. By utilizing an approach described previously in [25, 29], we estimated the unimolecular electron transfer rate constant k_s ($2.52\pm0.15\ s^{-1}$). This value is ten times higher than the electron-transfer rate of native LOx with O_2 as electron acceptor which leads us to the conclusion that the incorporation of MoO_3 nanowires in the enzyme greatly increases its maximum turnover rate.

Figure 2c demonstrates the chronoamperometric response of the MoO_3 nanowire-based biosensor, at an applied voltage of 0.5 V. The biosensor exhibited a fast and sensitive response to changes in L-lactate concentration, and 90% of the steady current density was achieved within 10 s. Figure 2d shows a typical calibration curve obtained from the Au–MoO_3–LOx–Nafion electrode at an operating potential of 0.5 V. The current density increased linearly as a function of the L-lactate concentration. The calibration curve exhibited an excellent linear relationship (correlation coefficient R=0.9920) in the range of 0.5 to 8 mM, with a sensitivity of 0.87 μA/mM. The detection limit for L-lactate was determined to be approximately 0.15 mM, based on multiple measurements taking into account the standard deviation of blank noise (95% confidence level, k=3, n=5).

The reproducibility and long-term stability of Au–MoO_3–LOx–Nafion electrodes were also evaluated as shown in Fig. 3. The reproducibility of the sensor was studied by measuring the sensitivity of ten electrodes in L-lactate concentrations ranging from 0.5 to 8 mM (Fig. 3a). The storage stability of Au–MoO_3–LOx–Nafion electrode was determined over a period of 16 days. The sensor was stored in PBS at 4 °C and was tested every 4 days. The results demonstrate that the response was almost the same over the entire investigation period, implying that the electrode can be used for long-term routine applications. The experiment was repeated five times to measure the change in the response of current density. The results reveal that the relative standard deviation of the potential responses is 0.5%, demonstrating that the MoO_3 sensor is stable and suitable for repeated detection of L-lactate (Fig. 3b).

In order to use these biosensors for L-lactate detection in real samples, it is necessary for the sensor to be insensitive to other electroactive substances. Interference studies into discrimination against possible interferents were carried out with the present biosensor. The responses to typical interferents, such as ascorbic acid, uric acid, cysteine, and succinic acid, were measured at their maximum physiological concentrations in a serum. It was found that uric, cysteine, ascorbic and succinic acid did not cause any significant interference in the response of the biosensor. These results indicate that the biosensor developed in this work has ultra-high sensitivity for detecting L-lactate and selectivity from electroactive substances at normal concentrations in serum samples.

Conclusions

Using a hydrothermal method, we synthesized MoO_3 nanowires with an average diameter of 100 nm and a length in the tens of micrometers. An L-lactate biosensor was then fabricated by immobilizing LOx on MoO_3 nanowires. We showed that MoO_3 nanowires form an attractive matrix for LO_X immobilization; they exhibit high affinity, sensitivity,

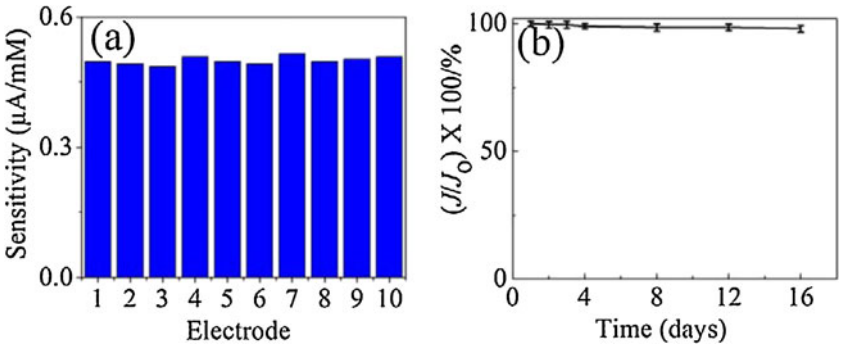

Fig. 3 **a** Sensitivity values measured on ten different MoO_3 nanowires electrodes in lactate concentrations ranging from 0.5 to 8 mM and **b** variation in amperometric response taken over 2 weeks for MoO_3 nanowire electrodes with the addition of 2 mM lactate

and fast response in L-lactate detection. This simple method of fabricating MoO_3–LOx biosensors can also be exploited to immobilize other enzymes and bioactive molecules on various 1D metal oxide nanostructures to form versatile electrodes for biosensor studies.

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