A Novel L-Lactate Sensor Based on Enzyme Electrode Modified with ZnO Nanoparticles and Multiwall Carbon Nanotubes

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Abstract—A highly sensitive and stable L-Lactate sensor based on the synergic action of multi-wall carbon nanotube (MWCNTs) and ZnO nanoparticles has been developed. The unique sandwich-like layer structure (PDDA/LOD/ZnO/MWCNTs) provides a favorable microenvironment to keep the bioactivity of LOD and prevent enzyme molecule leakage. Therefore, the proposed L-Lactate biosensor exhibited good analytical performances including high sensitivity and selectivity with satisfactory stability to amperometric determination of L-Lactate. The results indicated that the proposed PDDA/LOD/ZnO/MWCNTs film was an attractive matrix for the immobilization of LOD enzymes to fabricate biosensors.

I. INTRODUCTION

Since the first report of glucose biosensor in 1962 [1], extensive efforts have been made to improve the performance and stability of enzyme electrodes. Modified electrodes with various configurations and the performance of the constructed biosensors have attracted great interests. The ideal immobilization method for the enzyme should employ the mild chemical conditions and the short immobilization time to allow enough quantities of enzyme to be immobilized. In addition, the electrode materials with a large surface-to-volume ratio can increase the amount of enzyme immobilized and minimize the barriers for mass transportation. The combination of nanomaterials and biotechnology open a promising field for the development of the third generation biosensor, which is based on the direct electron transfer between the enzyme and the electrode. The nanomaterials have their unique advantages in immobilized enzyme. They could keep activity of enzyme due to the desirable microenvironment, and enhance the direct electron transfer between the enzyme’s active sites and the electrode. Up to now, many inorganic nanomaterials, such as gold nanoparticles and nanoclusters [2,3], Au/polyaniline nanocomposite [4], CNTs [5,6], nanocrystalline diamond [7], calcium carbonate nanoparticles [8] and Ag dendritic nanostructures [9], have been used as signal transducers and platforms for the enzyme immobilization in biosensors. In biosensors based on CNTs, the CNTs play multiple roles: (1) a substrate to immobilize enzyme, (2) electrocatalytic oxidation or reduction of H2O2 at the CNT surface to reduce overvoltage and avoid interference from other co-existing electroactive species, and (3) an enhanced signal because of its fast electron transfer and large working surface area [10].

The ZnO nanostructure also has great potential in application for sensor construction in terms of its biomimetic and high electron communication features [11-12]. Immobilization of cytochrome c on nanoporous ZnO film has already been reported. The ZnO with a high isoelectric point (IEP ~ 9.5) should be suitable for the adsorption of proteins or enzyme with low IEP [13]. Positively charged ZnO nanorods matrix not only provided a friendly microenvironment for negatively charged lactate oxidase (LOD) (IEP ~4.2~ 4.5) to retain its activity but also promoted the direct electron transfer effectively between LOD and the electrode. The experiments indicated that both conductive and biomimetic properties of ZnO nanorods played important roles in the electrochemical behavior of the adsorbed enzyme. Zhang et al. have reported a reagentless uric acid biosensor based on ZnO nanorods [14]. Recently, GOx was immobilized on ZnO nanocomb and nanorods to construct an amperometric biosensor for glucose biosensing [15-17] showing a sensitivity of 13 ~ 23 µA/mA.cm².

In this work, L-Lactate oxidase (LOD) was immobilized on the surface of ZnO nanoparticles due to their large difference in the IEP, while the ZnO nanoparticles were deposited on the negatively charged MWCNTs layer. A cationic polydiallyldimethylammonium chloride (PDDA) layer was coated on the LOD layer. The unique sandwich-like layer structure (PDDA/LOD/ZnO/MWCNTs) formed by self-assembling provided a favorable microenvironment to keep the bioactivity of LOD and to prevent enzyme molecule leakage. The excellent electrocatalytic activity toward H2O2 of the fabricated PDDA/LOD/ZnO/MWCNTs electrode indicated that the polyelectrolyte-protein multilayer and ZnO nanoparticles do not affect the electrocatalytic properties of MWCNTs, enabling sensitive determination of L-lactate. The synergic effect of MWCNTs and ZnO nanoparticles were revealed.
II. Experimental

A. Apparatus

Electrochemical experiments were performed with a CHI 630A workstation (CH Instruments) at room temperature in a conventional three-electrode system containing a 3.0 mm-diameter pyrolytic graphite (PG) disk working electrode modified with PDDA/LOD/ZnO/MWCNTs (home made), a platinum wire as the auxiliary electrode, and the Ag/AgCl as reference electrode. SEM, TEM and XRD images were recorded by a JEOL JSM-6700F Electron Microscope (Japan), HRTEM (Hitachi H-9000) and a D/MAX 2550V with Cu Kα radiation, respectively.

B. Reagents

LOD (EC 1.1.3.2, 34 units/mg, from Pediococcus species), uric acid and ascorbic acid glucose, PDDA were purchased from Sigma-Aldrich. Other reagents were of at least analytical-reagent grade. All solutions were prepared using doubly distilled water.

C. Preparation of ZnO/MWCNTs / PG electrode

The preparation process of the electrode was shown in Fig. 1.

![Figure 1](image)

Figure 1. Schematic illustration of the preparation process of a PDDA/LOD/ZnO/MWCNTs/PG electrode.

The PG wafer (3-mm in diameter, 1-mm in thickness) was polished sequentially with metallographic abrasive paper (No. 6), slurries of 0.3- and 0.05-µm alumina, and then sonicated sequentially in acetone, 1 mol/L (M) of NaOH, HNO₃ (1:1, v/v) and doubly distilled water. After rinsing with cellulose and C₁₀H₁₈O with 3:97 mass ratio, were mixed into sonicated sequentially in acetone, 1 mol/L (M) of NaOH, was polished sequentially with metallographic abrasive paper and double distilled water, it was sonicated with absolute ethanol and double distilled water for about 1 min, respectively.

MWCNTs were obtained by catalytic decomposition of CH₄ with La₂NiO₄ catalyst [18]. The purified MWNts (3 g) and the organic solvent (2 g), which was composed of ethyl cellulose and C₁₀H₁₈O with 3:97 mass ratio, were mixed into a kind of black slurry. Then the mixture was transferred onto the PG wafer using a microsyringe and allowed the solvent (water) to evaporate at ambient temperature. The as-prepared MWTNT films with a thickness of about 8 µm were annealed at atmospheric pressure of nitrogen at 500 °C for 10 min to remove the organic and other impurities. The growth of ZnO nanostructures on MWCNTs films followed the vapor-solid self-catalyzing mechanism. A mixture of an equal amount of ZnO powder and graphite powder, into which a little high purity zinc powder was added, was prepared as a reaction source and was placed at the end of a long quartz boat. In the same boat, a PG wafer with MWNT film was placed downstream site in the vapor flow direction at proper distances from the source, within desirable temperature regions correspondingly, to collect the products. The entire assembly was then placed into a horizontal-tube furnace and heated to 750 °C. It was kept for 15-30 min, under a pressure maintained by a constant high purity argon-gas flow of 500 SCCM (SCCM denotes cubic centimeter per minute at STP). After the reaction, the sample was taken out from the furnace and cooled down to room temperature. The PG electrode was prepared by putting the PG wafer (geometric area: 3.14×10⁻² cm²) with the MWCNTs and the ZnO nanoparticles layers into a glass tube, and fixed it by epoxy resin. Electrical contact was achieved by adhering a copper wire to the PG wafer with the Wood’s alloy.

D. LOD immobilization

LOD solution was prepared by dissolving 14.7 mg LOD in 1.0 mL phosphate buffer solution (PBS, 0.1 M, pH 6.8). 1.0, 2.0, 3.0 and 4.0 µL of the solution were dropped onto the ZnO/MWCNTs/PG electrodes surface, respectively and allowed to dry in air at room temperature. Finally, 4.0 µL 0.5% PDDA was coated on the enzyme electrode to eliminate the possible fouling and prevent the leaching of the enzyme. In this way, four electrodes with 0.5, 1.0, 1.5 and 2.0 units LOD were obtained. The enzyme electrodes were kept dry in refrigerator at 4 °C when not in use.

III. Results and Discussion

A. Characterization for MWCNTs and ZnO nanoparticles

Figure 2(a) shows a scanning electron microscopy (SEM) image of the cast MWNT film. It can be seen that the outer diameters of the nanotubes ranged from 20 to 40 nm and the typical length varied from 5 to 20 µm. No residuals can be found and the surface of nanotubes was smooth and tidy. The transmission electron microscopy (TEM) image of MWNTs was shown in the inset of Figure 2(a) indicated that the inner diameters of MWNT were in the range of 10 to 20 nm. The SEM images of MWNT films after the growth of ZnO nanostructures are shown in Figure 2(b). The walls of MWNTs were densely decorated by a great number of ZnO nanoparticles. The crystallinity of the ZnO nanostructures grown on MWNTs film was also examined by x-ray diffraction (XRD), and the result was shown in Figure 2(c). Clearly, similar to the bulk ZnO, the nanostructured ZnO were highly crystallized and exhibited a typical wurtzite hexagonal structure with unit-cell constants of a = 0.324 nm and c = 0.521 nm. It is worthy noting that the peak at 2θ = 26° originated from the graphite layers of the carbon nanotube film.

B. Cyclic voltamograms

Firstly, we evaluated the effect of enzyme loading on the L-lactate sensing. The experiment results illustrated that sensitivity of the L-lactate sensor was increased with the increase of the loading amount of LOD due to the catalytic ability of LOD. However, the linear range was decreased due to the limit of maximum current. We chose eclectically 2.0 U
LOD loading amount as the condition for sequent experiments. Secondly, the dependence of the response current for the sensor with 2.0 U LOD on pH values of the PBS buffer from 5.0 to 8.0 was tested. The maximum of response current was obtained at pH 6.8. Thirdly, the response time of the sensor with 2.0 U LOD was 6 s for the concentration of L-lactate changing from 0 to 1.0 mM and the relaxation time was 8 s via a time dependent curve at an applied potential of 400 mV, indicating the establishment of stable mass transport within 8 s. The L-lactate biosensor with 2.0 U LOD used to detect in 1.0 mM L-lactate standard solution for six times under the same conditions and the relative standard deviation (R.S.D.) of measurements was 2.85 %, illustrating a good reproducibility.

Figure 2. (a) SEM and i TEM (inset) image of the MWCNT, (b) SEM image of the high density ZnO nanostructures grown on MECNTs and (c) XRD pattern of ZnO nanostructures grown on MWCNTs.

Figure 3 shows the cyclic voltammograms for different LOD-immobilized electrodes in the absence or presence of L-lactate at a scan rate of 50 mV s$^{-1}$. MWCNT/PG electrode and ZnO/MWCNT/PG electrode exhibited significant electrocatalysis to the oxidation and reduction of H$_2$O$_2$ starting around 200 mV and reaching the maximum current values at 400 mV and -30 mV, respectively. The peak potential difference was 430 mV, implying that the redox reaction of H$_2$O$_2$ at the PDDA/ZnO/MWNT/PG electrode was irreversible. The above results indicated that the PDDA/ZnO/MWNT/PG multi-layer hybrid component electrode did not affect the electrocatalytic properties of CNTs remarkably. The oxidation peak potential of H$_2$O$_2$ at the ZnO/MWCNT surface was shifted from original 0.6 V to 0.4 V and amperometric responses were enhanced from original 12.5 µA to 21 µA.

C. Calibration curves and Michaelis-Menten constant

Fig.4 presents the amperometric responses of the PDDA/LOD/ZnO/ MWCNT/PG electrode at an applied potential of 400 mV to successive addition of lactate (0.2 mM /step) in 0.1 M PBS (pH 6.8) under continuous stirring. Insets: (a) Michaelis–Menten plot (Lineweaver–Burk plot. The straight lines are linear fits to the plots. A linear response range, sensitivity and detection limit (3σ) were 0.2 – 2.0 mM, 7.3 µA /mM and 0.16 mM, respectively. The apparent Michaelis-Menten constant ($K_{M}^{app}$) was also calculated to be 2.4 µM from the Lineweaver-Burk plot (Inset (b) in Fig.4).

Figure 3. Cyclic voltammograms obtained using different LOD-immobilized electrodes. (a) ZnO/MWCNT/PG electrode in the absence of L-lactate 0.1 M PBS solution (pH6.8). (b) PG electrode (c) MWCNT/PG electrode (d) ZnO/MWCNT/PG electrode in 1.0 mM of L-lactate solution. Scan rate: 50 mV/s.

D. Thermal Stability

Enzymes or proteins are susceptible to thermal denaturation; however, when they are immobilized onto the conducting surface, their thermal behavior will differ from that they are in the “free”state [19]. Thermal stability is a
measure of the practical features of the biosensor to withstand elevations in temperature, frequently in excess of those that normally denature the native enzyme [20–21]. The thermal stability of the proposed sensor was investigated between 10 and 40 °C. The amperometric responses continued to increase with the increase of temperatures and exhibited the ideal Arrhenius temperature-dependant relationship (inset of Fig. 5). The effect of temperature on the LOD–glutaraldehyde–BSA–modified sensor was also investigated under the same condition for comparison. In this case, immobilized LOD almost lost its activity at 40 °C and showed no amperometric response. Therefore, the excellent thermoresistance of the proposed sensor was ascribed to the construction of multilayer films. The hydrophobic ZnO nanoparticles provided a favorable environment for the immobilized LOD, which greatly enhanced the thermal stability of the sensor.

![Image](83x394 to 269x532)

Figure 5. Amperometric response of PDDA/LOD/ZnO/MWCNT/PG at 10, 20, 25, 30, 35 and 40 °C in 1.0 mM L-lactate in 0.1 M PBS (pH 6.8). Inset: plot of current vs. temperature

E. Anti-interferences

![Image](92x222 to 260x327)

Figure 6. Amperometric responses of the PDDA/LOD/ZnO/MWCNT/PG electrode to L-lactate (1.0 mM) and interferences (5.5 mM Glu, 0.4 mM UA, 0.2 mM PA, 0.06 mM AA and 0.2 mM CY) in 0.1 M PBS solution (pH 6.8)

Fig. 6 shows that the amperometric responses of the sensor to the interference species including glucose, uric acid, paracetamol, ascorbic acid and cysteine were negligible compared with that to L-lactate (1.0 mM), which indicates the proposed L-lactate biosensor has a good anti-interference ability. The good anti-interferences ability can be attributed to apply lower working potential due to the excellent electrocatalytic activity toward H2O2 of the fabricated PDDA/LOD/ZnO/MWNTs electrode.

F. Accuracy

To test the accuracy of the PDDA/LOD/ZnO/MWNT /PG sensor, several assays were made on serum samples. The L-lactate concentration was determined by the calibration curve and is presented in Table 1. Control experiments were carried out with a spectrophotometric method by a local hospital. As shown in Table 1, the results illustrated good consistent and precision between the two methods. In addition, the recovery tests were also carried out and the results are listed in Table 2. The recovery ranged from 96.5% to 104%.

![Image](336x222 to 531x342)

Figure 7. Long-term stability of the L-lactate sensor

The responses of the L-lactate sensor with 2.0 U LOD for 1.0mM L-lactate were measured 10 times a day (storage in a refrigerator when not in use). The average values over 10 measurements are plotted against the number of days from the preparation of the sensor. It can be seen from Fig.7 that a decrease in response current of the L-lactate sensor was observed during the first several days, which might arise from to the loss of un-immobilized enzymes. The response current decreased only 4.9% of the original value over 120 days for the L-lactate sensor. This long-term stability is attributed to
the synergetic effects of MWNTs and ZnO nanoparticles.

IV. CONCLUSION

A highly sensitive and stable L-lactate sensor based on the synergetic effect of MWNTs and ZnO nanoparticles has been successfully fabricated. The unique multilayer structure (PDDA/LOD/ZnO/MWCNT/PG) provided a favorable microenvironment to keep the bioactivity of LOD and the excellent electrocatalytic activity of MWNTs toward H₂O₂, enabling sensitive determination of L-lactate with good reproducibility and freedom of interference from other co-existing electroactive species. It showed a good agreement with the spectrophotometric method for the determination of L-lactate in serum samples, implying it could be applied to the real samples at low cost but with good precision.

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