A sensitive biosensor for lactate based on layer-by-layer assembling MnO_2 nanoparticles and lactate oxidase on ion-sensitive field-effect transistors

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A sensitive enzyme-based FET biosensor for lactate has been obtained by introducing MnO_2 nanoparticles at the gate surface *via* a layer-by-layer assembling method.

Ion-sensitive field-effect transistors (ISFETs) have received great attention in the rapidly developing field of biosensors.[1–8] Enzyme field-effect transistors, ENFETs, are based on biocatalytic reactions affecting the charges at the gate surface, and producing an electronic signal dependent on the concentration of substrates. Since the first reported ENFET based on glucose oxidase (GOD),[1] a lot of ENFETs were developed by using a wide variety of enzymes,[2] such as GOD,[5–7] horseradish peroxidase,[8] urease,[9] acetylcholine,[9] lactate dehydrogenase[10] and so on. However, a lactate biosensor based on the immobilization of lactate oxidase (LOD) on ISFET had not been reported previously. As is well known, lactate oxidase (LOD) works as a catalyst for changing lactate to pyruvate by oxidation, with O_2 being reduced to hydrogen peroxide. Due to the close pk_a values of lactic acid (3.86, 25 °C) and pyruvic acid (2.49, 25 °C), it seems that the ISFET could not be used to construct a sensitive lactate biosensor.

In the last decade, the application of nanometer materials has received great attention in the field of biotechnology and bioanalytical chemistry due to their unique and particular properties. As is well known, MnO_2 powder usually works as a catalyst to stimulate the disproportionation of H_2O_2 to O_2 and H_2O. However, MnO_2 nanoparticles were found to have special reaction activity in our previous work: they could react with hydrogen peroxide to form Mn^{2+} and O_2 whilst consuming two hydrogen ions.[11] Obviously, the pH change induced by the hydrogen ions consumed in the reaction could be monitored by the ISFET. This method could be utilized to develop oxidase-based FET biosensors, especially those biosensors in which little or no pH changes occurred in the enzyme (such as LOD) reactions.

Layer-by-layer assembly (LBL) developed by Decher is one of the most promising methods of thin film deposition based on alternate electrostatic adsorption of charged components (linear polyions, nanoparticles, and proteins).[12] The thin films are adsorbed onto a substrate from an aqueous solution through a sequential dipping process. Due to its simplicity and versatility, it has been successfully applied to the preparation of thin films of nanoparticles[13,14] and various proteins.[15,16] However, it has not been introduced to construct biosensors based on ENFET, to our knowledge.

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In this paper, we immobilized lactate oxidase and MnO_2 nanoparticles in poly(dimethyldiallylammonium chloride) (PDDA) films *via* layer-by-layer self-assembly to construct $(PDDA/MnO_2/PDDA/LOD)_n$ multilayer films for developing lactate ENFET. MnO_2 nanoparticles were prepared as previously reported.[11] ISFET transducers with a Si_3N_4 gate (20 μm × 400 μm) were purchased from the Institute of Electronics, Chinese Academy of Science (Beijing, China). The sensitivity of the ISFET is about 50 mV pH^{-1} in a pH range of 2 to 12. In pH 7.4 phosphate buffer solution (PBS), LOD is negatively charged. This negatively charged enzyme makes it adsorb onto polyelectrolyte (PE) multilayer films, beginning with a positively charged PE. Initially, the amino group was introduced onto the gate surface of the ISFET to make it positively charged in acidic solution by 10% (3-aminopropyl)trimethoxysilane at 50 °C in a water bath for 2 h. In order to obtain a densely charged surface for the stable adsorption of enzyme layers, the substrates were immersed in 2 mg mL^{-1} poly(sodium 4-styrenesulfonate) (PSS) and 2 mg mL^{-1} PDDA solutions alternatively, forming a PSS/PDDA "precursor" film. With the surface sufficiently overlaid with positive charges, the enzyme molecule or MnO_2 nanoparticles can be deposited by immersing the gate surface into a 1.4 mg mL^{-1} LOD solution or 20 mg mL^{-1} MnO_2 nanoparticle solution. Sequential repetition of the steps, that is, deposition of PDDA, nanoparticles, PDDA and enzyme, for up to 6 times led to the systematic stacking of PDDA/MnO_2/PDDA/LOD sandwich layers. Fig. 1 shows the idealized structure of the multilayer films. For comparison, multilayer films without MnO_2 nanoparticles were prepared similarly, thus PSS/$(PDDA/LOD)_n$ modified ISFETs were obtained.

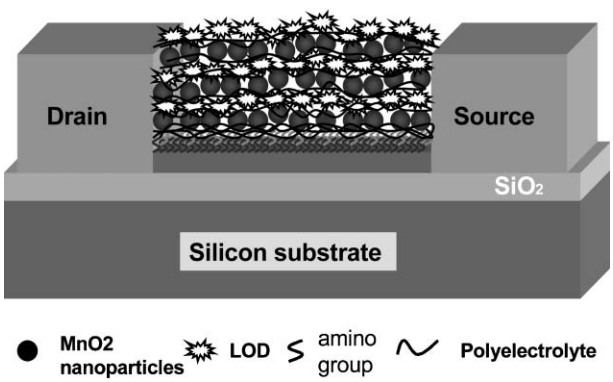

Fig. 1 Structure of an ISFET. The MnO_2 nanoparticles and LOD are layer-by-layer self-assembled on top of the sensitive membrane.

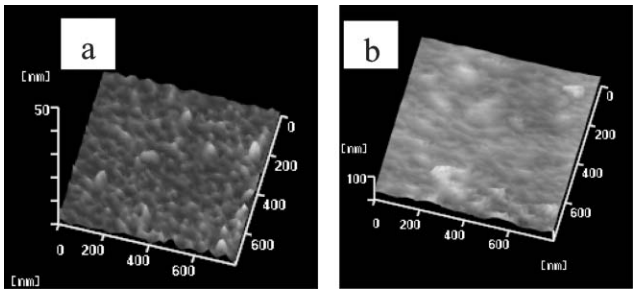

Fig. 2 AFM images of a precursor film of PSS/PDDA on a (3-aminopropyl)trimethoxysilane modified silicon surface (a) and a PSS/PDDA/MnO_2/PDDA/LOD film on a (3-aminopropyl)trimethoxysilane modified silicon surface (b). The AFM images were performed with an atomic force microscope (SPA-300 HA, Seiko Instrument, Inc., Japan).

Fig. 2 shows the morphologies of silicon slides after the deposition of PSS/PDDA and further deposition of MnO_2 nanoparticles, PDDA and LOD. A layer of dense PE film distributed on the whole silicon surface appeared (Fig. 2a). After the deposition of MnO_2 nanoparticles, PDDA and LOD, a homogeneous layer with some big "islands" was obtained (Fig. 2b), indicating the formation of a LBL film.

ENFETs were operated in a constant source current and drain-source voltage mode. The steady-state output voltages ascribed to a saturated calomel electrode (SCE) were recorded by a CHI660 Electrochemical Workstation (CH Instruments, USA) with a pre-amplifier. The sensor was immersed in a small glass cell filled with 2.0 mL of PBS. Before use, the sensor was soaked in 10 mM PBS (pH 7.4) for 20 min to equilibrate the membrane system. All experiments were carried out at 25 $\pm$ 0.1 °C. Magnetic stirring was used during measurements to ensure homogeneity of the solutions.

A typical response curve of the (PDDA/MnO_2/PDDA/LOD)$_3$ modified ENFET is shown in Fig. 3 (curve a). With the addition of lactate, the concentration of H^+ near the sensitive gate surface decreased, which induced the open circuit potential to shift to a more negative value. The response time is less than 100 s. For comparison, we also prepared a (PDDA/LOD)$_n$ modified ENFET. With the addition of lactate, a small reverse response occurred, indicating a small increase of the local pH in the sensitive biomembrane, which is due to the somewhat small pk_a value of pyruvic acid (2.49) compared to that of lactic acid (3.86). The calibration curves of the two kinds of ENFETs are shown in Fig. 3, inset. Without MnO_2 nanoparticles, the sensitivity of the ENFET is limited to 0.34 mV mM^{-1} (curve b). By introducing MnO_2 nanoparticles into the multilayers, the sensitivity of the ENFET is increased to 16.84 mV mM^{-1}, which is 50 times higher than that without MnO_2 nanoparticles. The dynamic range is extended up to 6.0 mM, and the linear range is 1.0 $\times$ 10^{-5}–3.6 $\times$ 10^{-3} M with a detection limit of 8.0 μM.

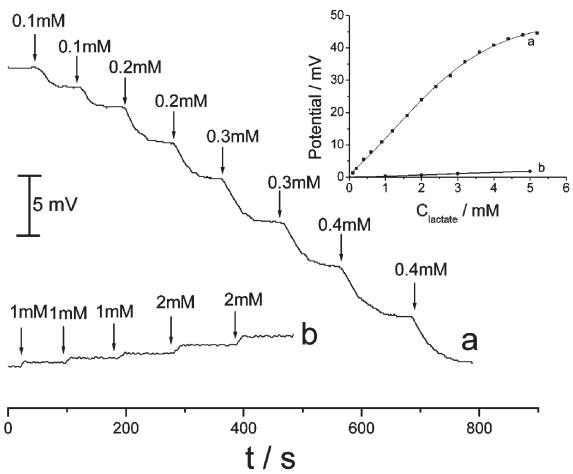

Fig. 3 Successive response of the three-multilayer film based ENFET with (a) and without (b) MnO_2 nanoparticles to lactate in 10 mM PBS (pH 7.4), containing 100 mM NaCl. Inset: calibration curve of the ENFET with (a) and without (b) MnO_2 nanoparticles to lactate.

The effect of the number of (PDDA/MnO_2/PDDA/LOD)$_n$ films on the performance of the biosensor was investigated by control experiments. The response of the biosensor with one to six layers was investigated. Modified with 2 and 3 multilayer films, the ENFET exhibited the largest response to 0.10 mM lactate. Meanwhile, the linear range increases as the number of multilayer films increases from one to three. An additional coating of four to six multilayer films induces decreases in the linear range. Thus the ENFET modified with the three-multilayer film exhibits an optimal performance in its response to lactate.

Since the response of the sensor is based on the local pH change of the ISFET sensitive gate surface, it would depend on the buffer concentration of the sample. A higher buffer concentration results in a smaller change of pH caused by the oxidation of hydrogen peroxide. The experimental results showed a dramatic decrease of the biosensor's response to lactate with an increase of buffer concentration. 10 mM PBS was selected to get a comparatively higher response and also to keep the solution at a certain buffer capacity.

Investigation of the influence of the buffer pH on biosensor performance is very important to optimize measurement conditions, since the activity of the immobilized LOD is pH-dependent. With a variation of the solution pH from 5.5 to 8.5, a decrease in response is observed. This phenomenon can be ascribed to a change in the reaction capability of the MnO_2 nanoparticles upon a different pH. An acidic environment is more favorable for the reaction of MnO_2 nanoparticles with hydrogen peroxide.[11]

Usually, the ionic strength of the solution affects the response of the ENFET. A variation of the response of the ENFET with different NaCl concentrations could be expected. Here, the performance of this modified ENFET showed independence from the concentration of NaCl. This phenomenon is consistent with our previous results.[11] However, large NaCl concentrations dramatically reduced the response of the ISFETs without MnO_2 nanoparticles. This indicated that the activity of the enzyme will be inhibited with an increase of the concentration of NaCl and the presence of MnO_2 nanoparticles will counteract this inhibiting effect. Although the exact reason for this result is not clear and needs further investigation, it is a good property for biosensors used in samples with high salt concentrations.

Though the MnO_2 nanoparticles in the multilayer films immobilized on the gate surface of the ENFET will be consumed gradually during the measurements, the lactate biosensor still shows good reproducibility and stability. The relative standard deviation (rsd) of the biosensor response to 1.0 mM lactate was 2.35% for 11 successive measurements.

The proposed biosensor also demonstrates acceptable long-term stability. When it was stored dry at 4 °C and tested at weekly intervals, its response was stable for 1 month, and the biosensor remained at about 89% of its original sensitivity after 2 months. The consumption of MnO_2 nanoparticles during the detections shows negligible influence on the long-term performance of the proposed lactate ENFET.

In summary, a layer-by-layer deposition technique has been proposed to prepare lactate biosensors on the gate of an ISFET. MnO_2 nanoparticles were introduced as an oxidant to react with H_2O_2 rather than a catalyst, which results in a sensitive pH change in the sensitive membrane of the ENFET with the addition of lactate. The resulting multilayer ENFET responds sensitively to low lactate concentration. It could be expected that the nanometer materials will greatly facilitate the appearance of various types of biosensors with excellent performances.

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