An electrochemical biosensor for the determination of lactic acid in expiration

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Abstract

We have successfully developed an electrochemical biosensor which could detect low level concentration of misty lactic-acid (LA) directly. In our method, enzyme lactate oxidase (LOD) was immobilized within mesoporous silica (FSM8.0) which was formed on screen-printed Prussian Blue (PB)-modified working electrode and the top of that was covered with hydrophilic porous membrane. The resulting biosensor showed an excellent sensitivity (150nM-1.1mM in solution) and can detect misty LA directly. This result indicates that our method provides a high-performance biosensor to determine LA levels in exhaled breath and that our approach is ideal for various medical applications and might improve the detection or management of disease.

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1. Introduction

The importance of lactic-acid (LA) measurement has increased recently because of its relation to specific pathological states such as shock, respiratory insufficiencies, and heart disease and because of its involvement in glucose metabolism [1]. So its measurement is very important to realize our quality of life, i.e., to achieve our comfortable and healthy life. However, current sensors for LA are for detecting LA in blood, and the determination of LA levels in exhaled breath, which is non-invasive and condensate as a
surrogate for blood-based LA sensors, is not a success because of its poor sensitivities. In this paper, we report an electrochemical biosensor which could detect low level concentration of misty LA directly.

2. Experimental

2.1. Chemicals and reagents

Lactate oxidase (LOD, E.C. 1.1.3.2, from Microorganism, 80 units/mg) was purchased from Toyobo Co., Ltd. (Osaka, Japan). Dococyltrimethylammonium chloride \([C_{22}H_{45}N(CH_3)3Cl]\) \((C_{22}TMA)\) was kindly donated by the Lion Corporation (Tokyo, Japan). Sodium silicate \((SiO_2/Na_2O = 2.07)\), tetraethyl orthosilicate \((TEOS)\), and lactic-acid \((LA)\) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,3,5-Triisopropylbenzene \((TIPB)\) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Several inks for the fabrication of screen-printed carbon electrodes, namely C2030519P4 \((carbon/graphite ink)\), C2070424D2 \((carbon mediate ink containing Prussian Blue \((PB)\))\), C61003P7 \((Ag/AgCl ink)\), and D2081009D6 \((dielectric ink)\) were purchased from Gwent Electronic Materials Ltd. (Torfaen, UK). Denatured polyvinyl alcohol \((\text{D-polymer, DF-20})\) and adipoyl dihydrazide \((\text{ADH})\) were obtained from Shin-Etsu Chemical Co., Ltd (Tokyo, Japan). Hydrophilic porous membrane \((\text{ARcare} \^{\text{TM}} 92205)\) was purchased from Adhesive Research, Inc. (Pennsylvania, USA). Ltd Glass epoxy sheets \((0.3 \text{ mm thickness})\) were supplied by Sumitomo Bakelite Co., Ltd (Tokyo, Japan). All other reagents were of analytical grade and were obtained from commercial sources.

2.2. Synthesis of mesoporous silica powder \((FSM8.0)\)

Mesoporous silica \((\text{MPS})\) powder, FSM8.0, with a pore diameter of 8.0 nm, was prepared from sodium silicate using dococyltrimethylammonium chloride and 1,3,5-triisopropylbenzene \((\text{TIPB})\) as we previously reported \([2]\). 16 g of C22TMA was added to 200 mL of water at 70 °C, and to this mixture was added 60 g of TIPB; the resultant mixture was vigorously stirred for 30 min at 70 °C. It was then added to 200 mL of water at 80 °C in the presence of 54.31 g of sodium silicate; the pH of this mixture was adjusted to 8.5 by slowly adding aqueous 2 M HCl. After the suspension was stirred for 3 h at 70 °C, the solid product was filtered out, washed three times with 300 mL distilled water at 70 °C, and dried. The sample was calcined at 150 °C for 2 h in air, and the calcined product \((300 \text{ g})\) was dissolved in a solution mixture of ethanol \((35.4 \text{ mL})\) and HCl \((1.7 \text{ mL})\). The suspension was then stirred for 5 h at 70 °C to remove the organic fraction. The solid product was filtered out, washed with ethanol three times, and dried; in this manner, we obtained FSM8.0.

2.3. Preparation of screen-printed carbon electrodes \((\text{SPCEs})\)

An LS-56TVA semi-automatic screen-printing machine \((\text{Newlong Seimitsu Kogyo Co., Ltd, Japan})\), equipped with a 250 threads per in. polyester screen, a polyurethane squeegee, and stainless steel flood blade was used to prepare the SPCEs. The SPCEs were printed in groups of 80 \((\text{reference, working, and auxiliary electrodes})\) onto glass epoxy sheets. Fabrication took place in four printing steps: (1) carbon ink was printed to form a conducting layer that also acted as the counter electrodes, (2) Ag/AgCl ink was printed in the positions for the reference electrodes, (3) carbon ink containing PB was printed on the working electrode to form an activated surface, (4) insulating ink was printed to cover the non-working and non-conducting region of the SPCE to define the working area. After each deposition, the electrodes were dried at 60°C for 30 min. The individual PB-SPCE was cut from the sheets into size 12 mm × 25 mm rectangles and the sampling unit composed of white PET \((\Theta 9.0 \text{ mm}, 12 \text{ mm} \times 12 \text{ mm} \times 1.6 \text{ mm})\),
which defined the working area and sample volume of 100μL, was bonded on top of each PB-SPCE with an epoxy adhesive. In a batch process, inexpensive PB-SPCEs with consistent quality were prepared. The working electrode was a 4 mm-diameter disk, and the auxiliary electrode and Ag/AgCl reference electrode were large and small portions of a 7.8 mm × 1.0 mm curved line, respectively.

2.4. Fabrication of the lactate sensor (HPM/LOD-FSM8.0/PB-SPCEs)

The LOD-immobilized FSM8.0, LOD-FSM8.0 conjugate, was prepared as follows [2]: An FSM8.0 powder (100 mg) was added to 4.0 mL of 10 mg/mL LOD solution (citrate buffer, pH 4.3). The suspension was then shaken for 24 h at 4°C to establish an adsorption equilibrium. The LOD-FSM8.0 conjugate was collected by centrifugation, and then washed with distilled water. The amount of LOD adsorbed into the pores of the FSM8.0 was estimated to be 232.5 mg/g of LOD-FSM8.0, as determined by spectrophotometric analysis.

The sensing layer of the lactate biosensor was constructed by the following procedure: (1) A 32.5 mg (1000 μL) of LOD-FSM8.0 and 500 μL of 20% DF-20 solution (2 g in 10 mL of water) was mixed together and stirred for 30 min. Then, 50 μL of 10% ADH solution (1 g in 10 mL of water) was added and stirred for a further 5 min. The resulting mixture was deposited in 2 μL aliquots on the working electrode surface of the PB-SPCEs and allowed to dry for 3 h at room temperature. (2) the surface of the working area was covered with a layer of hydrophilic porous membrane (HPM) with an average pore size of c.a. 200μm and thickness of c.a. 100μm by cutting and then rinsing with 0.1 M phosphate buffer (pH 7.4) to give HPM/LOD-FSM8.0/PB-SPCEs (Fig. 1). The external view of obtained HPM/LOD-FSM8.0/PB-SPCEs was shown in Fig.1.

![Fig.1. The external view of HPM/LOD-FSM8.0/PB-SPCE biosensor.](image)

2.5. Apparatus and electrochemical measurements

Amperometric measurements were performed out by applying a constant potential, 0.0 V, to the working electrode of the biosensor with a portable potentiostat (Palm Instruments BV, Netherlands) at room temperature (25 °C). After the transient current had settled (t = 200 s), a jet of misty LA was splayed to the sampling unit by nozzle and the sensor response was measured until the resulting current reached a steady state value. All electrochemical experiments were carried out after rinsing the working area with 0.1 M phosphate buffer (pH 7.4). The experimental setup is depicted in Fig. 2.
3. Results and Discussion

3.1. Principle of operation and the role of each layer

The principle of operation is described by the following reactions [3]:

\[ \text{Lactic acid} + O_2 \xrightarrow{LOD} \text{pyruvate} + H_2O_2, \]  

where immobilized LOD catalyzes the oxidation of LA to pyruvate with the production of hydrogen peroxide (H$_2$O$_2$) in the presence of molecular oxygen. This H$_2$O$_2$ is then detected on the PB-modified working electrode as a measurable current, the magnitude of which is directly related to the LA concentration.

The structure of our biosensor is illustrated in Fig.3. Each layer plays an important role in the detection process to achieve high-performance LA sensor: (1) Screen printed carbon electrode is formed on the substrate by screen printing technology, which is simple to fabricate and use. (2) Activated electrode containing PB lowers operating potential of the sensor [3]. (3) The enzyme LOD molecule was immobilized in mesoporous silica materials whose pore diameter is adjusted to the size of LOD($\approx$8.0nm). This enables high sensitivity and high stability of the sensor [2,4-7]. (4) Hydrophilic porous membrane layer, which consists of a water-based polymeric acrylate, was formed on the top. It has many channels with pore size of typically 200 $\mu$m is pass through the direction perpendicular to the substrate, which realizes rapid diffusion flow in z-direction and leads to high sensitivity and rapid response.
3.2. **Calibration curve and detection limit of the HPM/LOD-FSM8.0/PB-SPCEs**

The operating range and detection limit of the sensor were studied. Fig.4 and Fig.5 show the calibration curve and typical response of the sensor to introducing of 150nM concentration of misty LA. The HPM/LOD-FSM8.0/PB-SPCE sensor output had a good linear relation over the lactate concentration of 150nM-1.1mM and the lower detection limit of 150nM at a signal-to-noise ratio of 3, and the response time to reach a steady output current was within about two minutes. Thus, the HPM/LOD-FSM8.0/PB-SPCE sensor displayed high-sensitivity, wide rage linearity and rapid response that could be successfully applied to the detection of misty LA. This indicates a high affinity between LA and LOD immobilized within the HPM/LOD-FSM8.0/PB-SPCE sensor, mainly due to its biocompatibility and large surface area.

![Fig. 4. Calibration curve of our sensor](image)

![Fig.5. Typical response for misty LA](image)

3.3. **Selectivity against interferents**

The interference effects due to the most common electrochemically active species (200μM; ascorbic acid, cholesterol, cysteine, glucose, glutamine acid, urea, and uric acid) were also evaluated. The relative response gives a measure of the sensor selectivity. As shown in Fig.6, it was found that our biosensor retained specificity for LA and produced smaller signals for all chemicals other than LA under the testing conditions. In particular, the responses to ascorbic acid was attenuated to only 2.1% of the 200μM lactic-acid response. These results indicate that interference from these electroactive species was suppressed due to the low operating potential of 0.0V, and the HPM/LOD-FSM8.0/PB-SPCE sensor has accurate responses in the presence of potentially interfering substances.

![Fig.6. Selectivity of our sensor](image)
3.4. Storage stability of the sensor

The storage stability of the HPM/LOD-FSM8.0/PB-SPCE sensor was investigated. The sensor was stored desiccated at 4°C when not in use. Under these storage conditions, the HPM/LOD-FSM8.0/PB-SPCE sensor exhibited good stability, and shows no decrease over a period of 200 days (Fig.7). Such excellent stability is crucial for any commercial biosensor device. The highly stable nature of this sensor is thought to result from the intimate integration of the enzyme with FSM8.0 and shows that FSM8.0 efficiently retains the activity of LOD.

![Fig.7. Storage stability of our sensor](image)

Therefore, we have realized a high-performance LA sensor which possesses a great potential to detect LA in expiration.

4. Conclusion

We have succeeded detecting low level concentration (150nM) of misty LA. And it is fabricated with a simple and cost effective way. We think that the excellent sensitivity of our sensor reported herein is accomplished by suitable enzyme-immobilization method and sensor structure. Our result means that it is possible to determine LA levels in exhaled breath; that is, construction of non-invasive LA sensor is possible. It shows that our approach is ideal for various medical applications to realize a high quality of life and might improve the detection or management of disease, such as potential diabetic patient management, drastically in the near future.

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