

N, *N*-Dimethylindoaniline Mediated-Amperometric Detection of L-lactate

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It was found that *N*,*N*-dimethylindoaniline works as an excellent electron-transfer mediator for lactate oxidase. The second-order rate constant (k_{med}) with lactate oxidase in solution was much higher than that of other typical mediators, such as ferrocene and potassium hexacyanoferrate(II). An enzyme electrode using this compound as a mediator showed a larger response current and a higher sensitivity to lactate than a Pt-based H_2O_2 detecting lactate sensor. This sensor demonstrated a clear independence on the oxygen concentration in a buffer and a quite low interference current by ascorbic acid, etc. A H_2O_2 detecting sensor was strongly interfered by acetaminophen and uric acid, while a mediator based sensor was slightly influenced by uric acid. This sensor also showed good durability, which retained more than 90% of the initial activity until day 11.

Biosensors, which utilize highly specific enzymatic reactions, have been the subject of considerable attention for practical use. A lactate sensor comprising lactate oxidase (LOD) is monitored as a current signal, thus indicating the concentration of lactate. Lactate sensors based on oxygen or hydrogen peroxide have been developed and successfully applied to clinical analysis.[1,2] However, this type of biosensor has some disadvantages, such as sensitivity to O_2 concentration, and significant interference signals by ascorbic acid etc.[3–5] In order to solve these issues, a highly sensitive biosensor was designed using an electron-transfer mediator. The mediator plays the role of an electron-acceptor in place of O_2, and transfers the electron to an electrode. As for mediators, metal-coordinated compounds and inorganic metal complexes have been commonly used because of their high durability.[6,7] Although organic mediators show good mediation performance, their stability is questionable, as compared to that of metal-related ones. However, organic species can be tuned to the mediation properties by chemical modifications, such as immobilization or derivatization. Some organic compounds are known to be mediators for the oxidation of LOD.[8–11]

In the present work, we have shown the electron-transfer property of *N*,*N*-dimethylindoaniline (DMIA) with LOD by means of an electrochemical method, and have described the lactate sensor performance using DMIA as a mediator.

Experimental

Materials. Lactate oxidase from *Pediococcus sp*. (Asahi chemical, 120–60 U/mg solid), L-lactate (WAKO), and graphite powder was used. The mediator compounds were examined without further purification. The buffer and all other chemicals were of analytical grade.

Electrochemical Measurements. A mediator solution (0.1–1 mM) of 0.1 M KCl/0.1 M phosphate buffer (pH 7.5) containing 30 mM L-lactate was prepared. 2 mL of this solution in the presence or absence of 10 μL of a LOD solution (0.2 mg/mL in 0.1 M KCl/0.1 M phosphate buffer) was placed in an electrochemical cell and bubbled with nitrogen for 5 minutes. Glassy carbon (GC: ϕ = 1.6 mm), silver/silver chloride (Ag/AgCl), and Pt electrodes were used as the working, reference, and counter electrodes, respectively. A cyclic voltammogram (CV) was carried out under nitrogen at 25 ± 0.1 °C with a sweep rate of 20 mV/s. The measurement was performed with a HOKUTO HA-501 or HA-502 potentiostat controlled by a computer via a D-A/A-D converter.

The various concentrations of the mediator solution (0.1, 0.3, 0.5, 0.7, and 1.0 mM) containing 30 mM of L-lactate were placed in an electrochemical cell and bubbled with nitrogen and CV, and their potential-step chrono-amperometry was performed with variable sweep rates of 1,5,10,20,50, and 100 mV/s using a HOKUTO HA-501 potentiostat. The catalytic limiting current (I_{cat}) and the diffusion-controlled peak current (Ip) (an example is shown in Fig. 2) were plotted against C_{med} and $v^{1/2}$ according to the following Eqs. 1 and 2, respectively,

$$\text{Slope A} = I_{cat}/C_{med} = nFA(k_{med}D_{med}C_{ez})^{1/2} \quad (1)$$

$$\text{Slope B} = Ip/v^{1/2}C = 0.4463nFA(nF/RT)^{1/2}D_{med}{}^{1/2} \quad (2)$$

where C_{med} and C_{ez} are the concentrations of mediator and enzyme, respectively, and n is the number of electrons involved in the reaction at the electrode, C is the concentration of the mediator in the absence of LOD, and D_{med} is the diffusion coefficient of the mediator. The k_{med} value can be calculated from the following Eq. 3.

$$k_{med} = 7.757n(\text{Slope A/Slope B})^2C_{ez}{}^{-1} \quad (3)$$

Preparation of Enzyme Electrode. Graphite powder was neutralized with 0.1 M phosphate buffer of pH 7.5. Dried carbon powder (100 mg) and the mediator (20 μmol) were mixed with a small amount of acetone in an agate mortar until the solvent was evaporated. The mediator/carbon powder (30 mg) was placed in the agate mortar, and 100 μL of phosphate buffer (pH 7.5) con-

taining 1 mg of LOD and 1 mg of Bovine Serum Albumin was added and mixed. After drying, 50 mg of epoxy resin and 2.5 mg of Triton X-100 were added and thoroughly mixed. This paste was printed on a platinum electrode which was 2 mm across in a diameter, and dried at room temperature under vacuum overnight.

Stopped-Flow Method. The enzyme electrode was set to a three-electrode flow cell, and a potential was applied against Ag/AgCl reference electrode. TOSOH HPLC system was used for the measurement using a buffer of pH 7.0 as a flow solution. The flow rate was 0.2 mL/min. A L-lactate solution of various concentrations was injected by an autosampler; the pump was stopped after 1 min from sample injection in order to expose L-lactate to the sensor for 2 min in the quiescent solution, followed by re-start to rinse the biosensor with the buffer.

Interference Effect Measurement. An interference effect of the sensor was carried out by injections of various substances of higher concentration than the normal value in blood.

Influence of O_2 Concentration. Lactate assays of mediator-based sensors were carried out by injections of various concentrations of lactate dissolved in oxygen-, air-, and argon-saturated buffer solutions, respectively.

Durability Measurement. The enzyme electrode mentioned above was covered with a Nuclepore® membrane. After 30 shots of lactate solution (2 mM) had been injected per day, the sensitivity was measured for 14 days at room temperature.

Results and Discussions

Electrochemical Measurement. Mediators were evaluated by an electrochemical method[4,12,13] as a function of the redox potential and the second-order rate constant for mediation (k_{med}) in solution. The results are summarized in Table 1. Figure 1 shows the chemical structure and cyclic voltammograms of DMIA in the presence of LOD. The addition of LOD led to an increase in the anodic current; the so-called "catalytic current" wave was observed of LOD to the oxidized form of the mediator. In order to fabricate an O_2-independent and less-interfered biosensor, a mediator showing a low redox potential and a high k_{med} value is favorable. Consequently, DMIA showed a lower redox potential and a remarkably higher k_{med} value than a sodium ferrocene carboxylate (FcCOONa) and osmium(0) tris(4,4′-dimethoxy-2,2′-bipyridine) complex (Os(DMO-bpy))$_3$.[14] On the other hand, 2,6-dichloroindophenol (DCIP) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), which are partial moieties of DMIA, showed relatively low k_{med} values in spite of an analogous redox potential.

Table 1. The Electrochemical and Mediating Properties of Several Mediators for LOD

Mediators	$E^{Oa)}$/mV vs Ag/AgCl	k_{med} $\times 10^4$ M^{-1} s^{-1}
FcCOONa	330	4.38
Ni-cyclam	434	0.78
$K_3[Fe(CN)_6]$	248	0.57
Os(DMO-bpy)$_3$[b)]	270	2.59
DCIP	−5	6.91
DMIA	0	15.10
TMPD	68	0.89

a) The E^O were estimated by CV from the midpoints of the anodic and cathodic peak potentials.
b) See Ref. 13.

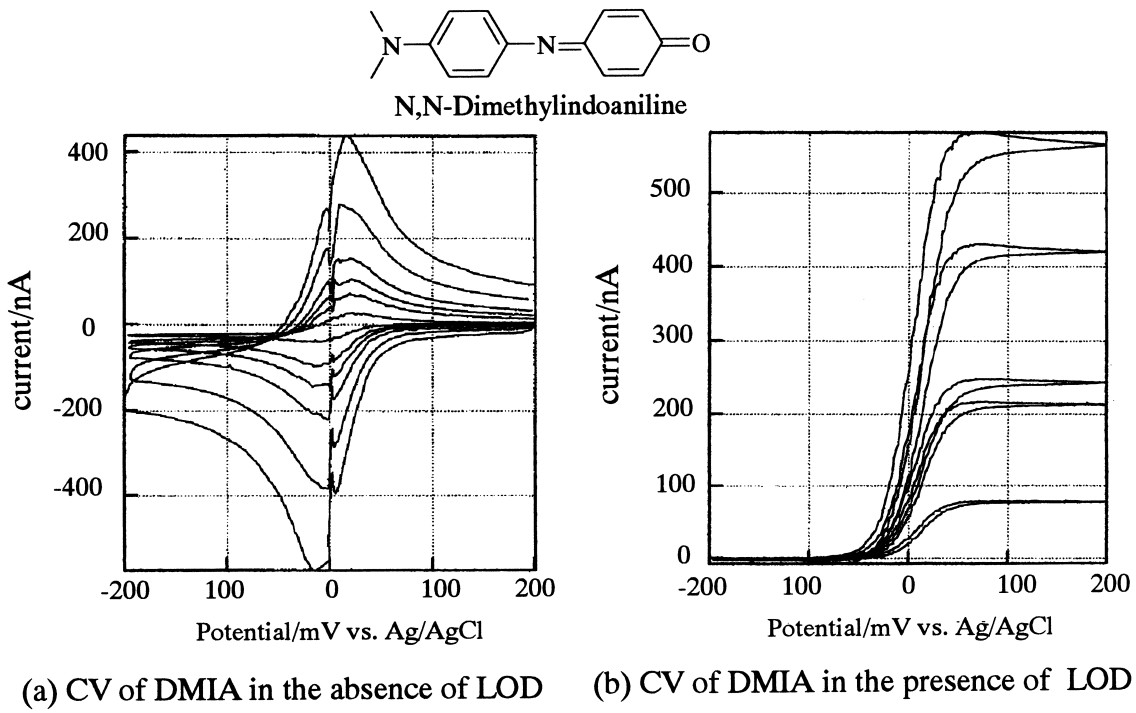

Fig. 1. Chemical structure and cyclic voltammograms of DMIA. (a) 1.0 mM of DMIA in 0.1 M KCl + 0.1 M PBS solution containing 30 mM L-lactate. Sweep rates:1, 5, 10, 20, 50, 100 mV/s. (b) 0.1, 0.3, 0.5, 0.7, and 1.0 mM of DMIA in buffer solution containing LOD and 30 mM L-lactate. Sweep rate: 20 mV/s.

This suggests that the chemical structure of mediators plays a crucial role in electron transport from LOD as well as the redox potential.

Enzyme Electrode. An enzyme electrode including selected mediators and LOD without any cover membrane was prepared according to Ikeda's method.[15] To reduce any interference effect like the oxidation of uric acid or ascorbic acid, the working potential was set to 200 mV against Ag/AgCl. The sensor performance was analyzed by the stopped-flow method and evaluated based on sensitivity and durability. The response current of this sensor was plotted against the lactate concentration. The sensitivity was evaluated from the initial slope of this calibration curve. The durability was estimated by the remaining activity of the sensor after 30 sample injections of 2 mM lactate solution. The results are summarized in Table 2. A LOD/DMIA-based sensor showed considerably higher sensitivity and better durability than others. This result indicated that this sensor showed an effective electron transportation from LOD to the electrode via the mediator.

Influence of Oxygen Concentration. Oxygen is a potential competitor for mediators in electron transfer from reduced enzymes. If oxygen significantly takes part in the recycle of LOD as an electron-acceptor in the sensor, the response current would be influenced by the oxygen concentration. Figure 2a shows a calibration curve for the LOD/DMIA sensor, where the response current is plotted against the lactate concentration under Ar, O_2, and air-saturated buffer solutions, respectively. As a comparison, a Pt-based H_2O_2 detecting sensor was also analyzed at 400 mV of the working potential.[16] The LOD/DMIA sensor demonstrated a slight dependence of the oxygen concentration in the sample solution, while a H_2O_2 detecting sensor was strongly influenced. In addition, a DMIA-based sensor showed better linearity in the range from 0 to 16 mM and a larger response current than the other.

Interference Effect Measurement. The interference effect was also examined by the injection of various substances of higher concentration than that in normal blood. Figure 3 shows the interference current ratio against 2 mM lactate response. It was found that the H_2O_2 detecting sensor strongly interfered by acetaminophen [*p*-(acetamido)phenol] and uric acid, while the mediator-based sensors were slightly influenced by uric acid. For example, the interference current ratios of the DMIA-based sensor were 4.0 (uric acid) and 0.0% (acetaminophen), respectively. However, those of the H_2O_2 detecting sensor were 48.7 and 78.0%, respectively.

Table 2. The Lactate Sensor Performance Using Mediators without a Covering Membrane

Mediators	Working potential	Sensitivity	Durability
	mV vs Ag/AgCl	nA/mM^{-1} s^{-1}	%
FcCCONa	400	3	1
Ni-cyclam	—[a)]	—	—
$K_3[Fe(CN)_6]$	—	—	—
$Os(DMO\text{-}bpy)_3$	400	none[b)]	none
DCIP	200	6	30
DMIA	200	92	70
TMPD	—	—	—

a) Not measured. b) Not detected.

Durability Measurement with Cover Membrane. Finally, an extended durability measurement of the lactate sensor was carried out using DMIA. The lactate sensor was covered with a Nuclepore® membrane. The result is shown in Fig. 4. When this membrane was used, the response current of the sensor dropped to about 1/8 of the original level, but still maintained an acceptable linearity against the lactate concentration. This sensor retained about 90% of the initial activity until day 11. At day 13, the sensor still retained a good linearity with 25% of its initial activity. Based on this experiment, it is possible to utilize long-life time lactate sensor applications by a further minor fabrication or modification that Wilkins or Heller has reported for the sensor.[4,7,17]

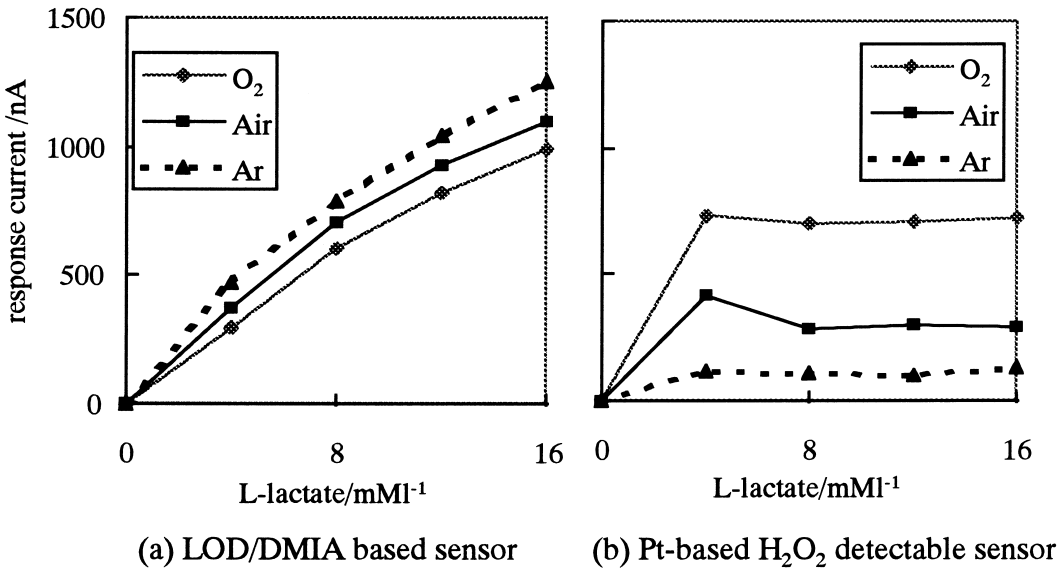

Fig. 2. Influence of oxygen concentration of lactate sensor based on DMIA (a) and H_2O_2 detecting Pt electrode (b) in O_2, Air, and Ar-saturated buffer solution, respectively.

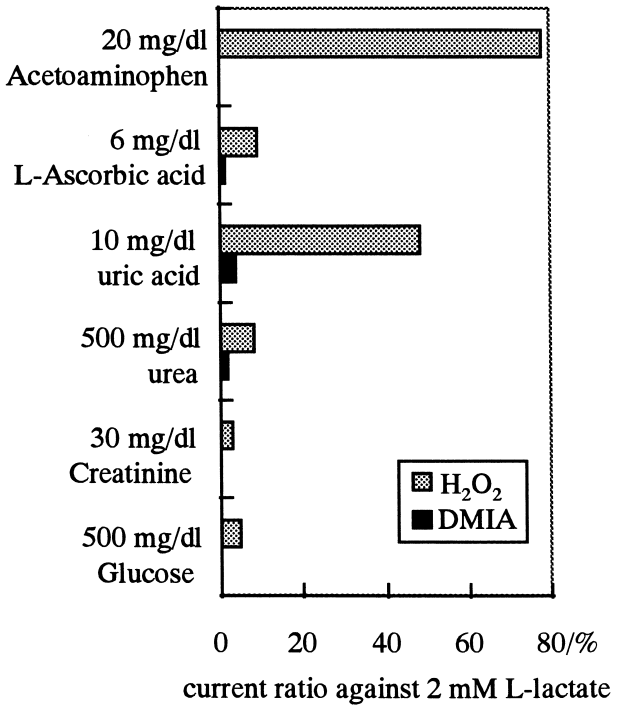

Fig. 3. Interference effects of lactate sensor. Pt-based H_2O_2 detecting sensor was tested at 400 mV and LOD/DMIA based sensor was at 200 mV.

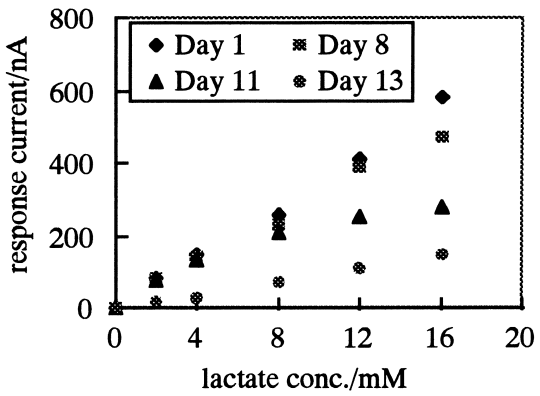

Fig. 4. Durability measurement of DMIA based lactate sensor covered with a Nuclepore® membrane.

Conclusions

It was elucidated that *N*,*N*-dimethylindoaniline worked as an excellent electron-transfer mediator for LOD. The combination of LOD/DMIA can be applied to lactate analysis with high sensitivity, independence of the oxygen concentration, and low interference effect. Since this sensor also demonstrated long durability, it has considerable promise to be utilized as a long-life time lactate sensor.

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