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Enzyme sensor for L-lactate with a chitosan-mercury film electrode

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Summary. An amperometric enzyme sensor composed of a mercury film electrode and an enzyme-immobilized chitosan membrane is developed. This biosensor is based on both a mercury film electrode detecting the consumption of dissolved dioxygen following enzymatic reaction, and a chitosan membrane. The latter provides an excellent permselectivity and excludes electroactive interferents. The detection range of this biosensor was $1.0 \times 10^{-5} - 3.0 \times 10^{-4}$ mol/l and the relative standard deviation, R.S.D. at 5.0×10^{-5} mol/l was 1.4% (n = 3). This biosensor was applied to the direct determination of L-lactate in human serum without pretreatment.

Many enzyme sensors based on a dioxygen-detecting electrode have been developed in the last decade [1–3]. These can be simply operated because NAD^+ (β-nicotinamide adenine dinucleotide) and mediators [4, 5] are not required. The preferred material for most of the dioxygen-detecting electrode is platinum. On the other hand, we have reported an amperometric biosensor for citrate with a mercury film electrode (MFE), which is based on the detection of pyruvate generated by enzyme reactions [6]. This type of biosensor is characterized by the case of electrochemical deposition and dissolution of the mercury film, and the reduction of dissolved dioxygen with MFE is more efficient than with a glassy carbon electrode.

The identification and quantitation of L-lactate are of primary importance in food processing, industrial and clinical fields. In our previous papers [7–9], details have been presented on the simple, selective and sensitive determination of L-lactate by differential pulse polarography with a soluble enzyme preparation or an immobilized enzyme reactor. In these studies, lactate oxidase [LO] has been covalently immobilized onto chitosan beads with a long-term stability. Furthermore, enzyme sensors using the permselectivity of a cellulose acetate membrane have been reported [10, 11]. It was conceived that chitosan membrane which, like the cellulose acetate membrane, is a polysaccharide, has a similar characteristic.

In this paper, we have studied the L-lactate sensor employing the permselectivity of the chitosan membrane as an excellent enzyme-immobilized support and the specialty of MFE with efficient reduction of dioxygen. This technique using LO is based on the consumption of dissolved electroreducible dioxygen. The reactions are as follows.

$$\text{L-lactate} + O_2 \xrightarrow{\text{LO}} \text{pyruvate} + H_2O_2 \quad (1)$$

$$O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2 \quad (2)$$

Additionally, this electrode consisting of the chitosan membrane and MFE might also be useful for the determination of dissolved dioxygen owing to its selectivity and lower applied potential.

Experimental

Apparatus

A Model 174A polarographic analyzer (EG & G, PAR, USA) and an X-Y recorder 7035B (Hewlett-Packard Co., USA) were used for the measurements. The base electrode used for the preparation of the enzyme sensor consisted of a glassy carbon electrode [GCE] (model No. 11-2012, 3.0 mm diameter, Bioanalytical Systems Inc., USA). The electrolysis system was composed of the enzyme sensor, an Ag/AgCl reference electrode (model No. 11-2020, Bioanalytical Systems, Inc., USA) and a platinum wire as a counter electrode, which were combined in a VC-2 voltammetry cell vial (model No. 11-1052, Bioanalytical Systems, Inc., USA). All measurements were performed at about 25° C.

Reagents

Lactate oxidase [LO] (no E.C., 9.53 mg/200 units) was purchased from Boehringer Mannheim GmbH, Germany. L-lactic acid (30 w/w % solution, Sigma Chemicals Co., USA) was of analytical reagent grade. Soluble chitosan was used as an enzyme-immobilized membrane-material and glutaraldehyde (25 w/v % solution) as the cross-linking reagent of chitosan and the activating reagent of the chitosan membrane (both from Wako Chemical Industries, Tokyo). Other chemials were also of analytical reagent grade. The water used for all solutions was purified using a double quartz distiller with deionized water.

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Preparation of the enzyme sensor

The non-polished GCE surface was electrochemically pretreated in 0.1 mol/l hydrochloric acid by potential sweep between +1.0 and −1.5 V vs. Ag/AgCl.

First, a chitosan membrane was preferentially made by dropping a chitosan solution before mercury deposition in order to avoid the disorder of the mercury film. Five µl of a mixture (1:1) of 10 w/v % chitosan solution in 0.2 mol/l citric acid-trisodium citrate buffer (pH 6.5) and 2.5% glutaraldehyde solution in the buffer was dropped onto the GCE [12]. After drying (about 1 h), a chitosan membrane was produced by cross-linking reaction. A mercury film was electrochemically grown onto the GCE surface in the chitosan membrane from 0.1 mol/l HNO_3 solution containing 0.5 mmol/l $Hg(NO_3)_2$ for 1 h at a constant applied potential (0 V vs. Ag/AgCl [6, 13]) by stirring.

In order to immobilize the enzymes, the chitosan membrane was activated by using 5 µl of 2.5% glutaraldehyde solution in the buffer dropped onto the membrane until drying (about 2 h). 5 µl of LO solution (400 units/ml) in the buffer was then dropped onto the membrane and dried (about 1 h) at ca. 5° C in a refrigerator. The construction of this L-lactate sensor is shown in Fig. 1.

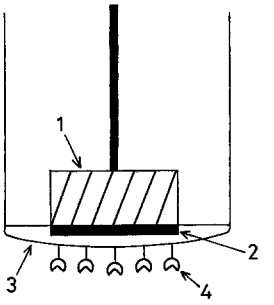

Fig. 1. Construction of the sensor. *1* GCE; *2* mercury film; *3* chitosan membrane; *4* enzyme

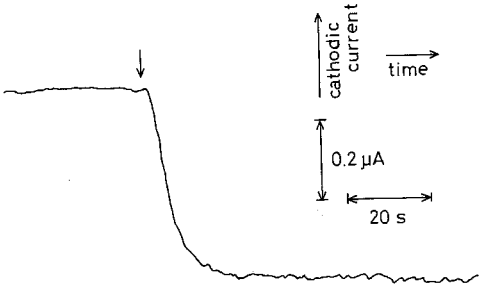

Fig. 2. Typical response curve. Sample: 100 µl of 10 mmol/l L-lactate injected into 10 ml of 0.2 mol/l citrate buffer (pH 6.5) at room temperature (final concentration: 100 µmol/l)

Recommended procedure

The L-lactate sensor was inserted into 10 ml of 0.2 mol/l citric acid-trisodium citrate buffer solution (pH 6.5) by stirring at −0.3 V vs. Ag/AgCl. When the background current reached a nearly constant value (after about 5 min), an adequate amount of sample was injected and then a current-time curve was recorded.

Results and discussion

Sensor characterization

Figure 2 shows a typical current-time curve for 0.1 mmol/l L-lactate using this sensor. The response current quickly reached a constant value 20 to 30 s after injection of the sample.The response current using a MFE remained constant at a negative potential of more than −0.3 V vs. Ag/AgCl, whereas the suitable applied potential using a bare GCE was more negative than −0.5 V. This means that the reduction of dioxygen on a MFE occurs at a low overpotential rather than that on a bare GCE. The use of a MFE is useful for lowering the applied potential. Thus the applied potential was set at −0.3 V.

The response was stable in the wide pH-range of 6 to 8 and remained constant during four weeks (about 100 measurements), when used at about 25° C and stored in the citrate buffer solution at about 5° C in a refrigerator. The response, however, gradually decreased after this period. This shows that the sensor has a more practical advantage since the immobilized enzyme is not affected by mercury which acts generally as an inhibitor and the MFE itself is also stable.

Selectivity

For this sensor we can consider three kinds of possible interferents: (1) enzyme inhibitors, (2) electrochemically active species and (3) oxidizable compounds which consume dissolved dioxygen. The concentrations of heavy-metal ions related to interferents (1) and (2) are in general in the range of µmol/l or less in physiological samples such as serum [14]; so it is considered that they do not affect the response of L-lactate. In the determination of L-lactate in serum, the main interferents may be proteins adsorbing onto the electrode surface, L-cysteine oxidized by reaction with mercury, and ascorbate consuming dissolved dioxygen. The permissible concentrations of these interferents are shown in Table 1 with the apparent increase of concentration caused by their presence on the response of 50 µmol/l L-lactate (corresponding to 1:100 dilution of serum by injecting a sample into the cell). These concentrations are higher than each level in serum.

Table 1. Sensor response for various interferents

Interferent	Permissible concentration	Detected concentration for 50 µmol/l L-lactate[a]/ (µmol/l)
Albumin	0.5 mg/ml	52.6 ± 5.5[b]
L-cysteine	10 µmol/l	53.0 ± 8.3[b]
Ascorbate	100 µmol/l	53.2 ± 9.4[b]

[a] 1:100 dilution of human serum level
[b] Number of measurements (n) = 3, 95% confidence limits from S.D.

Here, the apparent increment for L-cysteine is very low as compared with its concentration. The cyclic voltammograms of L-cysteine with both the bare MFE and chitosan-

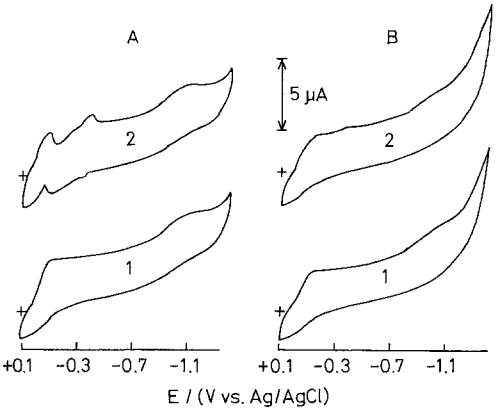

Fig. 3. Interference of L-cysteine in the cyclic voltammogram. **A** MFE; **B** chitosan-MFE; *1* 0.2 mol/l citrate buffer (pH 6.5) with O_2; *2* 1 + 100 μmol/l L-cysteine; scan rate: 5 mV/s

MFE are shown in Fig. 3. As can be seen in this figure, the cathodic wave of dioxygen was not affected by the chitosan membrane, whereas the anodic wave of L-cysteine was not observed because of its exclusion effect. This result, in particular, confirms the permselectivity of the chitosan membrane.

Calibration curve and detection limit

The response current was proportional to the concentration range of L-lactate between 10 and 300 μmol/l. The corresponding regression equation and the correlation coefficient (r) are

$$i/nA = 4.29\ C/(\mu mol/l) + 53.5$$

and

$$r = 0.999\ (n = 12).$$

Where i and C are the response current (nA) and L-lactate concentration (μmol/l), respectively. The relative standard deviation (R.S.D) at 50 μmol/l L-lactate was 1.4% (n = 3); the calculated detection limit was 6.4 μmol/l (S/N = 3). In a concentration range above 300 μmol/l, the response current deviated from the linearity. This might be due to the lack of dissolved dioxygen available for the enzymatic reaction, because the concentration of dissolved oxygen under atmospheric pressure is ca. 300 μmol/l [15].

Determination of L-lactate in human serum

Serum samples were not deproteinized, and 50 μl of the sample without dilution was directly injected into the cell and determined using a calibration graph. The results, based on triplicate measurements, are shown in Table 2. Reference values were measured by an F-kit for L-lactate (Boehringer Mannheim GmbH) using spectrophotometry. All results obtained were satisfactory within the experimental errors.

Table 2. Determination of L-lactate in human serum

Sample	Concentration[a] (mmol/l)	R.S.D. (%)	Reference[b] (mmol/l)
A (♀, 19)[c]	3.64 ± 0.48[d]	5.3	3.71 ± 0.61[d]
B (♀, 20)[c]	4.75 ± 0.27[d]	2.3	4.64 ± 0.39[d]
C (♀, 16)[c]	4.38 ± 0.18[d]	1.6	4.30 ± 0.35[d]

[a] n = 3
[b] Spectrophotometric measurement using F-kit (Boehringer Mannheim GmbH), n = 3
[c] Sex and age
[d] 95% confidence limits from S.D.

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