

Simultaneous Detection of Lactate Enantiomers Based on Diffusion-controlled Bioelectrocatalysis

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Amperometric biosensors were constructed for the simultaneous detection of lactate enantiomers. The enantioselectivity of the sensor is based on NAD-dependent L- and D-lactate dehydrogenases that, respectively, oxidize L- and D-lactates into pyruvate. The NADH formed during the enzymatic reduction was catalytically oxidized at Meldola's blue-adsorbed mesoporous electrodes. Stable amperometric measurements were performed in a two-electrode system using Ag/AgCl/sat. KCl as a counter electrode *via* a salt bridge. The response of the sensor reached a pseudo-steady state within 60 s. The agreement of the sensitivities for L- and D-lactates and the pseudo-steady-state characteristics of the sensors demonstrate that the current is strongly influenced by the diffusion of lactates at the edge of the electrode, enabling reproducible measurements. The pseudo steady-state characteristics are also realized at the chip-type electrode. The sensor was successfully applied for the detection of D- and L-lactates in horse serum.

Keywords Simultaneous detection, two-electrode system, bioelectrocatalysis, amperometric biosensor, lactate dehydrogenase

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Introduction

Lactate is a known biological marker for disease.^{1,2} Pyruvate is a metabolite produced in the anaerobic metabolism of glucose in muscles, where it is reduced to lactate and regenerates oxidized nicotinamide-adenine dinucleotide (NAD⁺) during homolactic fermentation. Lactate is a chiral compound with two enantiomers: L- and D-lactates; the former is the most abundant in the human body, whereas D-lactate is only produced by bacteria and acts as a major metabolic end-product of microbial carbohydrate fermentation. Both isomers are produced from and metabolized to pyruvate by the enzyme lactate dehydrogenase (LDH). Since this enzyme is enantiospecific, the production of D- and L-lactates requires D- and L-LDHs, respectively. Mammals have only L-LDH; therefore, the production of D-lactate in the human body is normally very limited. However, its concentration in human body rises with some disorders, for example, abnormal proliferation of intestinal bacteria due to antibiotic administration, intestinal palsy, and short bowel syndrome.^{3,4} In particular, when a patient with short bowel syndrome ingests a large amount of carbohydrates, the indigestible ones are carried to the colon, and the fermentation of lactic acid bacteria occurs.^{5,6} As a result, a large amount of D-lactate is produced, exceeding the metabolic ability of the colon, and thus, the concentration of this metabolite increases in the blood.

For pathological diagnosis, the development of novel analytical methods able to perform simultaneous and enantiospecific quantification of isomers is of great importance. A variety of

analytical methods have been reported for the enantiospecific determination of the lactate isomers, such as high-performance liquid chromatography,^{7,8} gas chromatography,^{9,10} and capillary electrophoresis.³ Compared to such analytical methods, electrochemical biosensors have several advantages: low cost, fast response, and no preprocessing required. The optical isomers of lactate can be easily determined using sensors with the corresponding enzymes.

Enzymatic biosensors enable highly selective and sensitive detection and are important analytical tools in the fields of medicine, agriculture, food safety, bioprocesses, and industrial monitoring.¹¹⁻¹⁴ Amperometric biosensors, such as commercial blood glucose sensors, convert the analyte concentration into a current (or electricity) based on bioelectrocatalytic reactions.^{15,16} In mediated-electron-transfer (MET) bioelectrocatalysis, a redox enzymatic reaction is coupled to an electrode reaction using a small redox active molecule, called a mediator. Extensive studies have been carried out to improve the detection range, sensitivity, stability, and reliability of amperometric biosensors.^{17,18}

In general, the steady-state current (I_s) is convenient for amperometric detections, and is obtained considering a serial resistance circuit in enzymatic biosensors, as follows:¹⁹

$$\frac{1}{I_s} = \frac{1}{I_d} + \frac{1}{I_p} + \frac{1}{I_k} + \frac{1}{I_e}, \quad (1)$$

where I_d is the diffusion-controlled current, I_p is the membrane-permeability-controlled current (if the electrode is covered with a membrane), I_k is the enzyme-reaction-kinetics-controlled current, and I_e is the electrode-kinetics-controlled current. The inverse of each term corresponds to the resistance. The process involving the lowest controlled-current is regarded as the rate-determining step. I_e becomes extremely large at potentials

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(for oxidation) that are more positive than the redox potential of the true electroactive species (the mediator in MET-reaction). Therefore, the electrode process is not the rate-determining step in usual amperometric biosensors. The value of I_k easily changes because of labile enzyme activity. To increase I_k , it is necessary to increase the amount of enzyme on the electrode surface and select a suitable mediator.^{20,21} The value of I_p also varies because of the difficulty in controlling membrane permeability and thickness. However, the process may be ignored in the absence of the membrane. Under the conditions of $I_d \ll I_k, I_p, I_c$, the diffusion-controlled conditions are satisfied, which is one of the ultimate requirements in amperometric biosensors.²² The steady-state I_d is realized by non-linear diffusion, for example at microelectrodes. In contrast, the diffusion current at planar electrodes is in principle a function of time. However, there is some contribution of non-linear diffusion in the mass-transfer to disk electrodes, and the contribution increases with time. Therefore, we may utilize non-linear diffusion on disk electrodes to obtain steady-state I_d and utilize for biosensing.

Moreover, amperometric measurements are generally carried out on a three-electrode system. However, this might be not convenient for miniaturized biosensors because of the complexity in an electric circuit and electrode arrangement, and large noise due to large resistance of the reference electrode. Hence, a two-electrode system is frequently utilized in commercial biosensors, though, in this system, it sometimes becomes difficult to control the working electrode potential because of an instability in the potential of the counter electrode as a pseudo-reference electrode.

In this work, we fabricated amperometric biosensors that selectively and simultaneously detect D- and L-lactates using a two-electrode system. Although the simultaneous detection of lactate enantiomers by two amperometric sensors in flow-injection analysis was reported by Vargas *et al.*,²³ the detection potential was too positive and was not convenient for avoiding the influence of reducing interferences in the system. We utilized NAD-dependent LDH that converts lactate into pyruvate by oxidation with NAD⁺. The proposed sensor enabled stable electrochemical measurements. The current response of the sensor was considered to be controlled by the diffusion of lactate. Because the substrate-diffusion-limited current without stirring is useful in disposable biosensors, chip-type electrodes were also constructed on polyethylene terephthalate (PET) films.

Experimental

Reagents and chemicals

Ketjen Black (KB, EC300J, BET area of 800 m² g⁻¹, and primary particle size of 40 nm) was kindly donated by Lion Corp. (Japan). Poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder was purchased from DuPont-Mitsui Fluorochemicals Co., Ltd. (Japan). L-LDH (EC 1.1.1.27, 6530 U mL⁻¹, L = dm³) from pig heart and D-LDH (EC 1.1.1.28, 694 U mg⁻¹) from microorganism were obtained from Toyobo Co., Ltd. (Japan) and used without further purification. Meldola's blue (MB) and potassium ferricyanide (K₃[Fe(CN)₆]) were acquired from Nacalai Tesque (Japan). β -NAD reduced form (NADH) and NAD⁺ were obtained from Oriental Yeast Co., Ltd. (Japan). D-Lactate and horse serum were purchased from Tokyo Chemical Industry (Japan) and Cosmo Bio Co., Ltd. (Japan), respectively. A photometric D-/L-lactic acid detection kit (ENZYTECTM) was acquired from R-Biopharm AG (Germany). Other chemicals

were obtained from Wako Pure Chemical Industries, Ltd. (Japan). All chemicals used in this study were of analytical grade, and all the solutions/suspensions were prepared with ultrapure water.

Preparation of mesoporous electrodes

The KB powder (40 mg) was mixed with PTFE (20 mg) and MB (4 mg) and homogenized in 3.5 mL of 2-propanol for 9 min at 0°C to prepare MB/KB slurry. A 3- μ L aliquot of the KB/MB slurry was applied on a glassy carbon electrode (GCE, 3 mm in diameter, BAS, Japan) and allowed to dry to prepare MB-adsorbed KB/GCE (MB/KB/GCE). A similar electrode (KB/GCE) was fabricated without MB following the same procedure.

Enzyme immobilization on KB/MB/GCE

A 20- μ L aliquot of ethanol was cast onto the MB/KB/GCE surface, and 20 μ L of L-LDH or D-LDH stock solution (10 mg mL⁻¹) was then cast onto the wet MB/KB/GCE surface. Ethanol was used to facilitate infiltration of the enzyme solutions into the mesoporous structure of the electrode.²⁴ The immobilized-L-LDH electrode (L-LDH/MB/KB/GCE) was kept at 4°C overnight in air and immersed in a 0.1 M (M = mol dm⁻³) phosphate buffer (pH 7.0) for 1 min to remove excess free enzymes from the surface before use. The D-LDH-immobilized electrode (D-LDH/MB/KB/GCE) was kept in a water-saturated atmosphere for 10 min at 4°C, washed with distilled water, and used immediately for electrochemical measurements.

Fabrication of electrode tips

Electrode tips were prepared as described in the literature,²⁵ with some slight modifications. Briefly, a negative electrode pattern was printed on an OHP film (PET with a thickness of 100 μ m) using a laser printer (ApeosPort-V C3375, Fuji Xerox Co., Ltd., Japan; see Sect. "Response of the chip-shaped electrode"). This pattern corresponded to an electrode with a diameter of 3 mm. A gold film with a thickness of 100 nm was prepared on the entire surface of the OHP film by sputtering with a desktop quick coater (SC-704, Sanyu Electron Co., Ltd., Japan), controlling the current and time. The patterned toner on the OHP film was removed by sonication in *N,N*-dimethyl-formamide. The prepared electrode tips were thoroughly washed with ethanol and water.

Electrochemical measurements

Chronoamperometry and cyclic voltammetry were performed using an electrochemical analyzer (CV-100W, BAS). The Ag|AgCl|sat. KCl system was also used as a counter electrode in the two-electrode setup. The effective surface area of the counter electrode was approximately 1 cm². To simplify the connection of the counter electrode in the measurement solutions, a saturated KCl-agar salt bridge of 1 mm diameter, and 10 cm length with a resistance of approximately 10 k Ω was used.

For chronoamperometry, sample solutions were slightly stirred for a short period using a magnetic stirrer, just after the addition of analytes, to equalize the concentration; however, all amperometric measurements were carried out under still conditions.

Photometric measurements

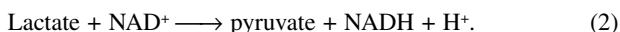
The photometric D-/L-lactic acid detection kit is based on the enzymatic reduction of NAD⁺ by lactate with each LDH facilitated with glutamate pyruvate transaminase. The sample solution, L-glutamic acid solution, NAD solution, GPT suspension, and D-LDH solution was mixed. The absorbance at

340 nm was recorded after 30 min. After the detection of D-lactate, L-LDH solution was added into the reaction mixture, and then the absorbance was recorded after 30 min.

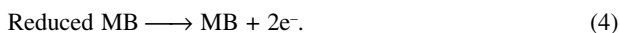
Results and Discussion

Catalytic oxidation of NADH at MB/KB/GCE

LDH oxidizes lactate into pyruvate and reduces NAD^+ into NADH:



Since the direct oxidation of NADH requires a large overpotential, several catalysts, such as phenothiazine derivatives and MET-bioelectrocatalysis with diaphorase, are frequently utilized to reduce the overpotential.^{26–30} In this work, MB was employed as a mediator because it has a large rate constant for catalytic NADH oxidation^{31,32} given by



KB was used to construct mesoporous structures suitable for physically trapping the mediator and enzyme for MET-bioelectrocatalysis.³³ In addition, a large surface area is convenient to increase the enzymatic reaction rate on the electrode surface. Note here that the diffusion layers in the close vicinity of the mesoporous surface overlap with each other with time, resulting in a simple diffusion layer on the projected surface area.

The cyclic voltammogram (CV) recorded at MB/KB/GCE in the absence of NADH (dashed line in Fig. S1, Supporting Information) showed a reversible redox couple at -0.13 V, corresponding to MB adsorbed on KB/GCE. The wave was stable during multiple scans, indicating that MB stably remained on the mesoporous electrode, *i.e.*, it did not leak during redox cycling. In the presence of NADH (solid line in Fig. S1), an increased peak was observed at -0.16 V in the anodic scan and the cathodic peak decreased due to the catalytic oxidation of NADH. Compared with the CV of the direct oxidation of NADH at KB/GCE (dot-dashed line in Fig. S1), an effective decrease in the overpotential was achieved. In addition, the diffusion-tail-like shape of the anodic wave indicates that the catalytic reaction of NADH by the adsorbed MB is sufficiently fast, and that the catalytic current is close to a diffusion-controlled state. In following experiments, the detection potential was set at 0.05 V for NADH determination. The detection potential was selected to oxidize the adsorbed MB completely and to avoid any side reactions at the electrode in body fluids, such as the oxidation of ascorbic acid and uric acid and the reduction of oxygen.

Catalytic oxidation of lactate at LDH/MB/KB/GCE

The formal redox potentials of the NAD^+/NADH and pyruvate/lactate redox couples are $E^{\circ}_{\text{NAD}} = -0.320$ V vs. SHE³⁴ and $E^{\circ}_{\text{pyr}} = -0.185$ V vs. SHE³⁵ at pH 7. Therefore, reaction (2) is uphill and is susceptible to inhibition from pyruvate and NADH. Many attempts to shift the equilibrium of reaction (2) toward the product side have been reported.^{32,36} In this work, an alternative solution was proposed: using an excess of NAD^+ .

A two-electrode system was used to simplify the electric circuit (for simultaneous detection with two working electrodes) and to reduce the electric noise. Two counter electrodes were

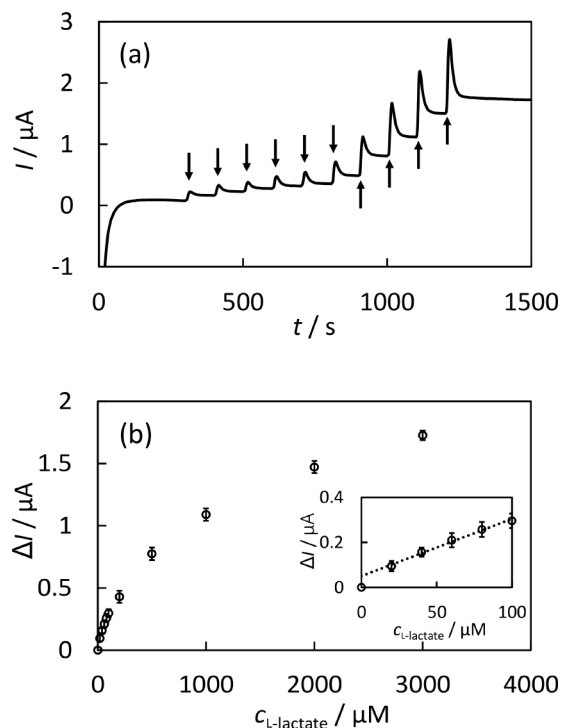


Fig. 1 (a) CA of the catalytic oxidation of L-lactate at L-LDH/MB/KB/GCE using an Ag|AgCl|sat. KCl counter electrode. The measurements were carried out at 0.05 V in a 0.1 M phosphate buffer (pH 7.0) containing 5 mM NAD^+ at 50°C . The arrows indicate the successive addition of L-lactate at final concentrations of 0.02 , 0.04 , 0.06 , 0.08 , 0.1 , 0.2 , 0.5 , 1 , 2 , and 3 mM. (b) Calibration curve obtained from the CA current at 60 s after the L-lactate injection. The error bars show 90%-confidence levels ($n = 3$). The inset shows a magnified view of the calibration curve.

evaluated in the two-electrode system: an Ag|AgCl ink-printed electrode and the Ag|AgCl|sat. KCl electrode. Although the response of the sensor was inhibited when the Ag|AgCl ink-printed electrode was used, such inhibition was not observed when the Ag|AgCl|sat. KCl electrode was used by connecting *via* a salt bridge. Utilization of the Ag|AgCl|sat. KCl electrode with a salt bridge as a counter electrode provided stable measurements. This is due to the following: 1) the low current density at the electrode, which stabilizes the electrode potential, and 2) separation from the measurement solution by the salt bridge, which protects the enzyme on the modified electrode from pollution (or inhibition) by components transferred from the Ag|AgCl electrode. The most possible inhibitor for the sensor is trace amounts of Ag^+ .

Figure 1(a) shows a chronoamperogram (CA) at L-LDH/MB/KB/GCE with successive additions of L-lactate in the two-electrode system under stationary conditions (except just after the addition of L-lactate where the solution was shortly stirred). Upon adding L-lactate into the solution, the current increased stepwise with the L-lactate concentration. This current is ascribed to the catalytic oxidation of L-lactate. Here a pseudo-steady state was defined as a position when the variation of the current within 1 s was lower than 0.3% of the total current, that is, $\Delta \leq 0.003 \text{ s}^{-1}$, where Δ is $-(dI(t)/dt)/I(t)$, with I and t being the current and time, respectively. According to the data in Fig. 1(a), the current reached the pseudo-steady state within 60 s after analyte injection. Although this is not a true steady state, but a pseudo-steady state, the currents are useful in

practical applications to minimize the measurement time. Figure 1(b) presents the calibration curve for L-lactate obtained from the pseudo-steady-state current at 60 s after the addition of the analyte. The slope of the linear part of the curve, that is the sensitivity of L-lactate sensor for L-lactate, was $0.0025 \pm 0.0004 \text{ A M}^{-1}$, and the upper limit of the linear part was about $100 \mu\text{M}$. The limit of detection (LOD) of this sensor is $3 \mu\text{M}$ (3σ). A similar calibration curve was obtained from bioelectrocatalysis with D-LDH in the two-electrode system for D-lactate (sensitivity of $0.0026 \pm 0.0006 \text{ A M}^{-1}$ and $\text{LOD} = 9 \mu\text{M}$ (3σ), Fig. S2, Supporting Information). Even though the LDH activity on the electrode was different for each isomer, the slopes of the two curves were almost the same. These characteristics are likely due to the fact that the response was controlled by diffusion of the analyte. This means that the system satisfied the $I_d < I_k \ll I_p, I_e$ conditions. The origin of the curved characteristics of the calibration curve seems to be increased contribution of the kinetics of the enzymatic reaction.

Pseudo-steady-state current by non-linear diffusion at GCEs

The origin of the steady-state condition at LDH/MB/KB/GCEs under stationary conditions has to be considered. At planar electrodes, the diffusion-controlled current of an analyte decreases with time because of an increase in the diffusion resistance (or the concentration polarization). In these experiments, conventional-size GCEs were used, and then planar diffusion is predominant in a relatively short time under the limiting current conditions. However, the contribution of non-linear diffusion of the analyte increases with electrolysis time at the edge of the electrode surface. The current (I) for the non-linear diffusion at a disk electrode is given by³⁷

$$I = 4nFDcr(0.7854 + 0.8862\tau^{-1/2} + 0.2146e^{-0.7823\tau^{-1/2}}), \quad (5)$$

where, $\tau = 4Dt/r^2$, n is the number of electrons of the analyte, F is the Faraday constant, D is the diffusion coefficient of the analyte, r is the radius of the disk electrode, and c is the bulk concentration of the analyte.

To evaluate the non-linear diffusion characteristics at the edge of the disk electrode, we monitored CAs of $[\text{Fe}(\text{CN})_6]^{3-}$ at a bare GCE and KB/GCE under stationary conditions (except just after the successive addition of the analyte). The current reached an almost steady state ($\Delta \leq 0.003 \text{ s}^{-1}$) within 60 s after injection (Figs. S3(A) to S3(D), Supporting Information). The pseudo-steady-state current increased linearly with the concentration of $[\text{Fe}(\text{CN})_6]^{3-}$, with slopes of -0.0013 and -0.0013 A M^{-1} for a bare GCE and KB/GCE, respectively (Figs. S2(E) and S2(F)). Therefore, the microstructure at a KB/GCE surface has no critical effect on the value of the pseudo-steady-state current. According to Eq. (5), the theoretical slope is 0.0013 A M^{-1} at $t = 120 \text{ s}$, considering $D = 7.30 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$,³⁸ $n = 1$ for $[\text{Fe}(\text{CN})_6]^{3-}$, $r = 1.5 \text{ mm}$ of the GCE, and $\Delta \leq 0.003 \text{ s}^{-1}$. The theoretical and experimental values are in good agreement. These results verify that non-linear diffusion at the edge of the electrode becomes predominant, even at a conventional GCE at extended electrolysis times.

On the basis of the discussion concerning the diffusion-controlled current of $[\text{Fe}(\text{CN})_6]^{3-}$, it was assumed that the steady-state characteristics of the catalytic oxidation current of lactates at LDH/MB/KB/GCEs are predominantly due to the non-linear diffusion of lactates toward the edge of the surface of LDH/MB/KB/GCEs. According to Eq. (5), the theoretical slope is 0.0022 A M^{-1} at 125 s , considering $D = 5.62 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$,³⁹ $n = 2$ for lactate, and $\Delta \leq 0.003 \text{ s}^{-1}$. This value also agrees with the experimental one (0.0025 A M^{-1} , Fig. 1(b)), confirming that

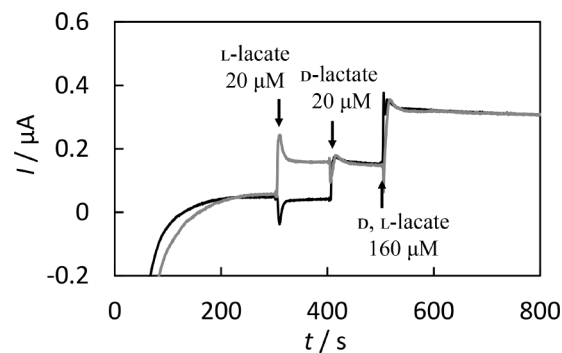


Fig. 2 Simultaneously recorded CAs of the catalytic oxidation of D- (black line) and L-lactates (gray line) at 0.05 V with D- and L-LDH/MB/KB/GCEs. The measurements were carried out in a 0.1 M phosphate buffer (pH 7.0) containing 5 mM NAD^+ at 50°C . The arrows indicate the addition of lactates.

Table 1 Simultaneous detection of D- and L-lactates^a

Inject sample	D-Lactate	L-Lactate
20 μM of D- and L-lactates	$(19 \pm 2) \mu\text{M}$	$(20 \pm 2) \mu\text{M}$
Racemic mixture of 160 μM lactates	$(81 \pm 4) \mu\text{M}$	$(84 \pm 5) \mu\text{M}$

a. The error is given in a 90%-confidence level.

the pseudo-steady-state catalytic current is predominantly determined by the diffusion of lactate.

Simultaneous detection of lactate enantiomers

The simultaneous detection of D- and L-lactates was performed with two working electrodes, D- and L-LDH/MB/KB/GCEs, to investigate the selectivity of the fabricated lactate sensors. The black and gray lines in Fig. 2 show CAs simultaneously recorded at D- and L-lactate sensors, respectively, in the two-electrode system; the arrows indicate the addition of lactates. These sensors responded only to the corresponding substrate; the D-lactate sensor showed no response when L-lactate was added and *vice versa*. The performance of the sensor in the simultaneous measurements is listed in Table 1. The selectivity of the sensors is due to the substrate specificities of the LDHs. Here the selectivity of a sensor is defined as the LOD of the interference substance. The sensitivity of L-LDH/MB/KB/GCE for D-lactate was $(2 \pm 8) \times 10^{-5} \text{ A M}^{-1}$ (data not shown). The sensitivity corresponds to the LOD of D-lactate on L-LDH/MB/KB/GCE of 2.5 mM . On the other hand, the sensitivity of D-LDH/MB/KB/GCE for L-lactate was $(6 \pm 1) \times 10^{-5} \text{ A M}^{-1}$ (data not shown). The sensitivity corresponds to the LOD of L-lactate on D-LDH/MB/KB/GCE of $300 \mu\text{M}$.

Application in serum samples

To demonstrate the practical applicability of the sensor, a selective determination of lactate enantiomers was carried out for a horse serum sample. Measurements were conducted within the linear range of the calibration curve after dilution of the sample with 0.1 M phosphate buffer (pH 7.0) to 11 times for D-lactate or 200 times for L-lactate. The lactate concentration was determined by the standard-addition method. The concentrations of lactates detected by the developed sensor were compared to those detected by the commercially available photometric determination kit; the results are summarized in

Table 2 Lactate determination in horse serum using two different methods^a

Method	D-Lactate	L-Lactate
Electrochemical biosensor	$(1.7 \pm 0.2) \times 10^2 \mu\text{M}$	$(5.2 \pm 0.4) \text{ mM}$
Photometric kit	$(1.7 \pm 0.2) \times 10^2 \mu\text{M}$	$(5.5 \pm 0.4) \text{ mM}$

a. The error is given in a 90%-confidence level.

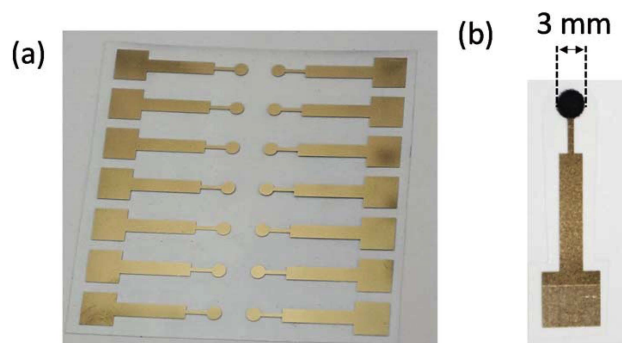


Fig. 3 (a) Photograph of the chip-shaped electrodes constructed on an OHP film after gold sputtering and toner removing. (b) Photograph of a sensor chip after modification with a MB/KB slurry.

Table 2. The concentrations of D- and L-lactates determined by the two methods were in good agreement. Compared to the photometric assay of lactates, the developed amperometric lactate sensor shows advantages, such as rapid and cheap measurements.

Response of the chip-shaped electrode

We attempted to realize diffusion-limited bioelectrocatalysis at the edge of chip-shaped electrodes constructed on a film, because chip-shaped electrode is practical for disposable biosensors. The chip-shaped electrodes constructed are shown in Fig. 3. KB, MB, and LDH were immobilized on the chip. Figure 4(a) shows a CA of the catalytic oxidation of L-lactate recorded at the modified chip. The measurements were carried out in a 0.1 M phosphate buffer (pH 7.0) containing 5 mM NAD⁺ at 50°C. It took more than 60 s to reach a pseudo-steady state at the chip-shaped electrode. Most probably, it was difficult to maintain a steady position for the chip in solution, which caused a stirring effect due to slight movement. Figure 4(b) presents the calibration curves. The current sampling was carried out at 100 s after injection of the analyte. Although the current decay seems to be affected by the streaming, the quasi-steady-state current showed a linear relationship with the L-lactate concentration, with a slope of 0.0024 A M^{-1} , and an upper limit of $100 \mu\text{M}$ in the linear range. The slope was also in agreement with the value obtained using Eq. (5). Therefore, after the decay of the effect of streaming, the current was controlled by the diffusion of lactate around a disk electrode. Realization of the quasi-steady-state current on the chip-shaped electrode allows fabricating diffusion-controlled disposable biosensors.

Conclusions

We have developed amperometric enantioselective lactate sensors constructed on a two-electrode system; they show good

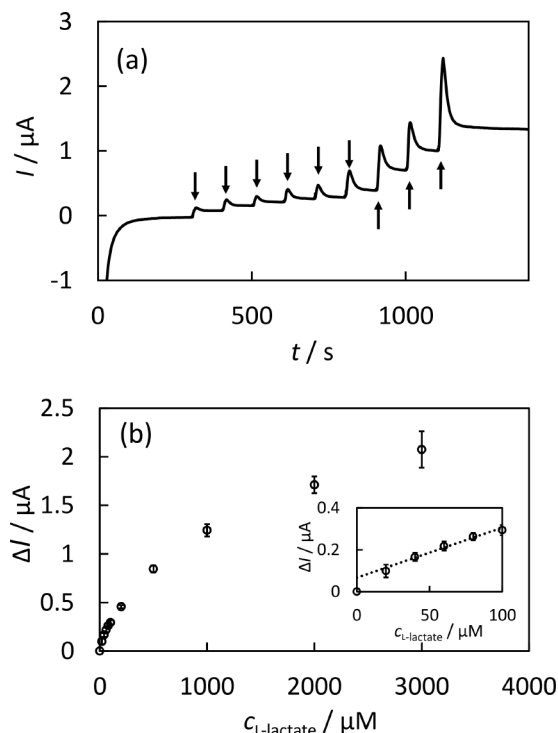


Fig. 4 (a) CA of the catalytic oxidation of L-lactate recorded by a modified tip of chip-shaped electrode at 0.05 V. The measurements were carried out in a 0.1 M phosphate buffer (pH 7.0) containing 5 mM NAD⁺ at 50°C. Arrows indicate the successive additions of L-lactate at final concentrations of 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.5, 1, 2 and 3 mM. (b) Calibration curve obtained from the CA current at 100 s after the L-lactate injection. The error bars show 90%-confidence levels ($n = 3$). The inset shows a magnified view of the calibration curve.

selectivity for D- and L-lactates. The low current density on the Ag|AgCl|sat. KCl counter electrode and its separation from the working electrode *via* a salt bridge allow for the stable amperometric two-electrode detection of lactate. The sensors allowed for the simultaneous detection of D- and L-lactates in a real sample. It is noted that the pseudo-steady-state responses of the sensors are strongly affected by non-linear diffusion of the lactate at the edge of the electrode. These characteristics enable reproducible measurements that are unaffected by changes in enzyme activities. The pseudo-steady-state responses were also realized on the tip electrode fabricated on the film. Therefore, the proposed strategy for the pseudo-steady-state responses is applicable to disposable biosensors.

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Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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