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Biosensors & Bioelectronics 17 (2002) 13–18

BIOSENSORS & BIOELECTRONICS

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Development of a screen-printed amperometric biosensor for the determination of L-lactate dehydrogenase level

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Received 16 August 2000; received in revised form 25 April 2001; accepted 6 August 2001

Abstract

We attempted to develop a screen-printed biosensor for the amperometric determination of L-lactate dehydrogenase (LDH) level on the basis of NAD^+/NADH-dependent dehydrogenase reaction. The printing ink for the working electrode consisted of L-lactate, NAD^+, composite polymer of hydroxyethyl cellulose with ethylene glycol, 3,4-dihydroxybenzaldehyde (3,4-DHB) as an electron transferring mediator, and graphite as the conducting material. The 3,4-DHB was electropolymerized on the carboneous working electrode by potential cycling between -200 and $+300$ mV vs. Ag/AgCl reference electrode. Through the electrocatalytic reaction with immobilized 3,4-DHB, the NADH generated by the LDH reaction could be efficiently oxidized at lower potential than the unmodified carbon electrode. The analytical performance of the electrode was characterized in terms of linear sensing range and detection limit for LDH. The response from the developed biosensor was linear up to 500 U/l of LDH, and the detection limit of 50 U/l was observed at the signal-to-noise ratio of 3.

Keywords: Enzyme electrode; Amperometric biosensor; 3,4-Dihydroxybenzaldehyde; Thick-film electrode; NADH

1. Introduction

Biosensors have attracted a considerable attention as the potential successor to a wide range of analytical techniques. Especially, biosensors based on electrochemical transducers have been of particular interests because of their advantages, such as high sensitivity, rapid response, relatively simple instrumentation, and operational convenience. In this respect, studies on the electrochemical biosensors have mainly focused on the analysis of organic compounds using the specific enzymes (Pamidi and Wang, 1996; Park et al., 1999; Xin and Wightman, 1997). However, only a few studies on the analysis of proteins and enzymes have been reported (Moser et al., 1997). In the clinical diagnostic fields, it is necessary to analyze the titer of enzymes/proteins in circulatory systems, which are released from organs in case of disorders, such as tissue injury. One of these enzymes/proteins is lactate dehydrogenase (LDH), and the determination of its level can be used as an indicator in the clinical diagnosis including the phase of liver disorder or the symptom of myocardial infarction (Huijgen et al., 1997).

In our previous report (Yoon and Kim, 1996), we have applied screen-printing technology (Hilditch and Green, 1991; Wring and Hart, 1992) to the mass production of a disposable electrochemical biosensor for the determination of lactate concentration based on NAD^+/NADH-dependent LDH reaction. In the NAD^+/NADH-involved enzyme reaction, use of electron transferring mediators facilitates the electrochemical oxidation/reduction at lower operating potential, reducing the interference by other electro-oxidizable compounds. It was reported that *o*-quinone and its derivatives, which are immobilized on the surface of carbon electrodes, are adequate for the electrocatalytic oxidation of NADH (Degrand and Miller, 1980; Jaegfeldt et al., 1981, 1983; Pariente et al., 1994; Tse and Kuwana, 1978; Ueda et al., 1982). Abruña et al. constructed a stable redox-active electropolymerized

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0956-5663/02/$ - see front matter
PII: S0956-5663(01)00272-X

film by electro-oxidation of 3,4-dihydroxybenzaldehyde (3,4-DHB) on a glassy carbon electrode and established an aldehyde biosensor based on the determination of NADH which is generated from the aldehyde dehydrogenase reaction (Pariente et al., 1994, 1995).

In this work, as an extension of our previous study, we fabricated a disposable screen-printed electrode for the amperometric determination of LDH level. For the efficient bioelectrocatalytic oxidation of NADH, we employed 3,4-DHB as an electron-transferring mediator and immobilized the molecule on the carboneous working electrode by potential cycling. The electrochemical properties of electrodeposited 3,4-DHB were characterized, and the analytical performance of the resulting LDH biosensor was investigated.

2. Experimental

2.1. Materials

LDH (EC 1.1.1.27, rabbit muscle, 850 U/mg, Sigma), β-nicotineamide adenine dinucleotide from yeast (β-NAD, Sigma), β-nicotineamide adenine dinucleotide, reduced form (β-NADH, Sigma), L-lactate (lithium salt, Sigma), hydroxyethyl cellulose (HEC; Aldrich), ethylene glycol (EG; Aldrich), graphite (Aldrich), and 3,4-DHB (Fluka) were used as received. The base substrate of thick-film electrodes was a flexible polyester film with 0.35 mm thickness. Printing inks for base electrode were purchased from Acheson Colloids Co. (USA); Electrodag 427SS (silver ink), Electrodag 423SS (graphite ink), and Electrodag 452SS (UV curable dielectric ink) were used as received. The Ag/AgCl pseudo-reference electrode ink was purchased from MCA Services (UK). Potassium phosphate buffer (0.1 M, pH 8.2) as a supporting electrolyte was prepared with doubly distilled water. All solutions were prepared just prior to use with distilled and deionized water. All other reagents used were of analytical grade.

2.2. Preparation of ink for working electrode

The printing ink for the working electrode contained graphite, L-lactate, NAD^+, 3,4-DHB, HEC, and EG. Polymeric solution was prepared by dissolving HEC and EG in 0.1 M potassium phosphate buffer (pH 8.2) and mixing thoroughly after adjusting each portion to 2 and 6% (w/w), respectively. Five milligrams of 3,4-DHB were dissolved in the prepared polymeric solution, and then L-lactate, NAD^+ and graphite were added and mixed homogeneously. The final concentrations of graphite, L-lactate, and NAD^+ were 10.5, 26.2, and 10.5% (w/w), respectively. Working ink prepared from 1 ml of polymeric solution was used for printing about 100 screen-printed electrodes.

2.3. Fabrication of thick-film electrodes

Thick-film electrodes were fabricated on a polyester sheet by using the conventional screen printer (Model MSP 150 M, Minong Co., Korea). The screen having the mesh number of 160 was used for the carbon-based working electrodes, and 250 for other printing steps (Minong Co.). Polyester films were cut into pieces of 100 mm × 130 mm, and eight electrodes were printed onto each piece. The dimension of each electrode was 25 mm × 50 mm × 0.38 mm. Each electrode was constructed in a three-electrode configuration, consisting of working, counter, and Ag/AgCl pseudo-reference electrodes. The silver conducting paths were applied to polyester sheet, and the carbon-based ink for working and counter electrodes was printed at the end of silver conducting paths, and then Ag/AgCl pseudo-reference electrode ink was applied to the remaining silver path. In each case, the printed layer was cured for 20 min at 70 °C. The insulation ink was printed over the substrate surface except for three electrodes region, and polymerized using an UV crosslinker (Spectronics, USA). Finally, ink for the working electrode (2 mm × 2 mm) was printed, and the electrodes were dried and stored at 4 °C under vacuum before use.

2.4. Measurements

All electrochemical measurements were carried out with a BAS CV-50W electrochemical analyzer (USA). Electrochemical characterization of the working electrode was performed using the standard three-electrode configuration composed of an Ag/AgCl reference electrode (3 M NaCl, BAS) for a consistent potential maintenance and a platinum gauze electrode instead of the printed electrodes. Cyclic voltammetry and time-based amperometry were conducted in a reaction cell of 2 ml at room temperature. Prior to each measurement, 3,4-DHB in the working electrode ink was electropolymerized by potential cycling between −200 and +300 mV vs. Ag/AgCl for 10 min in 0.1 M phosphate buffer (pH 8.2) (see Section 3.1, vide infra). The amperometric determination of the LDH level was carried out at +300 mV vs. Ag/AgCl after spiking with the stock solution. The LDH solution was prepared freshly just prior to use.

3. Results and discussion

3.1. Electrochemical characteristics of electrodeposited 3,4-DHB at the thick-film carbon electrode

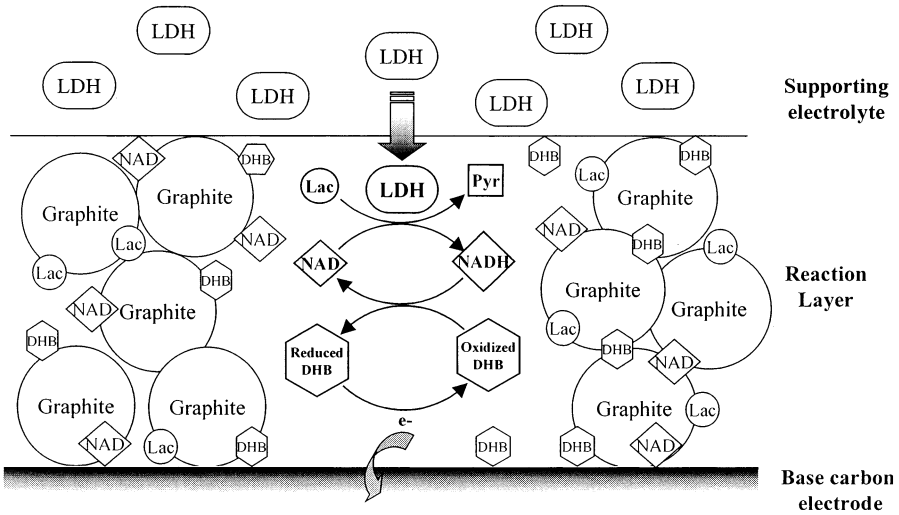
The reaction scheme for thick-film LDH biosensor is depicted in Scheme 1. The reaction layer of printed ink for the working electrode was composed of graphite,

electrodeposited 3,4-DHB, NAD^+, and L-lactate, which are confined in the composite polymeric binder. The LDH, target analyte, diffuses into the reaction layer from supporting electrolyte and converts the film-entrapped L-lactate to pyruvate with concomitant NAD^+ reduction to NADH. The NADH generated in this reaction is reoxidized to its oxidized couple, NAD^+, through electrocatalytic oxidation mediated by surface immobilized 3,4-DHB.

For the amperometric measurement of LDH level, we first electrodeposited 3,4-DHB on the working electrode by potential cycling. From optimization studies regarding the potential range, cycling sweep rate, electrolyte concentration and pH, and 3,4-DHB content, the electrodeposition of 3,4-DHB was conducted by potential cycling between -200 and $+300$ mV vs. Ag/AgCl at 20 mV/s in 0.1 M potassium phosphate buffer (pH 8.2; Fig. 1(A)). At the first scan during the electrochemical deposition of 3,4-DHB, a large anodic current was observed above $+150$ mV, and a small cathodic current at $+90$ mV on a reverse scan, as can be seen in Fig. 1(A). The oxidative current (anodic) of 3,4-DHB exponentially decreased as the deposition cycle iterated, indicating that the surface of working electrode was gradually covered with electrodeposited 3,4-DHB. And concurrently, the redox traces were developed around $+100$ mV, and the redox peak slightly increased with the deposition cycle. The anodic peak was developed at $+140$ mV, which is due to the oxidation of immobilized moieties to triol (Gui et al., 1991), and the cathodic peak was at $+90$ mV. As can be seen in the inset of Fig. 1(A), electrodeposition of 3,4-DHB mainly occurred during the early stage of potential cycling. Gradual filling of 3,4-DHB at the surface defect seemed to proceed from the slight increase in the peak current of the multiple-scan voltammograms. After potential cycling for 10 min (12 cycles under the optimized condition), electrodeposition of 3,4-DHB was found to be almost completed. The cyclic voltammogram of modified electrode (Fig. 1(B-b)) showed the typical redox wave for the electrodeposited 3,4-DHB compared to the background voltammogram of the unmodified electrode (Fig. 1(B-a)).

Cyclic voltammograms of the 3,4-DHB modified electrode at different scan rates are plotted in Fig. 2(A), and the dependency of the peak current on the potential sweep rate is shown in Fig. 2(B). A relatively small anodic–cathodic peak potential difference, ΔE_p, was observed, and the peak currents from cyclic voltammograms exhibited a linear proportionality to potential sweep rate up to 50 mV/s, which indicates that redox-active functionalities were immobilized on the electrode surface. And a rapid diffusion-controlled charge transfer occurs only at slow scan rates. But, anodic peak current began to deviate from linearity at higher scan rates above $+50$ mV/s, due to rather slow electron transfer reaction under fast potential scans (Pamidi and Wang, 1996).

The electrochemical properties of electrodeposited 3,4-DHB at the working electrode in this work are similar to the immobilized 1,2-hydroquinones on the surface of pyrolytic graphite (Tse and Kuwana, 1978) and 1,4-dihydroxynaphthalene onto a carbon paste electrode (Papouchado et al., 1968). In those electrodeposition processes, electrochemically oxidized *o*-quinones with an electron-withdrawing substituent like carbonyl-, carboxyl- or nitro-group were susceptible to nucleophilic attack by hydroxyl- or carbonyl-moiety present at carboneous electrode material in competition with hydroxyl group in solvent, and finally immobilized to the working electrode via ester or ether bond formation (Lorenzo et al., 1998). In this regard, electrodepo-



Scheme 1. Reaction scheme of the thick-film LDH biosensor. Abbreviations used: LDH, L-lactate dehydrogenase; Lac, L-lactate; Pyr, pyruvate; NAD, β-nicotineamide adenine dinucleotide; NADH, β-nicotineamide adenine dinucleotide, reduced form; DHB, 3,4-dihydroxybenzaldehyde.

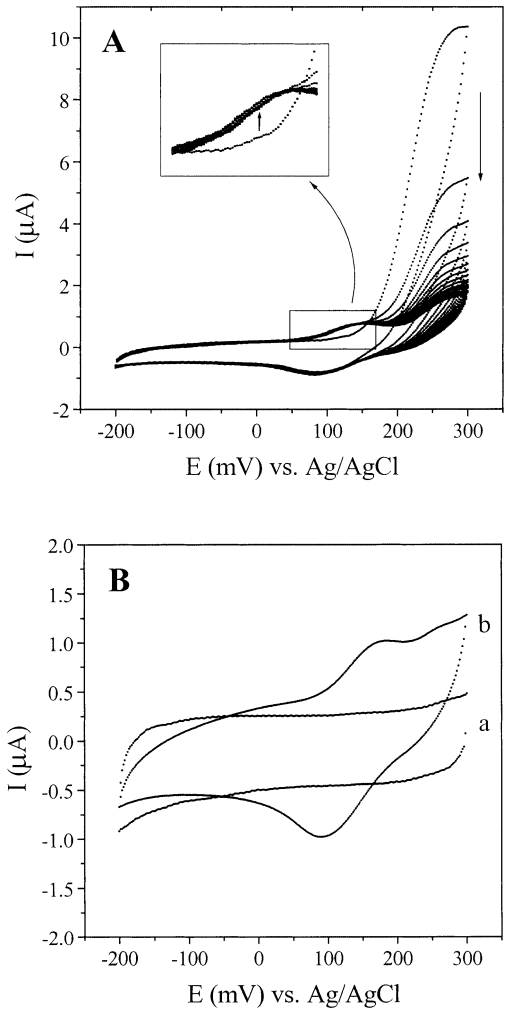


Fig. 1. (A) Electrodeposition process of 3,4-DHB by potential cycling between −200 and +300 mV at 20 mV/s. (B) Cyclic voltammogram of the working electrode before (a) and after electrodeposition of 3,4-DHB (b). Potential scan rate was 5 mV/s. Electrodeposition and electrochemical measurements were performed in 0.1 M phosphate buffer (pH 8.2).

sition of *o*-hydroquinone and the stability of the immobilized moieties were known to be strongly affected by pH of the solution (Pariente et al., 1994, 1996). The electropolymerization step of 3,4-DHB was conducted in neutral range of pH 8.2 in this work.

3.2. Electrocatalytic oxidation of enzymatically generated NADH at the thick-film electrode

From the reaction scheme depicted in Scheme 1, the LDH level in electrolyte can be determined by measuring the anodic current generated through the electrochemical oxidation of NADH which is produced from NAD^+ in proportion to the LDH level. Thus, we first examined the electrocatalytic oxidation of NADH at the 3,4-DHB modified electrode. Cyclic voltammetric tests in the absence of NADH represented an unmediated redox wave of the surface-confined 3,4-DHB (Fig. 3(a)). When NADH was added to the electrolyte, the anodic current increased significantly from the potential of as low as +50 mV and reached a catalytic saturation around +150 mV, showing a proportional increment in peak current to the NADH concentration (Fig. 3(b) and (c)). This strongly suggests the efficient NADH electrocatalysis at the 3,4-DHB modified electrode. From the above results, it is evident that the 3,4-DHB modified electrode is adequate for the electrocatalytic oxidation of NADH, leading to a lower operating potential than the conventional carbon-based electrodes (Moiroux and Elving, 1978; Yoon and Kim, 1996).

On the basis of the above observation, we tried to generate the enzyme-catalyzed and mediated signal with the developed LDH biosensor. From the LDH-mediated reaction scheme, we reasoned that LDH molecules

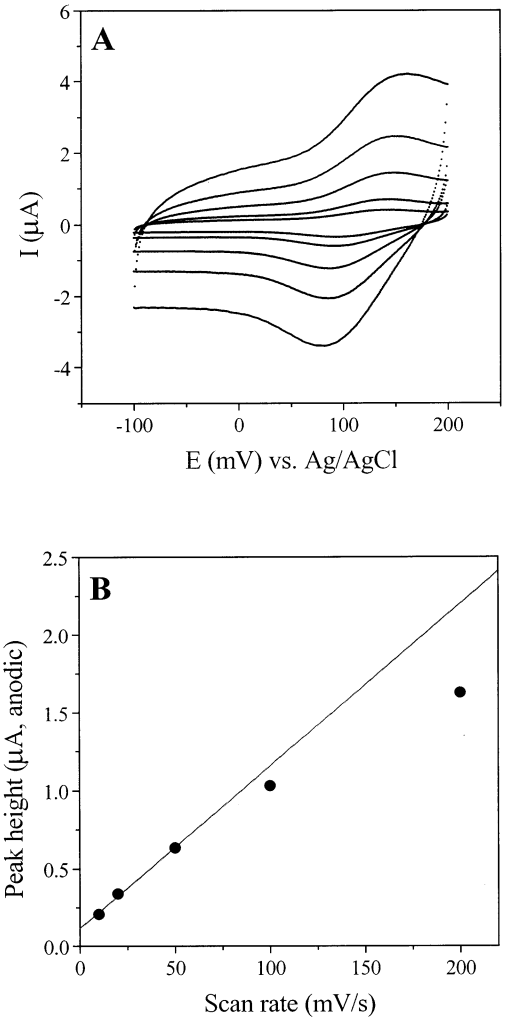


Fig. 2. (A) Cyclic voltammograms of the 3,4-DHB modified electrode at different potential scan rates: 10, 20, 50, 100 and 200 mV/s (from inside voltammograms) in 0.1 M phosphate buffer (pH 8.2). (B) Dependence of the anodic peak currents on the potential sweep rate.

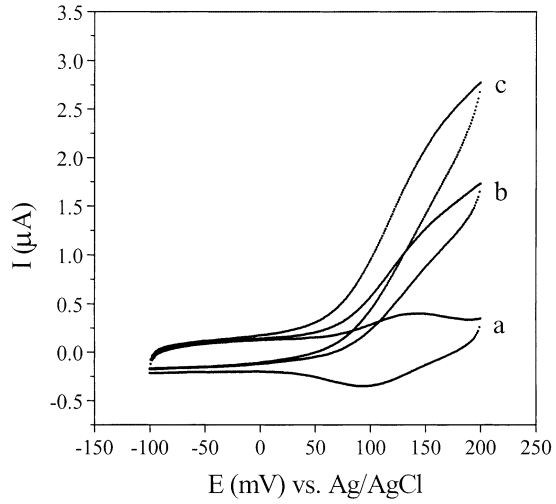


Fig. 3. Electrochemical oxidation of NADH at the 3,4-DHB modified electrode in the absence of NADH (a); in the presence of 0.5 mM NADH (b) and 1 mM NADH (c). Potential scan rate was 10 mV/s.

in electrolyte favorably diffuse into the working electrode film, and convert NAD^+ and L-lactate to NADH and pyruvate, respectively. Finally, the produced NADH is electrocatalytically oxidized to NAD^+ via electron-mediation with 3,4-DHB, generating the electro-oxidative current (Scheme 1, vide supra). In cyclic voltammograms from the bioelectrocatalyzed reaction of NADH (Fig. 4), background voltammogram in the absence of LDH showed an unmediated redox wave as in Fig. 4(a). After injection of LDH in electrolyte, enhancement in the anodic current as a function of LDH concentration, which is typical for the electrocatalytic oxidation of NADH, was observed (Fig. 4(b) and (c)). From this result, we believe that LDH molecules in

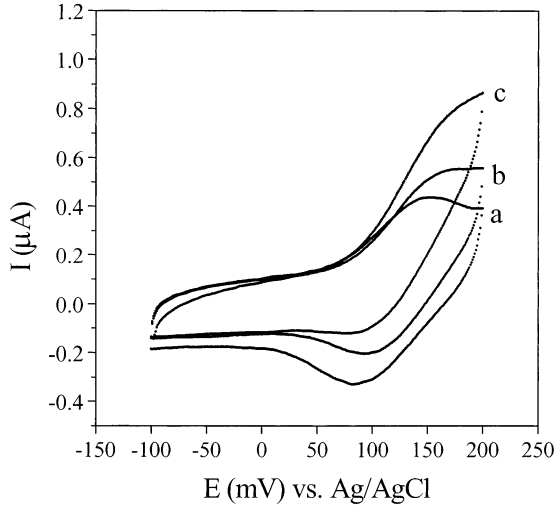


Fig. 4. Electrocatalytic oxidation of enzymatically generated NADH at the working electrode in the absence of LDH (a); in the presence of 0.2 mg/ml LDH (b) and 0.4 mg/ml of LDH (c). Potential scan rate was 10 mV/s.

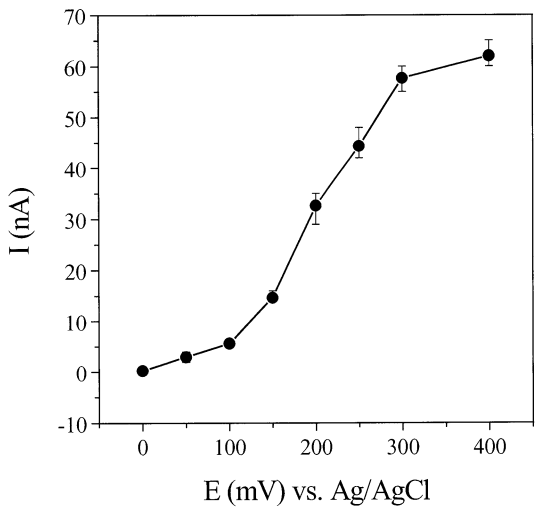


Fig. 5. The amperometric response of the thick-film biosensor to the LDH concentration of 200 U/l as a function of the applied potential.

the sample solution diffuse to the film surface and catalyze the reaction in the presence of substrate (L-lactate) and cofactor (NAD^+), generating NADH which is then electrocatalytically oxidized and detected.

3.3. L-lactate dehydrogenase biosensor

In order to determine the operating potential for the measurement of LDH, anodic signal dependency on the applied potential was investigated with time-based amperometry under fixed potential. After applying operating potential to the electrode, the residual capacitive current was allowed to diminish. When the background current reached the minimum level, LDH samples of predetermined concentration (200 U/l) were injected and analyzed. The signal from the biosensor gradually increased between $+100$ and $+300$ mV and leveled off around $+300$ mV (Fig. 5). Therefore, all the measurements were performed at $+300$ mV vs. Ag/AgCl reference electrode. Typically, 90% response of the steady-state anodic current was obtained in 7 s after sample injection, and gradual signal decay was noticed, which is presumably due to the deactivation of LDH or local insufficiency of L-lactate and NAD^+ confined at the working electrode. It is likely that enzyme reaction by LDH occurs mainly at the vicinity of the film surface, resulting in local insufficiency of L-lactate and NAD^+ because LDH does not penetrate into the film but just diffuse to the film surface of working electrode. However, the biosensor developed in this work seemed sufficient for one-shot determination of LDH level as a disposable-type biosensor. The calibration curve and linear detection range of the developed LDH biosensor are shown in Fig. 6. The response was linear up to 500 U/l (peak current (nA; anodic) = 0.294 (LDH, U/l) + 2.228, $r = 0.998$), and the detection limit was estimated

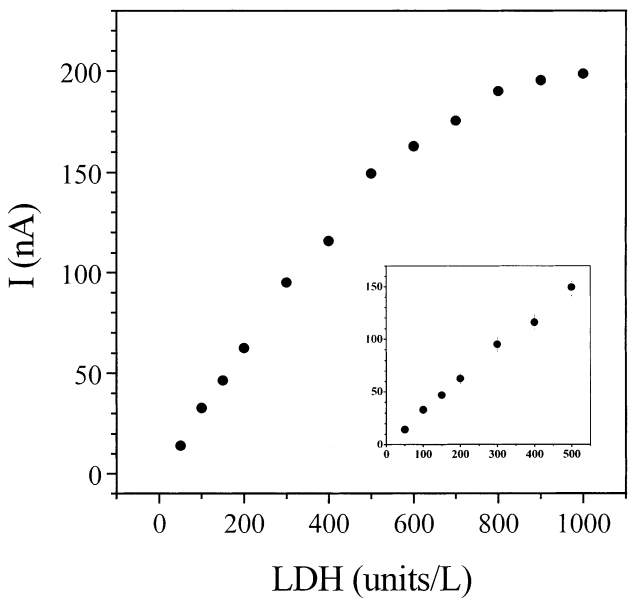


Fig. 6. Calibration curve of the LDH biosensor. The inset indicates calibration curve obtained for the linear range. Steady-state electrocatalytic responses were registered at applied potential of +300 mV vs. Ag/AgCl.

to be 50 U/l at the signal-to-noise ratio of 3. When considering clinically normal range of LDH (40–90 U/l) in serum, the analytical applicability of the LDH biosensor developed in this work seems promising.

Acknowledgements

This work was supported by the Ministry of Health and Welfare and the BK21 Program of the Ministry of Education, Korea.

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