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Signal Amplification by Substrate Recycling on Polyaniline/Lactate Oxidase/Lactate Dehydrogenase Bienzyme Electrodes

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Abstract

The bienzyme electrodes were fabricated by coimmobilization of lactate oxidase (LOD) and lactate dehydrogenase (LDH) onto electrochemically prepared polyaniline (PANI) films. These PANI/LOD/LDH bienzyme electrodes were shown to provide signal amplification by substrate recycling, making it possible to detect L-lactate at lower concentrations (0.1–1 m*M*). The PANI/LOD/LDH bienzyme electrodes were found to be stable for about 21 d at 4–10°C.

Index Entries: Lactate oxidase; lactate dehydrogenase; substrate recycling; coimmobilization; bienzyme electrode.

Introduction

The concept of enzymatic recycling of an analyte in order to get amplified signal has been successfully applied to several systems *(1,2)*. Substrate recycling refers to the exploitation of enzyme catalysis when amplification is based on the cycling of the two enzyme-catalyzed reactions *(3)*. The recycling is produced on accumulation of one of the products and results in significantly large signal amplification. The amplification involves two coupled reactions, one oxidizing the coenzyme and the other reducing it. In this context, several enzymes have been coimmobilized in order to get an amplified signal by substrate recycling *(4–7)*. Raba and Mottola *(8)* have illustrated the determination of L-lactate utilizing enzymatic amplification by substrate recycling in a dual-enzyme reactor containing lactate oxidase

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(LOD) and lactate dehydrogenase (LDH) with the mediation of reduced flavin adenine dinucleotide as the hydrogen donor. In such a system, the substrate recycling permits the generation of H_2O_2 beyond the stoichiometric limit, which is detected at a stationary platinum ring electrode, making it possible to detect L-lactate concentrations as low as 0.3 fmol/L.

It has been suggested that the amplification depends on several experimental conditions that need to be optimized for a given system and take longer time for the assay *(9)*. Lowry and coworkers *(10,11)* developed highly sensitive batch measurements of coenzymes at concentrations of 10^{-9} to 10^{-13} *M* by applying the concept of recycling. Schubert et al. *(5)* have shown the association of cytochrome-b_2 and LDH. Casimiri and Burstein *(12)* coimmobilized L-lactate oxidase and L-lactate dehydrogenase on a film mounted on an oxygen electrode for highly sensitive determination of L-lactate. The detection limit of L-lactate has been shown to decrease from 10 μ*M* to 20 n*M*. Durliat et al. *(13)* fabricated the bienzyme electrode based on LDH and diaphorase to assay L-lactate in the concentration range of 0.2–8 m*M*.

Recently, a few examples of coimmobilization of several enzymes in conducting polymer films have been reported. The redox characteristics of conducting polymers are known to be useful for the development of novel enzyme-based biosensors in which rapid electron transfer occurs without mediators *(14,15)*. The creatinine electrode was fabricated by coimmobilization of creatininase, creatinase, and sarcosine oxidase in polypyrrole matrix *(16)*. Yao et al. *(17)* observed that the coimmobilization of LOD and LDH in poly(1,2-diaminobenzene) films provides a highly sensitive detection of L-lactate owing to amplification of signal by substrate recycling. Among the conducting polymers, polyaniline (PANI) has been widely utilized for various applications including biosensors *(18,19)*, because of its stability, solubility in a variety of solvents, and flexibility in chemical structure. Gerard et al. *(20)* have recently reported the immobilization of LDH on electrochemically prepared PANI films. Bartlett et al. *(21)* have fabricated a reduced nicotinamide adenine dinucleotide (NADH) biosensor based on PANI.

The rapid, accurate, and selective assay of L-lactate is necessary in clinical biology and in the food-processing industries. It is also important in the control of some fermentors and in monitoring the growth of certain cells. Scheller et al. *(4)* have demonstrated enhancement of the sensitivity up to 1000-fold using an enzyme thermistor based on LOD coimmobilized with catalase and LDH. Such an enzyme thermistor lowered the detection limit to <5 pmol of lactate. Zhang et al. *(22)* fabricated an amperometric tetrathiafulvalene-mediated sensor sensitive to reduced NADH based on coimmobilized LOD and LDH. We have recently reported the results of our preliminary studies on PANI/LOD, PANI/LDH, and PANI/LOD/LDH electrodes for determination of L-lactate *(23)*.

The present work relates to the detailed studies pertaining to the PANI/LOD/LDH bienzyme electrodes fabricated from coimmobilization

of LOD and LDH on electrochemically prepared PANI films. An attempt was also made to study the surface morphology, and the effects of pH, temperature, and storage time on the response of these bienzyme electrodes.

Materials and Methods

Preparation of PANI Films

The PANI films were prepared electrochemically in potentiostatic mode at a potential of 0.8 V by 0.1 *M* aniline in 1 *M* HCl using a three-electrode system (electrochemical interface: Schlumberger SI 1286). Indium-tin-oxide (ITO)-coated glass plates were used as working electrodes, and a platinum plate and Ag/AgCl were used as the counterelectrode and reference electrode, respectively. The area used for deposition of the films was about 1 cm^2. The PANI films thus obtained were found to have electrical conductivity in the range of 10^{-1} S/cm. The films were thoroughly washed with phosphate buffer prior to use.

Immobilization of Enzymes

The stock enzyme solutions were prepared in 0.1 *M* phosphate buffer (pH 7.0) with a working concentration of 40 U/mL for LOD from *Pediococcus* sp. (EC1.1.3.2) and 200 U/mL for LDH type XI from rabbit muscle (EC1.1.1.27). For coimmobilization of LOD and LDH, these enzyme solutions were mixed in a ratio of 1:10. Twenty-five microliters of the mixture were physically adsorbed on the PANI films followed by thorough washing with phosphate buffer. These PANI/LOD/LDH bienzyme electrodes were dried overnight prior to use.

Scanning Electron Microscopy Studies

Scanning electron microscopy studies were conducted on the PANI/LOD/LDH electrodes using a scanning electron microscope (LEO 440) for morphological changes that occur owing to the adsorption of enzymes (LOD and LDH) onto the PANI electrodes. These changes may significantly affect the redox behavior of the PANI electrodes.

Response Studies

The amperometric response measurements were carried out by applying a potential of 200 mV using a Keithley Electrometer (Model 617). The reaction solution consisted of lactate and NADH (0.05 *M*) in phosphate buffer. Prior to response measurements, the steady-state current was achieved by polarizing the working electrode at 200 mV in the presence of NADH in phosphate buffer. As soon as L-lactate was introduced into the reaction solution, the lactate was converted to pyruvate by LOD, which in turn was recycled to lactate in the presence of LDH. The H_2O_2 thus produced could be detected at the applied potential utilizing PANI as the electron-transferring medium. The scheme for the recycling reaction is shown in Fig. 1.

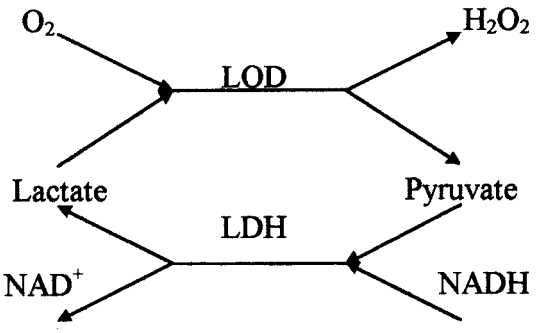

Fig. 1. Schematic of the lactate/pyruvate recycling on PANI/LOD/LDH bienzyme electrode.

Results and Discussion

Scanning Electron Microscopy Studies

Figure 2 presents the scanning electron micrographs (SEMs) obtained for the PANI films immobilized with enzymes LOD and LDH. Figure 2A is the SEM of the electrochemically deposited PANI film on the ITO-coated glass plate. The fibrous structure of PANI is known to be advantageous for physical adsorption of different enzymes *(24–26)*. Figure 2B is the SEM of the PANI films immobilized with LOD. Small globules along with the fibrils indicate the presence of LOD on the PANI films. Figure 2C is the SEM of PANI films immobilized with LDH enzyme. Flakelike structures along with the fibrils of PANI may be attributed to the presence of LDH molecules. The SEM in Fig. 2D reveals the presence of globules, flakes, and fibrils in LOD- and LDH-immobilized PANI films. The observed increased density of flakes over that of globules indicates a higher concentration of LDH molecules on the PANI surface.

Amperometric Response Measurements

The response measurements were carried out at room temperature in phosphate buffer (0.1 *M*) as a function of L-lactate concentration in the presence of NADH (0.05 *M*). Prior to the measurements, the electrodes were polarized at a working potential of 200 mV until the steady-state current was achieved. The reaction solution consisted of lactate and NADH (0.05 *M*) with a total volume of 5 mL. The amperometric response measurements were carried out by applying the bias voltage of 200 mV at different lactate concentrations. The following biochemical reactions occur on the bienzyme electrode:

$$\text{Lactate} + O_2 \xrightarrow{\text{LOD}} \text{Pyruvate} + H_2O_2 \quad (1)$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+ \quad (2)$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2\,e^- \quad (3)$$

$$PANI_{(OX)} + 2\,e^- \longleftrightarrow PANI_{(Red)} \quad (4)$$

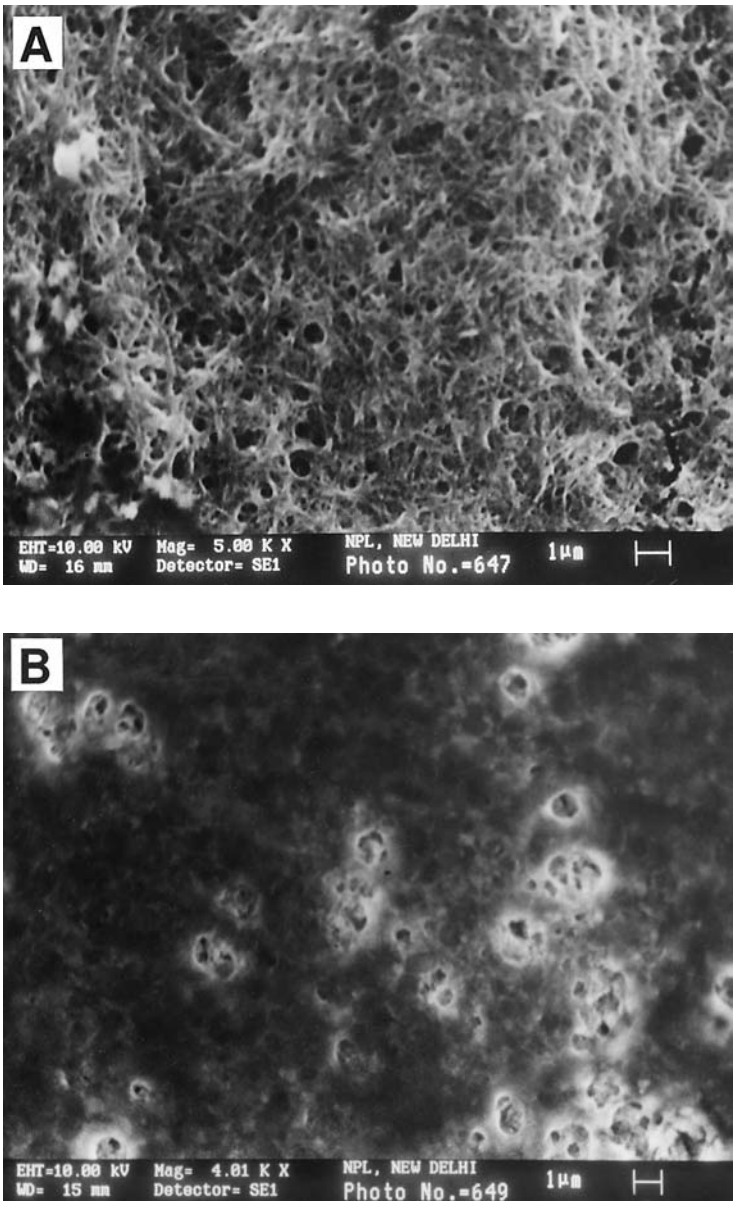

Fig. 2. SEMs of **(A)** electrochemically prepared PANI on ITO-coated glass plates, **(B)** PANI films immobilized with LOD *(Figure 2C, D continued on next page)*.

Hydrogen peroxide formed in reaction 1 was detected amperometrically at 200 mV. The continuous availability of H_2O_2 is owing to the repeated cycling of L-lactate between reactions 1 and 2. As a result of such cycling, chemical amplification occurs, and the accumulation of product (H_2O_2) results in the enhanced signal, making it possible to detect substrate (lactate) at very low concentrations. Figure 3 shows amperometric response current obtained as

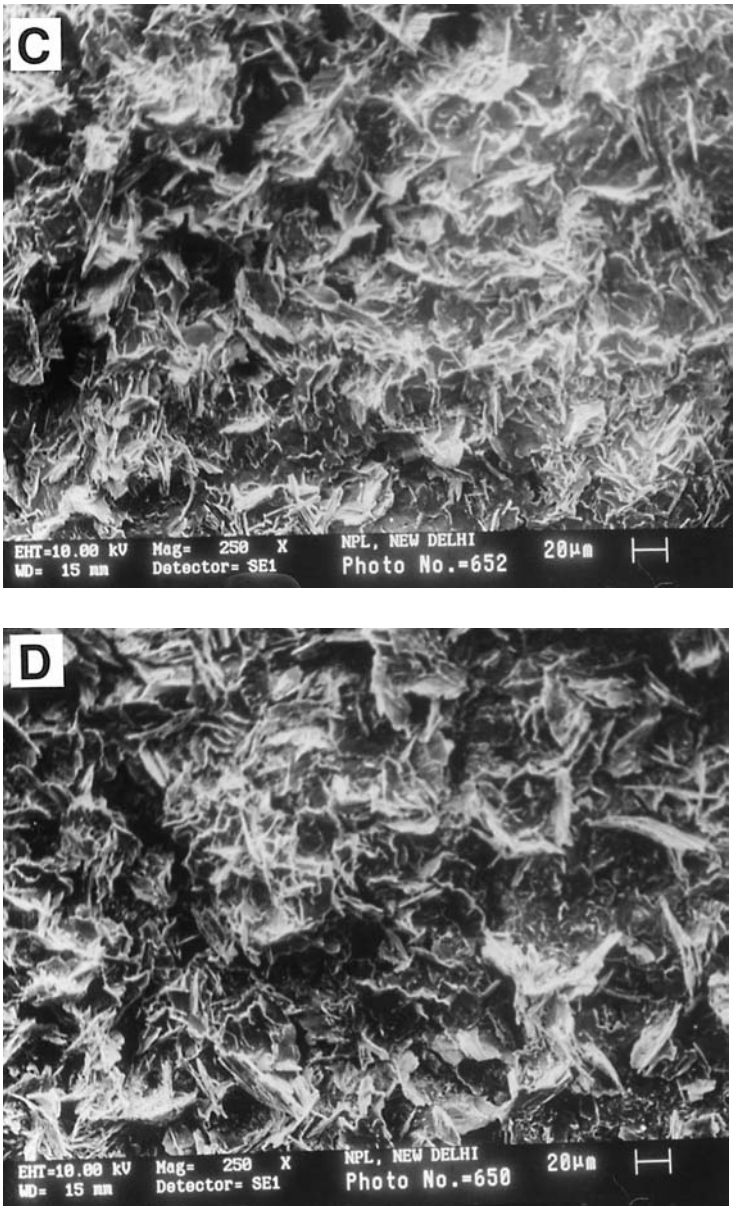

Fig. 2. *(continued from previous page)* **(C)** PANI films immobilized with LDH, and **(D)** PANI films coimmobilized with LOD (1 U) and LDH (10 U).

a function of lactate concentration. The curve shows a linear behavior from 0.1 to 1 m*M* lactate, and then it reaches a plateau, implying the steady-state response. Each measurement was carried out in triplicate, and each point in the graph designates the mean of the measurements. The apparent Michaelis-Menten constant (K_m^{app}) obtained for the PANI/LOD/LDH system was found to be 1.9 m*M* lactate.

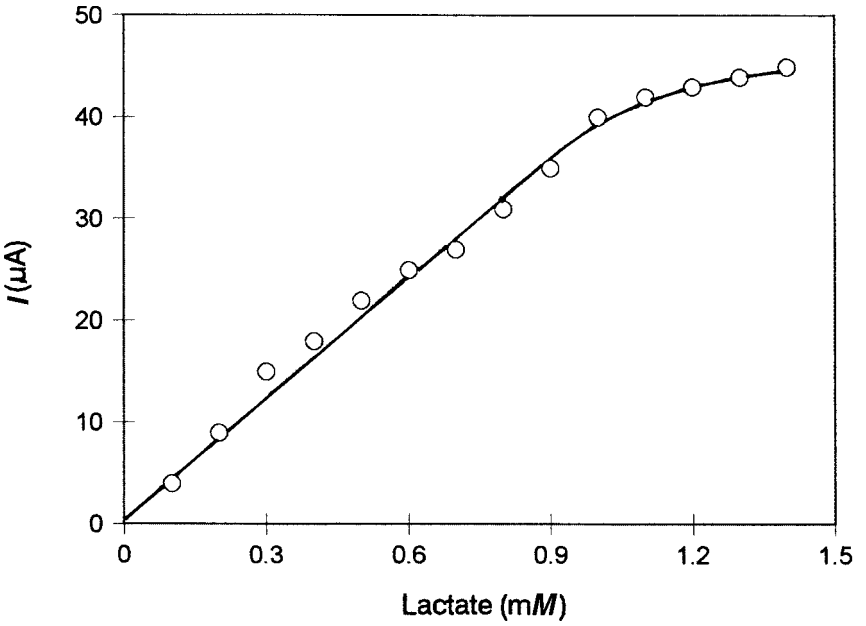

Fig. 3. Response curve obtained for PANI/LOD/LDH bienzyme electrodes at 200 mV as a function of lactate concentration in the presence of NADH (0.05 *M*).

Effect of pH on Response Measurements

The pH of the medium is known to significantly affect the affinity of the enzymes with the substrate. Figure 4 exhibits the effect of pH of the medium on the response current obtained at 200 mV for 1 m*M* L-lactate in the presence of NADH (0.05 *M*). The coenzyme NADH is required for recycling of lactate and pyruvate. It can be seen that the response current increases from pH 5.5 to 7.0, whereafter it started to decrease. Interestingly, the response current did not significantly decrease at higher pH values. This may perhaps be owing to the total effect of pH on the activity of both the enzymes.

Effect of Temperature on Response of PANI/LOD/LDH Electrodes

The relation between temperature and maximum response current of the PANI/LOD/LDH electrodes at 200 mV in the presence of lactate (1 m*M*) and NADH (0.05 *M*) is shown in Fig. 5. The response current increased with increasing temperature between 25 and 35°C and then started to decrease as the temperature was further increased. The maximum response was obtained between 35 and 40°C. It was also found that the response current was highly stable at and below the optimum temperature; however, it was found to be unstable at higher temperatures (above 40°C). These results indicate that the PANI/LOD/LDH bienzyme electrodes can be used for determination of lactate from 25 to 45°C.

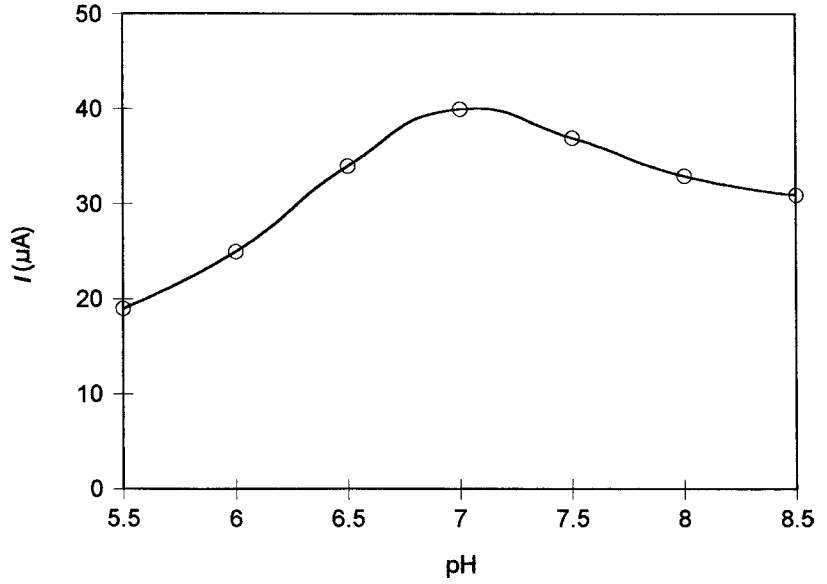

Fig. 4. Effect of pH on the response of PANI/LOD/LDH bienzyme electrodes at 200 mV in the presence of lactate (1 m*M*) and NADH (0.05 *M*).

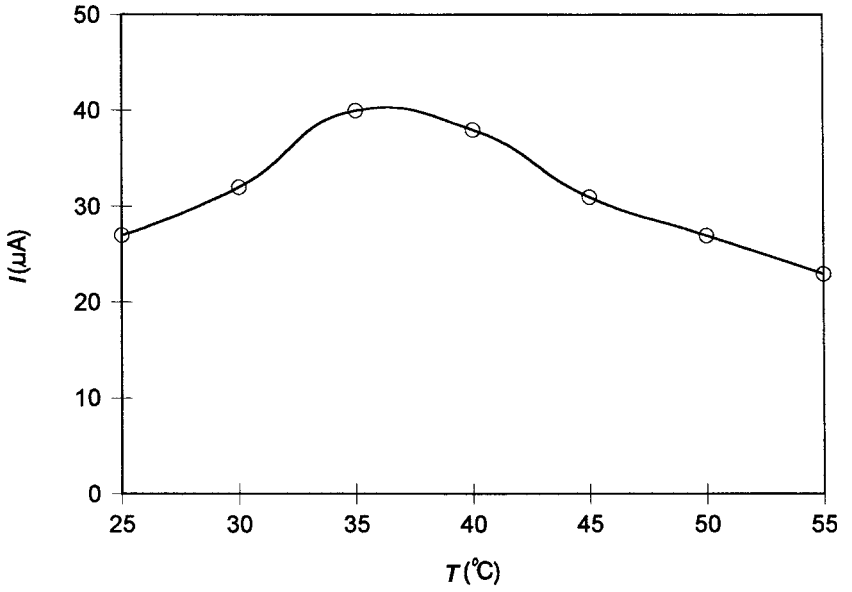

Fig. 5. Effect of temperature on the response of PANI/LOD/LDH bienzyme electrodes at 200 mV in the presence of lactate (1 m*M*) and NADH (0.05 *M*).

Effect of Storage Time

The PANI/LOD/LDH electrodes were stored at 4–10°C, and the response was observed at an interval of 3 d. Figure 6 shows amperometric response obtained at 200 mV as a function of storage time. It was found that

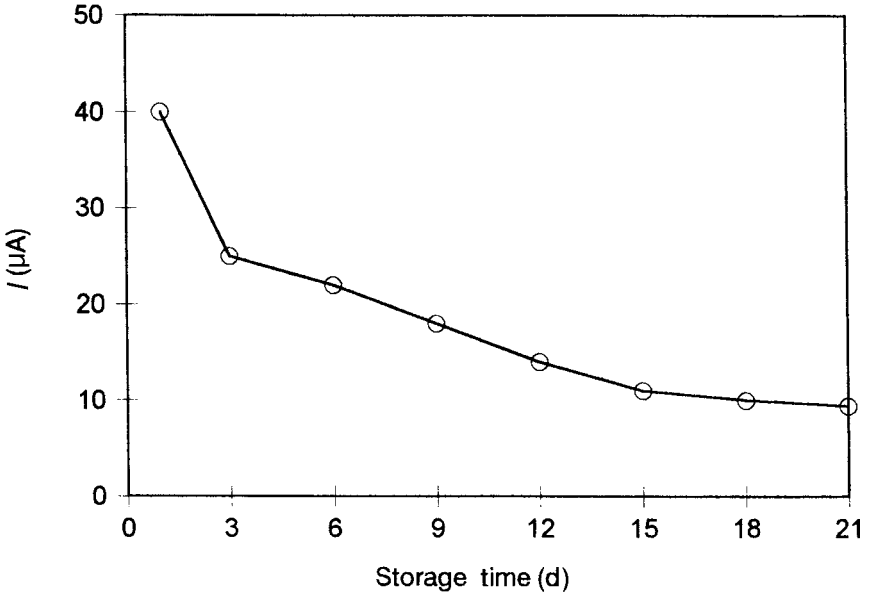

Fig. 6. Effect of storage time on the response of PANI/LOD/LDH bienzyme electrodes at 200 mV in the presence of lactate (1 m*M*) and NADH (0.05 *M*).

the maximum response obtained decreased rapidly within about 3 d, after which it decreased slowly up to about 21 d. This may be attributed to the decrease in enzyme activity taking part in the biochemical reactions. These results reveal that these bienzyme electrodes can be utilized for estimation of lactate up to about 21 d.

Conclusion

It has been shown that the PANI/LOD/LDH bienzyme electrodes can be utilized for the signal amplification for estimation of L-lactate at lower concentrations. The bienzyme electrodes showed linearity from 0.1 to 1 m*M* lactate, with detection limit of 1×10^{-5} *M* at optimum pH of 7.0. The apparent Michaelis-Menten constant for these electrodes was found to be 1.9 m*M* lactate. These PANI/LOD/LDH bienzyme electrodes were found to be stable for about 21 d at 4–10°C.

Experiments regarding the stability of PANI/LOD/LDH bienzyme electrodes beyond 3 weeks are presently in progress in our laboratory. We are also investigating the effects of various interferents such as ascorbic acid, uric acid, glucose, and glutamic acid on the amperometric response of these electrodes.

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