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Immobilization of Lactate Dehydrogenase on Tetraethylorthosilicate-Derived Sol-Gel Films for Application to Lactate Biosensor

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Abstract

Tetraethylorthosilicate (TEOS)-derived sol-gel films were utilized for the immobilization of lactate dehydrogenase (LDH) by physical adsorption and sol-gel/LDH/sol-gel sandwich configuration. An attempt was made to ascertain the optimum pH and temperature for the immobilized LDH. It was shown that TEOS-derived sol-gel films containing physically adsorbed LDH exhibited linearity from 0.5 to 4 m*M*, whereas those containing LDH in sandwich configuration showed linearity from 0.5 to 3 m*M* L-lactate. These sol-gel films, immobilized with LDH, were found to be stable for about 4 weeks at 4–10°C.

Index Entries: Immobilization; lactate dehydrogenase; tetraethylorthosilicate (TEOS); sol-gel; lactate; biosensor; physical adsorption; sandwich configuration.

Introduction

Chemical and biochemical sensors are the subject of extensive research and development because of their wide application in health care, veterinary medicine, and fermentation processes *(1–3)*. Biosensors offer the prospects of simplified, virtually nondestructive analysis of turbid biological fluids. Among all these, biosensors for medical care have demanded the greatest attention for technical development. The major advantage of this technology lies in its exploitation of biological specificity, which facilitates the device to discriminate a low concentration of analyte in complex

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matrices. A variety of biosensors for estimation of glucose, alcohol, urea, cholesterol, lactate, and so on have been fabricated *(4–8)*.

A number of matrices such as conducting and nonconducting polymeric films, membranes, gels, carbon, graphite, and silica have been used for the immobilization of biomolecules *(9–12)*. A biomimetic phospholipid/alkanethiolate bilayer immobilizing uricase and an electron mediator on gold electrode has been fabricated for amperometric determination of uric acid *(13)*. The preparation of the urea electrode based on perfluoroalkylated enzyme (urease) immobilized for use with an ammonia gas–sensing electrode has also been reported *(14)*. Ramanathan et al. *(15)* have reported that the exchange of bulky anions such as *p*-toluene sulfonate and ferricyanide dopant ions in polypyrrole with chloride ion in solution leads to greater porosity and maximization of glucose oxidase (GOD) immobilization in polypyrrole. Casimiri and Burstein *(16)* have fabricated a highly sensitive electrode for the estimation of lactate by coimmobilization of lactate oxidase (LOD) and lactate dehydrogenase (LDH) onto a film mounted on an oxygen electrode.

Sol-gel-derived films have emerged as a new class of materials well suited for the immobilization of biomolecules. Sol-gel processing offers a simple and versatile route for combining the favorable properties of inorganic materials with the bioactivity of enzymes and proteins *(17,18)*. This inorganic material is particularly attractive for fabrication of biosensors since it possesses physical rigidity, chemical inertness, and high photochemical and thermal stability, and experiences negligible swelling in aqueous and organic solvents *(19,20)*. Above all, these materials are optically transparent and, therefore, are highly useful for a variety of biosensing applications.

MacCraith et al. *(21)* fabricated a fiber optic oxygen sensor based on fluorescence quenching of evanescent-wave-excited ruthenium complexes in sol-gel-derived porous coatings. Wang et al. *(22)* used a sol-gel organic-inorganic hybrid material based on silica sol and poly(vinyl alcohol) grafting 4-vinylpyridine copolymer as a matrix for enzyme immobilization. Several biosensors for the estimation of analytes such as glucose, urea, hydrogen peroxide, and cholesterol *(23–26)*, reported in the literature, are based on the sol-gel technique. Ramanathan et al. *(27)* have reported the immobilization of LDH on tetraethylorthosilicate (TEOS)-derived sol-gel films and studied the enzyme assay as a function of time, pH, and pyruvate concentration.

The determination of lactate is important in the diagnosis of respiratory insufficiencies, cardiac disorders, and metabolic disorders. Its determination is also useful in food, dairy industries, and sports medicine.

In the present investigation, we attempted to immobilize LDH on TEOS-derived sol-gel films on glass plates by physical adsorption and in sandwich configuration. In addition, we systematically studied the merits

and demerits of this method of immobilization under varying pHs, temperatures, buffer systems, and lactate concentrations.

Materials and Methods

Preparation of TEOS-Derived Sol-Gel Films

TEOS was procured from E-Merck and was used without further purification. The stock solution was prepared by mixing TEOS (4.5 mL), deionized water (0.4 mL) (Milli-RO IOTS; Millipore), and 2,4-dinitrophenyl hydrazine (1 mL in 0.01 *M* HCl). The contents were placed in a stoppered glass container and stirred at 300 rpm with a magnetic stirrer for about 5 h. The clear solution obtained was used as the stock solution. The solution was further diluted in methanol (1:3). About 100 µL of the diluted stock was placed on a glass plate (area of about 4 cm^2) and spun at 2800 rpm. These films were then dried at 200°C for 10–15 min.

Immobilization of LDH

The stock solution (10 mg/mL) of LDH (EC 1.1.1.27, Type XI extracted from rabbit muscle) was prepared by dissolving LDH in phosphate buffer (0.05 *M*, pH 7.0). For physical adsorption of LDH, 50 µL of a 1:100 dilution of the stock solution was applied onto the sol-gel films and they were left at 8–10°C for about 24 h. For entrapment of LDH in sol-gel/LDH/sol-gel sandwich configuration, one layer of sol-gel was spun-cast over the adsorbed films.

Activity Measurements

The activity of the sol-gel/LDH and sol-gel/LDH/sol-gel films was determined by a photometric assay using an ultraviolet-visible (UV-VIS) spectrophotometer (Shimadzu 160A). The reaction mixture consisted of 2 mL of buffer (Tris and glycine, 0.05 *M*), 1 mL of lactate, and 100 µL of 0.02 *M* nicotinamide adenine dinucleotide (NAD). The activities of LDH, physically adsorbed and in sandwich configuration, were estimated at 37°C by putting these films in a cuvet and measuring the increase in absorbance at 340 nm (NADH) at an interval of 60 s for 10 min.

Results and Discussion

Activity Measurements

Since the sol-gel films are optically transparent in the 200- to 900-nm range, the spectrum of the reaction solution remains unaffected owing to the presence of TEOS-derived sol-gel films. LDH immobilized on sol-gel films catalyzes the following reaction:

$$\text{Lactate} + \text{NAD} \xleftrightarrow{\text{LDH}} \text{NADH} + \text{Pyruvate} \qquad (1)$$

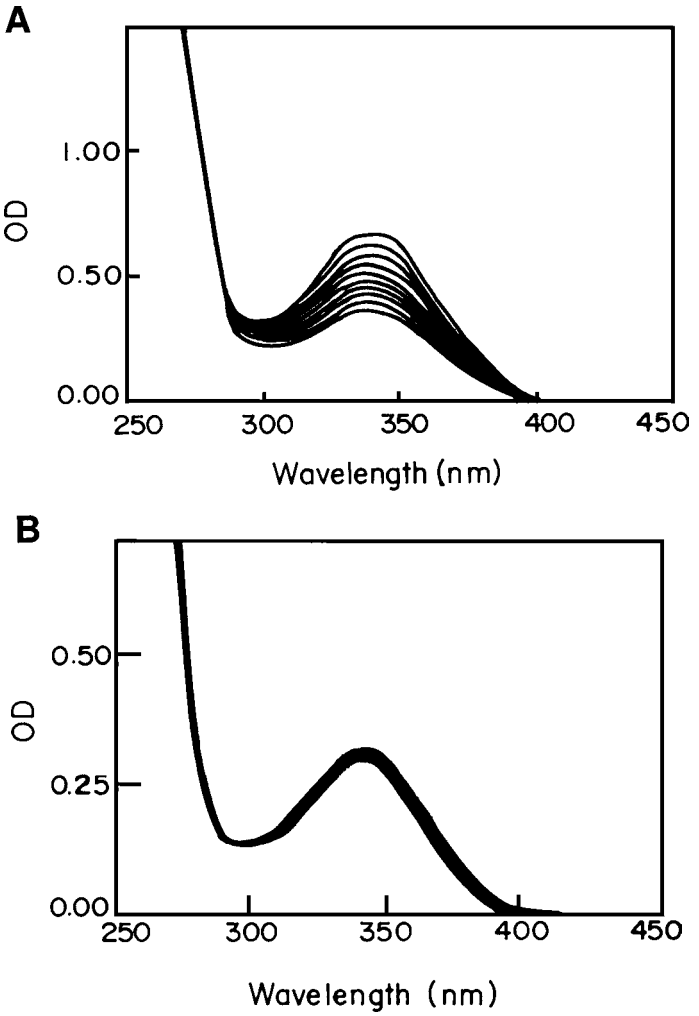

Fig. 1. Absorbance of NADH (340 nm) as a function of time in **(A)** sol-gel/LDH films and **(B)** sol-gel/LDH/sol-gel films. OD, optical density.

NADH produced in the reaction can be monitored at 340 nm. 2,4-Dinitrophenyl hydrazine present in the sol-gel film, traps pyruvate during the reaction and is converted to hydrazone, thus inhibiting the backward reaction.

The results of UV-VIS measurements carried out on TEOS-derived sol-gel films containing immobilized LDH (physically adsorbed and sol-gel/LDH/sol-gel configuration) are shown in Fig. 1. Figure 1A shows that there is a rapid increase in absorbance of NADH (340 nm) for physically adsorbed films as compared with sol-gel/LDH/sol-gel films (Fig. 1B). The observed data in each of the spectra, recorded at 60-s intervals, shows a uniform response implying that the diffusion of the substrate/ product through the supercoat plays an important role in the activity/response measurements. This is in conformity with the observed lesser increase in

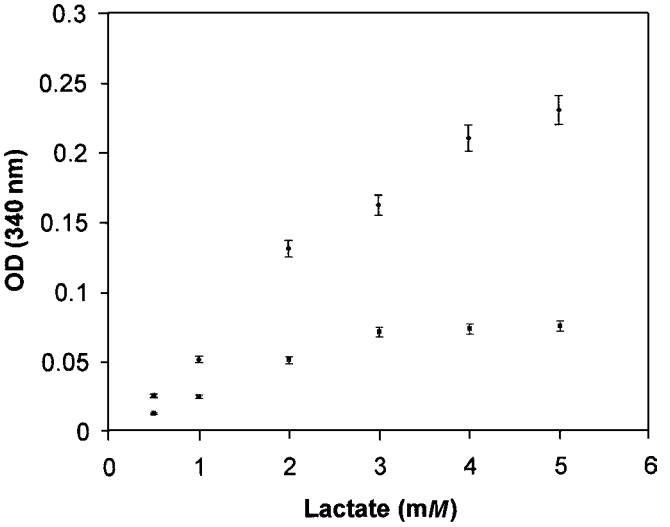

Fig. 2. Response obtained for sol-gel/LDH films (◆) and sol-gel/LDH/sol-gel films (■) as a function of L-lactate concentration in the presence of NAD (0.02 *M*) in phosphate buffer. OD, optical density.

absorbance at 340 nm (NADH) recorded within 60 s for sol-gel/LDH/sol-gel films as compared with sol-gel/LDH films.

Response Measurements

Figure 2 shows the response of LDH immobilized on TEOS-derived sol-gel films to varying lactate concentrations. It can be seen that the sol-gel/LDH/sol-gel films exhibited lower enzyme activity as compared with the LDH/sol-gel films. The absorbance was monitored in L-lactate and NAD (0.02 *M*) at 340 nm (for NADH produced during the reaction). The linearity in the case of sol-gel/LDH films was found to be from 0.5 to 4 m*M* and 0.5 to 3 m*M* L-lactate in the case of sol-gel/LDH/sol-gel films. The values of apparent Michaelis-Menten constant (K_m^{app}) were found to be 6.2 m*M* for the physically adsorbed and 8.8 m*M* for sol-gel/LDH/sol-gel films. The higher K_m found for the sandwich configuration was owing to the lesser diffusion and the reduced affinity of the enzyme (LDH) with the substrate molecules. However, the physically adsorbed sol-gel/LDH films had direct contact with the reaction solution, resulting in lower K_m^{app}. The response characteristics of the LDH/sol-gel and sol-gel/LDH/sol-gel films are given in Table 1.

pH Dependence of Adsorbed and Entrapped LDH/Sol-gel Films

The buffer system can affect the activity and stability of an enzyme owing to charge, anion activation, or surface charge effects. Figure 3 shows the effect of different buffer systems (0.05 *M*), Tris (pH 7.5–12.0), and glycine (pH 8.0–13) on the activity of the TEOS-derived sol-gel films immobi-

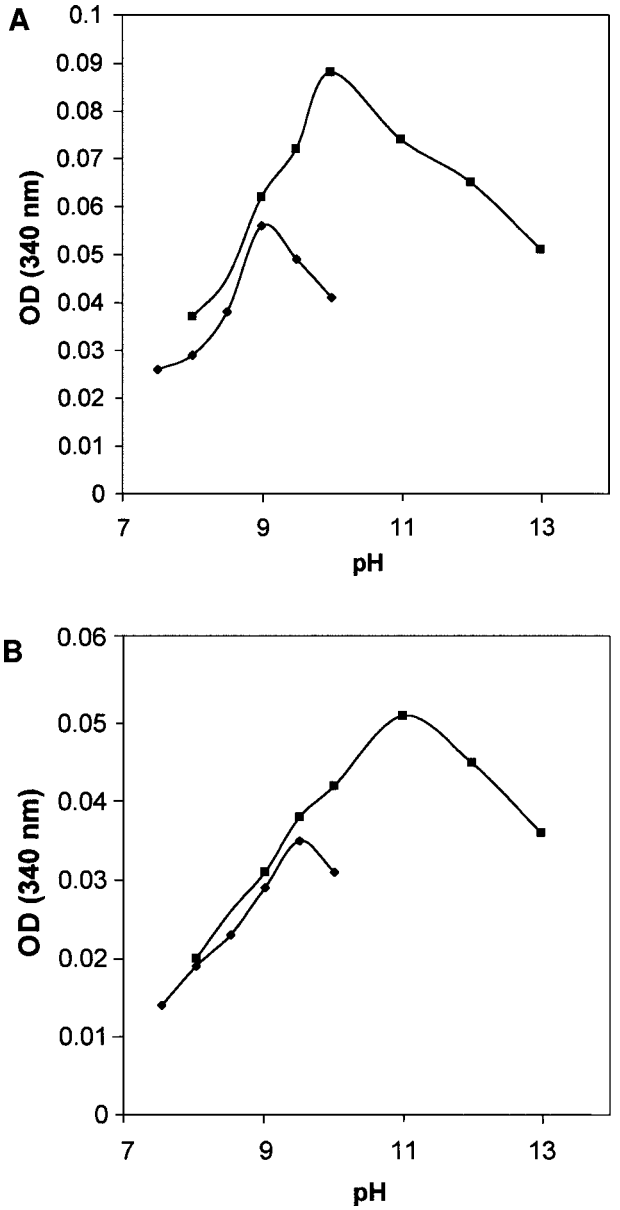

Fig. 3. Effect of pH (0.05 *M*) for Tris (◆) and glycine buffer (■) on the activity of **(A)** sol-gel/LDH films and **(B)** sol-gel/LDH/sol-gel films. OD, optical density.

Table 1
Response Characteristics of LDH/Sol-gel and Sol-gel/LDH/Sol-gel Films

System	Detection limit (m*M*)	Linearity (m*M*)	K_m (m*M*)	Stability (d)
Sol-gel/LDH	0.05	0.5–4	6.2	25
Sol-gel/LDH/sol-gel	0.1	0.5–3	8.8	30

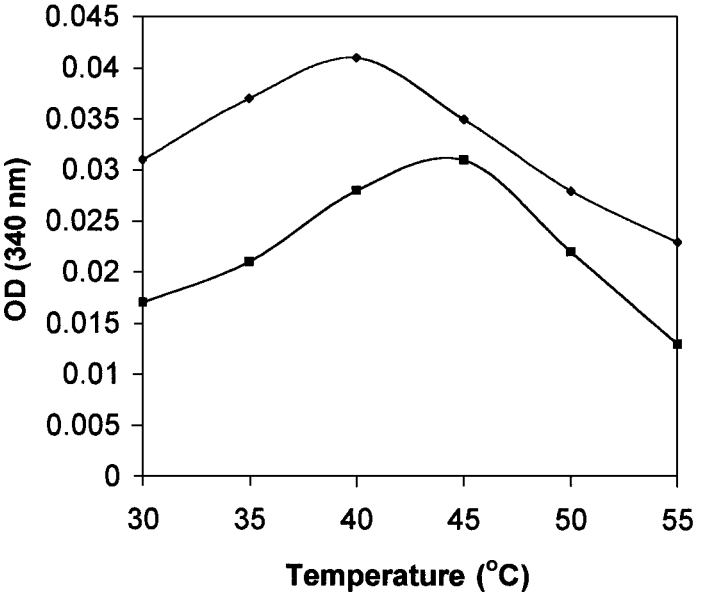

Fig. 4. Effect of temperature on the response of sol-gel/LDH films (◆) and sol-gel/LDH/sol-gel films (■). OD, optical density.

lized with LDH. The response of sol-gel/LDH and sol-gel/LDH/sol-gel films to L-lactate (1 m*M*) containing NAD (0.02 *M*) was investigated in the presence of Tris and glycine buffers, respectively. Figure 3A shows the absorbance at 340 nm for NADH produced as a result of reaction catalyzed by LDH adsorbed on the sol-gel films. These sol-gel/LDH films show a maximum response at pH 10.0 and 9.0 in the presence of glycine and Tris buffer, respectively. Figure 3B shows the pH profile obtained for sol-gel/LDH/sol-gel films. The optimum pH was found to be 11.0 and 9.5 in glycine and Tris buffers, respectively. It can be seen that this optimum shifts toward a more alkaline range (pH 11.0 and 9.5) as compared with that obtained for the sol-gel/LDH films, in which case the optima were obtained at pH 10.0 and 9.0. This may be attributed to the influxing/effluxing effects of the substrate and products through the sol-gel matrix resulting in the change in the microenvironment of the enzyme. Note that the high activity obtained in the glycine buffer was irrespective of the method of immobilization. These results indicate that the sol-gel films immobilized with LDH can be used for the real-time analysis from pH 9.0 to 11.0 in both adsorbed and sandwich configurations.

Effect of Temperature

The effect of temperature on response to 1 m*M* lactate and NAD (0.02 *M*) is shown in Fig. 4. The maximum response in the physically adsorbed and sandwiched films was found to be at about 35°C. In both cases, the response was found to decrease after about 40°C. The experiments were repeated from 30 to 55°C three times with a fresh sensor each time, and the plotted

values represent the average of these measurements. Since the sensor response is known to depend on the enzyme (LDH) activity and/or the diffusion properties of the substrate (lactate and NAD) and products (pyruvate and NADH), it appears that beyond 35°C, perhaps partial denaturing of the enzyme results in the observed decreased response.

Conclusion

It has been demonstrated that LDH can be immobilized on sol-gel films by physical adsorption and in sandwich configuration. It has been found that L-lactate can be estimated from 0.5 to 4 m*M* by photometric method using sol-gel/LDH and from 0.5 to 3 m*M* using sol-gel/LDH/sol-gel films. These sol-gel-based biosensing films were found to be stable for about 4 wk at 4–10°C. These results show that LDH-immobilized sol-gel films can act as efficient and stable sensing enzymatic films and, hence, can be utilized for the estimation of L-lactate.

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