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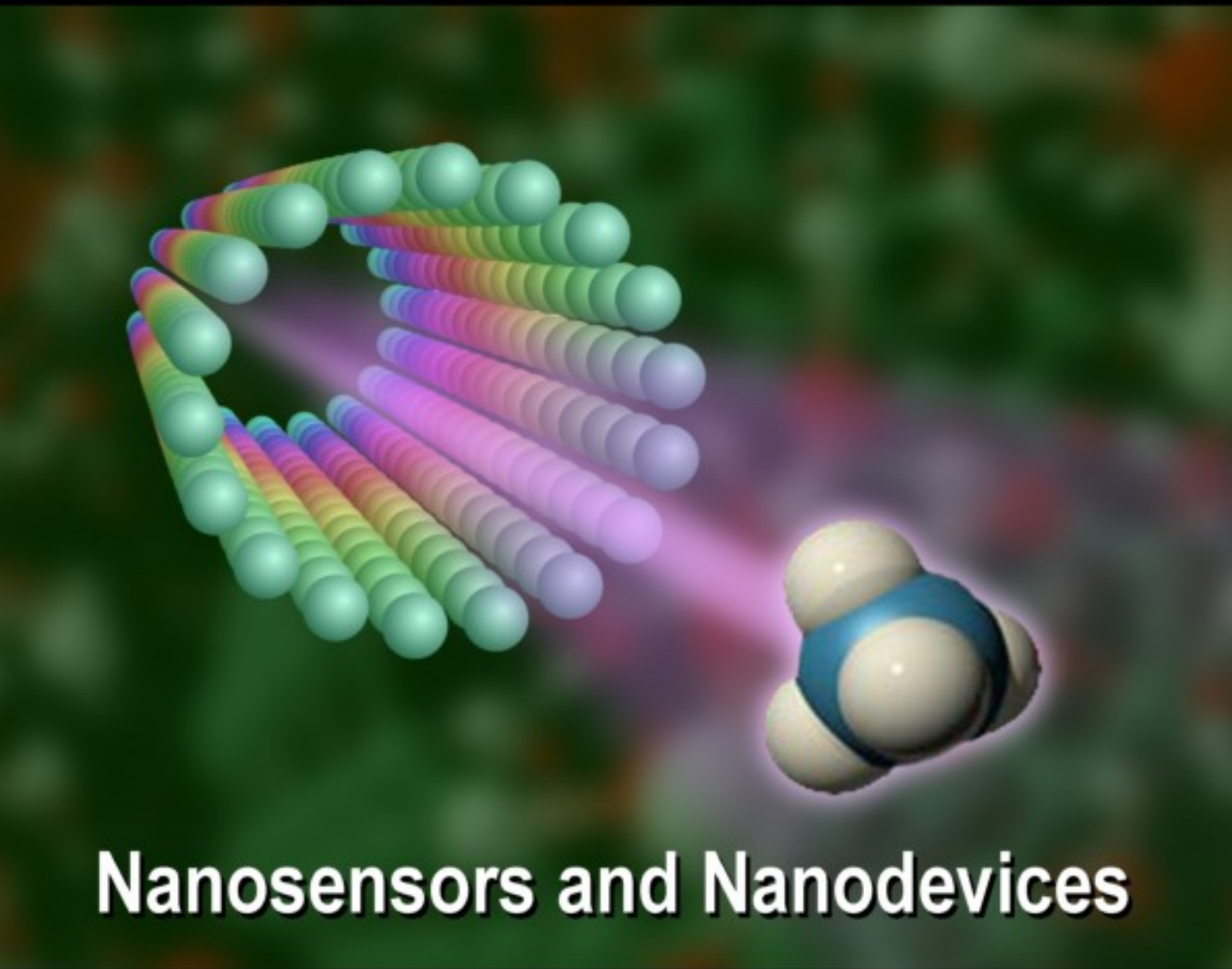

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Novel Fabrication of CA Membrane Bound Carbon Electrode for Bi-enzymatic Determination of Lactate

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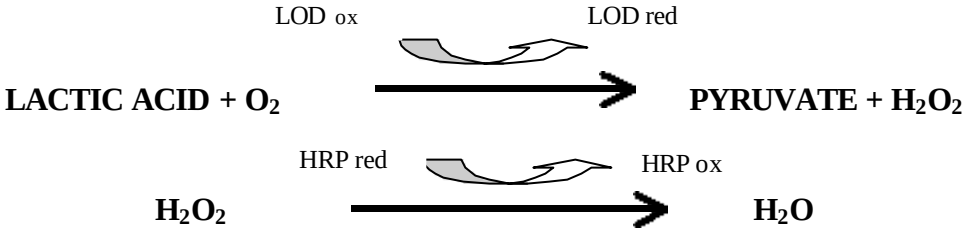
Abstract: Lactate oxidase from *Pediococcus species* has been immobilized onto cellulose acetate (CA) membrane to form an enzymatic membrane. HRP has been incorporated into carbon paste electrode. Enzymatic membrane was mounted over the HRP-carbon paste electrode with the help of dialyses membrane, which acts as working electrode. Lactate biosensor was constructed by connecting this fabricated working electrode to Ag/AgCl reference electrode along with platinum wire auxiliary electrode through potentiostate. The biosensor showed an excellent performance with a linear response range between 5µM to 1mML^{-1} of lactate with a correlation coefficient (r) of .94 for *n*=30, when compared to standard colorimetric methods. The optimum pH of the biosensor is 6.5 and incubation temperature is 25º C. This bi-enzyme electrode can be used for 150 determinants; over 45 days with out any considerable lose of activity, when stored at 4°C in 0.5M sodium phosphate buffer (pH 6.5). The response time was 1 second and no major metabolic interference was observed.

Keywords: Lactate oxidase, Horseradish peroxidase, Carbon paste electrode, Cellulose acetate membrane

1. Introduction

Lactate is formed from pyruvate in muscles and liver due to inadequate supply of oxygen and its normal range in blood is 0.5-2.5 mM. The determination of lactate in serum is essential in the diagnosis and medical management of various diseases such as tissue hypoxia, circulatory failure and hematologic disorder [1]. Recently, amperometric lactate determination has been exploited by constructing enzyme electrodes. Different immobilized enzyme biosensors for lactate monitoring have

been described. To develop such biosensors cytochrome b_2 [2], lactate monooxygenase (LMO) [3], lactate oxidase (LOD) has been immobilized on a poly-o-phenylenediamine film [4]. Bi-enzyme electrode using co-immobilized lactate oxidase (LOD) and lactate dehydrogenase (LDH) onto polyaniline (PANI) films [5] and cytochrome b_2 /LDH [6], soybean peroxidase (SBP)/LOD [7], have also been developed. LOD was preferred over LDH due to its simple reaction, which involves aerobic oxidation of lactic acid in to pyruvate. Lactate oxidase has also been immobilized onto various conducting polymer [8] for the preparation of biosensor. But all these electrodes suffer from one or other drawbacks such as less storage stability, poor electrical response, less processability. H_2O_2 generated in the reaction is monitored at high over potential (0.65V versus Ag/AgCl). But interference problems are seen as the other co-metabolites present in the sample may be oxidized and accuracy or linearity in the method is not achieved. Therefore, the present work was aimed to overcome these problems. HRP is incorporated into the carbon paste electrode for signal amplification and no over potential is needed for break down of H_2O_2. HRP has a greater affinity for peroxide, sensing it even at very small amounts [9]. The bi-enzymatic sensor based on LOD & HRP operates according to the following reaction.



2. Materials and Methods

2.1 Reagents and Materials

Lactate oxidase from *Pediococcus species*, Horseradish peroxidase from Horseradish, L(+) lactic acid, glutaraldehyde and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (CDI) from Sigma Chemical Co, USA. Cellulose acetate (CA) from Fluka (95% GC), Graphite/ carbon powder from wishler. Mumbai were used. All other chemicals were of analytical reagent grade. Amperometric measurements were conducted with a Patentiostat/ Galvanostat (Gamry Instrumentation USA, Model PS 708).

2.2 Fabrication of bi-enzymatic electrode

Cellulose acetate membrane was prepared by the method of Reddy [10] with slight modification. Commercially available lactate oxidase was co-immobilized with BSA on cellulose acetate membrane through glutaraldehyde coupling by the method of Reddy *et al* [10] with modification: Lactate oxidase (3mg; 0.85U/mg) and BSA (50 mg) were dissolved in 250µl distilled water. 25µl of enzyme solution and 12.5µl of 2.5% (w/v) of glutaraldehyde in distilled water were mixed rapidly and placed on a 4cm^2 portion of cellulose membrane (Inner membrane). A further 4cm^2 portion of cellulose acetate membrane (outer membrane) was then placed on enzyme membrane and 2 glass slides were used to compress both the membrane under mild finger pressure for approximately 5 min. The resulting laminate was then washed with buffer solution (0.05M sodium phosphate buffer, pH 7.0) to remove excess glutaraldehyde. About 200mg of carbon/graphite powder was activated by mixing it with CDI [11]. This mixture was allowed to react for 2h at 25C, after which it was rinsed with doubly distilled water and dried in a dessicator for 2.5 h, this material was denominated as GC. 1.5mg of HRP (150 U)

was added to the GC in phosphate buffer at pH 6.0. This mixture was shaken and allowed to react in a refrigerator for 16h, before drying in a desicator for 2h. This material was denominated as GCP.

In about 50mg of GCP, mineral oil is dropped until the consistency of past is obtained. This paste was molded in the form of an electrode using a plastic module. The LOD modified CA membrane was mounted over the HRP-carbon paste electrode with the help of dialyses membrane. This bi-enzymatic electrode construct acts as working electrode in the three electrode system.

2.3 Electrochemical measurements

Electrochemical measurements were carried out with a patentiostat/ Galvanostat (Gamry Instrumentation USA, PS 708). The Ag/AgCl electrode as a reference, platinum wire as auxiliary/ counter current and bienzymatic carbon past electrode as working electrode were used in the three-electrode system. All experiments were carried out in .05M sodium phosphate and sodium succinate buffer. The enzyme electrode was washed with sodium phosphate buffer (0.05M, pH 6.5) and stored at 4°C when not in use.

2.5 Colorimetric method

The assay of native/ free LOD was carried out, as described by Lockridge et al [12]. The reaction mixture contained 0.80ml reaction cocktail, 0.20ml dimethylaniline (0.2% DMA). The contents were mixed well and equilibrated at 37ºC for 2 min followed by addition of 0.02ml of dissolved enzyme. The contents were mixed again and preincubated at 37ºC for 2 min 2.0ml of dodecylbenzene sulphonic acid (0.25%DBS) was added, reaction mixture was mixed and absorbance was recorded at 565nm (A_{565}) against control in Spectronic-20 (Milton & Roy, USA) and the content of H_2O_2 generated in the reaction was calculated from standard curve between A_{565} vs. H_2O_2 concentration.

3. Results and Discussion

The optimal working conditions of this present electrode were as follows:

3.1 Applied potential dependence

The applied potential affects the biosensor response, which can be verified in Fig. 1. The response of the biosensor increased as the applied potential was shifted towards more negative values (-100, -150mv versues Ag/AgCl). For measurement, 100mV versus Ag/AgCl was used, because at the more negative potential values the reduction of oxygen in solution begins to interfere in the biosensor response. At -100mV versus Ag/AgCl, the interference of oxygen on the biosensor was not observed as oxygen reduction starts at more negative potentials. Moreover, higher negative potentials could lead to the formation of inactive HRP, decreasing the lifetime of the biosensor.

3.2 Optimum pH

To determine optimal pH for biosensor response, the pH of reaction buffer was varied from pH 5.5 to 7.5 using the following buffer, each at a final concentration of 0.05M; pH 5.5 to 6.0, sodium succinate buffer, pH 6.0 to 7.0 sodium phosphate buffer. An optimal electrode response was seen at pH 6.5, which is lower to that of free enzyme (pH-7.5). The decrease in optimum pH of LOD after

immobilization on CA membrane could be due to loss of $-NH_2$ groups on the surface of enzyme as result of glutaraldehyde coupling.

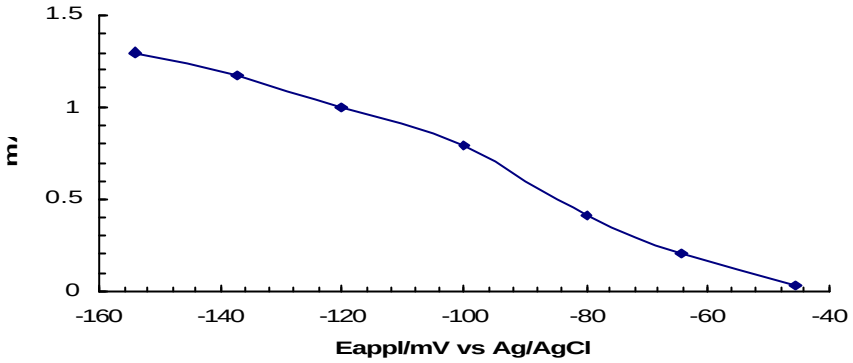


Fig.1. Applied potential dependence on bi-enzymatic biosensor response, 0.5M sodium phosphate buffer pH 6.5.

3.3 Incubation temperature for maximum activity

To determine optimum temperature for biosensor response, the reaction mixture was incubated at different temperature ranging from 20 to 40°C, in a temperature controlled chamber. Enzyme electrode showed maximum response at 25°C, which is lower than that of free enzyme.

3.4 Interference study

The biosensor response to consecutive addition of various possible interfering substances found in blood such as urea, uric acid, glycine, succinic acid, sodium dithionite and l- ascorbic acid was studied by adding each of them individually in the reaction mixture at their physiological concentration. Only uric acid caused 3% in biosensor response respectively. Rest had practically no effect.

3.5 Analytical Curve

To determine the effect of substrate concentration on membrane bound enzyme, the concentration of lactate was varied from 5µM to 3.0mM in reaction mixture. There was a linear relationship between lactic acid concentrations ranging from 5µM to 1.0 mML-1 of lactate (Fig. 2) with a correlation co-efficient (r) of .94 for n=30. The minimum detection limit of the present method is 0.01mM. The response time was only 1.0 s. The shelf life of biosensor was 45 days when stored in 0.05M, sodium phosphate buffer pH 6.5, at 2-8ºC. During this storage period, the biosensor was reused about 150 times, revealing a good reusability of present lactate biosensor (Table. 1).

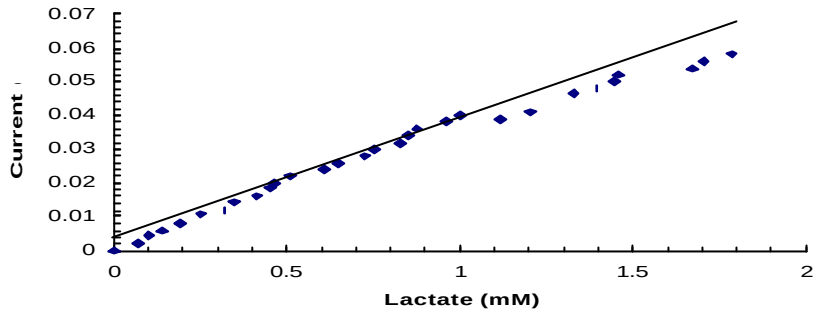


Fig. 2. Analytical curve for Lactate obtained with the bi-enzymatic biosensor, 0.5M sodium phosphate buffer, pH-6.5, E_{appl} = -100mV vs Ag/AgCl.

Table 1. Kinetic parameters of bi-enzymatic lactate biosensor.

Optimum pH	Optimum temp	Linearity	Response time	Stability	Reusability
6.5	25	5µM- 1mM	1sec	45 days	150

3.6 Lactate determination in serum

Blood samples from patients suffering from various diseases (tissue hypoxia, circulatory failure and hematological disorder) and apparently healthy persons were collected from the General Hospital, Sirsa and centrifuged at 5000rpm for 5min and their supernatant (serum) was collected. Lactate content was determined in these serum samples by the biosensor and the results were compared to those obtained from colorimetric method of Barker and Summerson [13] (Table. 2). A difference from 5 to 13 % between the results obtained from biosensor developed and the reference method can be observed. Although the difference is high, the results are always higher than those obtained with the reference method (colorimetric). The HPR incorporated in the carbon post is highly sensitive to H_2O_2 generated at the surface of the electrode, secondly due to direct contact of HRP to the carbon granules the is no transmission blockade and results in increasing the sensitivity and accuracy of the bi-enzymatic sensor and thus the biosensor should be more reliable. Moreover, the biosensor has a great advantage in its rapidity, low cost and the fact that it generates no residues.

Table 2. Lactate concentration found in serum samples with the biosensor and Colorimetric method.

Serum Samples	Colorimetric (mM)	Biosensor (mM)	Difference
Healthy	1.1 (± 0.07)	1.155(± 0.013)	5
Diseased	8.8(± 0.3)	9.944(± 0.6)	13

4. Conclusions

The bi-enzymatic biosensor for lactate showed a very good performance. A wide linear response range between 5µM to 1mML^{-1} of lactate in sodium phosphate buffer at pH 6.5 was verified. Although the

serum sample contains urea, uric acid, glycine, succinic acid, sodium dithionite and l- ascorbic acid, are not found as potential intereferents during the biosensor response. When the biosensor was tested in serum sample, the results showed a difference from 5 to 13% when compared to colorimetric method. Each time the response of biosensor is higher. The biosensor provides more rapid, sensitive, cost effective solution without generation of any chemical residue.

The biosensor described here showed better performance in terms of linearity, response time and sensitivity in comparison to potentionmetric sensors descried in the literature. These good characteristics can be assigned to the amplification system incorporated in the carbon paste, where peroxidase increases the signal due to preconcentrator and displacer properties, of the chemical equilibrium, improving the biosensor performance.

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