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Construction of an Amperometric Lactate Biosensor Based on Immobilization of Lactate Dehydrogenase Nanoparticles onto Pencil Graphite Electrode

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Abstract

Nanoparticles (NPs) of commercial lactate dehydrogenase from rabbit muscle were prepared, characterized and immobilized covalently onto pencil graphite (PG) electrode to fabricate an improved amperometric lactate biosensor (LDHNPs/PGE). The biosensor showed an optimum response within 2.5s at pH 7.0, temperature 35°C, at an applied potential of -0.4V. The wide linear response was produced by biosensor with in a concentration range of lactic acid (0.001 µM to 45 mM) and current (mA) under optimal conditions. The LDHNPs/PGE electrode showed high sensitivity (2.45 ± 2.0 µA cm^{-2} $µM^{-1}$); a lower detection limit (0.001 µM) and good correlation coefficient (R^2 = 0.99) with a standard enzymic colorimetric method. The evaluation study of biosensor offered a good analytical recovery of 98.01%, when lactic acid concentration was added in the sera sample. In addition, within and between batches coefficients of variation for working electrode were detected as 0.03% and 0.04%, respectively. The biosensor was applied for the detection of lactic acid in the serum of apparently healthy subject and patients affected from cardiogenic shocks. There was a 10% loss in initial activity of biosensor after its regular use over a time period of 180 days, while being stored at 4°C.

Keywords: Lactate; Lactate dehydrogenase (LDH); LDH nanoparticles; Lactate biosensor; PG electrode

Introduction

The lactate is generated during glycolysis of glycogen in muscle tissue to produce pyruvate, which is further reduced to lactate by lactate dehydrogenase enzyme in the absence of oxygen [1,2]. During this process, lactate generation rate is much higher than its consumption rate, thus creates a lactate concentration which is proportional to the level of physical exertion [3,4]. The circulation of lactate throughout the body is managed by monocarboxylate transporter and thus lactate can be traced in biological samples at typical conentration [5]. The normal lactic acid concentration found in blood was 4.5 to 19.8 mg/dL (0.5-2.2 mmol/L) [6] and its measurement is necessary for diagnosis and prevention of various clinical diseases including cardiogenic shock respiratory failure, liver disease, systemic disorders, renal failure, and tissue hypoxia [7-10]. A large number of methods are available for screening of lactate such as colorimetric method [11], spectrophotometric & titrimetric method [12], fluid chromatography method [13], cyclic voltammetry method and proton nuclear resonance method [14]. On the contrary, these methods have drawbacks as they required skilled personnel, expensive instrument set up, tedious and prolonged protocols. Therefore, to overcome these drawbacks biosensors and specifically amperometric biosensors are designed for lactate detection [14]. In fabrication of lactate biosensor two types of enzymes have been used: lactate oxidase (LOx) and lactate dehydrogenase (LDH). The biosensors based on LDH enzyme have superior performance than LOx enzymes, in terms of cost of an enzyme, its stability, and high catalytic activity [15,16]. In LDH based biosensors, the enzyme has been immobilized onto different nanocomposites, for detection of lactate in biological materials [17,18]. However, direct immobilization of native LDH enzyme onto nanocomposites may bring about their denaturation, prompting loss of their activity and stability. The issue was overcome by aggregating the enzyme molecules by synthesis of their nano-particles and immobilizing them onto electrodes. However, use of enzyme nanoparticles (ENPs) rather than native enzyme could enhance the diagnostic effectiveness of enzyme electrodes. Due to their unique electronic, optical, mechanical, electrical, thermal and catalytic (ability to facilitate electron transfer) properties, beside increased surface area, ENPs have shown great promises in improving enzyme electrodes [19,20].

Pencil graphite (PG) electrode offers various exciting features such as excellent conductivity, low cost, low noise and easy availability, which renders it a better option over electrodes such as Au, glassy-carbon and Pt electrodes [21]. Hence, we describe herein the fabrication of an ultrasensitive, rapid and cost-effective lactate biosensor based on nanoparticles (NPs) of lactate dehydrogenase (LDH) and PG electrode for detection of lactate in cardiogenic shocks patients. In the present study, the use of enzyme nanoparticles without incorporating nanomaterials facilitates a new simple approach towards the biosensor design in clinical applications.

Experimental Method

Materials

Lactate dehydrogenase (LDH) from rabbit muscle (25 U/mg), TRIS-buffer, NAD+, L-lactic acid, glycine, uric acid, glucose, ascorbic acid, acrylamide, potassium chloride, silica gel from SRL, Mumbai, sulphuric acid and hydrochloric acid from Qualigens Fine Chemicals, Mumbai were used. 6B graphite pencil (Make: Camlin, India) with a graphite rod of 2 mM diameter was purchased from the local stationary market. All other chemicals were of analytical reagent (AR) grade. Double distilled

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water (DW) (ohmic resistance: 1.8 $\times 10^{-5}$ for every ohm) was used throughout the study. Sera samples of apparently healthy subjects and persons suffering from cardiogenic shocks were collected from local PGIMS hospital.

Instruments used

Potentiostat/Galvanostat (Make: Autolab, model: AUT83785, made by Eco Chemie, The Netherland) with GPES (General purpose electrochemical software) software was used. Scanning electron microscopy (SEM) (Zeiss EV040, USA), UV Spectrophotometer (Make: Shimadzu, Japan, Model 1700), X-Ray diffractometer (XRD), (Make: 122 Rigaku, D/Max2550, Tokyo, Japan), Fourier Infra-red spectrometer (FTIR) and spectronic-20 (Thermo Scientific, USA), were utilized. The electrochemical impedance spectra (EIS) was recorded by FRA software in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 25 mL solution at -0.4 V between frequency range, 0.01 Hz–10 kHz.

Assay of LDH

The LDH assay is based on the principle of decrease in absorbance at 340 nm because of the oxidation of NAD+. The reaction mixture comprising 2.8 mL 0.2MTris-HCl buffer, pH 7.3 (Reaction buffer), 0.1 mL 6.6 mM NAD+ 0.1 mL 30 mM lactic acid was incubated in UV spectrophotometer at room temperature (25°C) for 4-5 min to accomplish temperature equilibration and set up a blank rate. The enzyme (1 mg/mL) was added to the reaction mixture and ΔA340/min from standard curve was recorded. The unit activity of the enzyme was calculated using the equation:

$$Units/mg = \frac{\Delta A340/\min}{6.22 \times mg\ enzyme/ml\ reaction\ mixute}$$

One unit of LDH is defined as the amount of enzyme required to reduce one micromole of NAD^+ per min per mL under the standard assay conditions [15,16].

The protein content of dissolved LDH was determined by Lowry method using bovine serum albumin (BSA) as standard protein [22].

Preparation of LDHNPs

The LDHNPs were prepared by desolvation method using ethanol [23]. To 2 mL LDH solution (1 mg/mL), 6 mL of desolvating agent (absolute ethanol) was added drop wise 0.1-0.2 ml/min under constant stirring at a speed of 500 rpm. The process was followed by the addition of 1 mL, 2.5% glutaraldehyde solution in the mixture under the identical stirring conditions at 4°C for 24 h to ensure complete crosslinking of respective ENPs. The glutaraldehyde provided intermolecular crosslinking of enzyme molecules via Schiff base. The synthesized ENPs were thiol functionalized by adding 0.2 mL of cysteamine solution (0.02 g/mL) to each suspension under constant stirring for 5-6 h. ENPs were precipitated from mixture by centrifugation NPs mixture at 1200×g for 10 min at 4°C. The process was followed by dispersion of ENPs in 2 mL 0.1M phosphate buffer, pH 7.3 and sonication for 5 min. It was assumed that alpha-NH_2 group of cysteamine reacts with excess unreacted –CHO groups of glutaraldehyde cross-linked ENPs to form Schiff base. Thus, the glutaraldehyde cross-linked ENPs get functioned with $-NH_2$ groups. These functionalized ENPs were stored at 4°C, until use.

Characterization of LDHNPs

LDHNPs were characterized by taking their images in a transmission electron microscope (TEM) and recording their FTIR (4000-500 cm^{-1}) and UV–visible spectra (200-600 nm).

Preparation of LDHNPs modified PG electrode/working electrode

LDHNPs were immobilized onto the surface of PG electrode to prepare a ENPs-based working electrode. The potential pretreatment was provided to the PG electrode by allowing its scanning over the potential of -1.1 to 0 V range in freshly prepared 0.2 M H_2SO_4 until the cyclic voltammogram feature of the clean PG electrode was developed. The PG electrode was placed in the LDHNPs suspension under mild stirring at 4°C for 12h to get physical adsorption of LDHNPs onto PG electrode. This immobilization could be possible by adsorption due to an electrostatic attraction between negatively charged -COOH groups of PG electrode and positively charged $-NH_2$ groups on the surface of ENPs, introduced by cysteamine di-hydrochloride. The LDHNPs/PG electrode was rinsed with 0.1M of phosphate buffer (PB, pH 7.0) carefully and stored in a PB buffer at 4°C, when not in use. The LDHNPs/PG electrode was washed with 0.1M sodium phosphate buffer (SPB, pH 7.0) and stored in a SPB at 4°C, when not in use. The characterization of LDHNPs/PG electrode was carried out by SEM and EIS before and after immobilization of ENPs.

Construction of lactate biosensor and its response measurements

An amperometric lactate biosensor was fabricated using LDHNPs/PG electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as an auxiliary electrode connected through galvanostat potentiostat. Figure 1 provides the schematic representation of fabrication of LDHNPs/PG electrode and electro-chemical reactions involved in its response measurement. The electrochemical optimizations, characterization and measurements of modified LDHNPs/PG electrode were carried out by using cyclic voltammetry and chronoamperometry. All electrochemical procedures were established in 0.1M phosphate buffer saline containing 0.1 mM (0.1 mL) lactate and optimized concentration of NAD^+ at an optimized scan rate. The current response was measured between 1s and 10 s at an interval of 2.5 s.

Optimization of lactate biosensor

The kinetic properties such as NAD^+ concentration, applied potential, scan rate, pH, incubation temperature, response time and effect of lactate concentration of LDHNPs/PG electrode were studied electrochemically in order to optimize the operational conditions of the biosensor. The concentrations of NAD^+ were varied from 0.1 to 8.5 mM to get the meaningful response at optimum concentrations. The effect of applied potential was studied between -1.1 to -0.1 V. Similarly, effect of scan rate was studied between 5 mV/s to 20 mV/s. To get the optimum pH, the pH was varied in the range pH 4.0 to 9.0 at an interval of pH 0.5 using the suitable buffers (strength- 0.1M) such as: pH 4.0 to 5.0 sodium acetate buffers, pH 5.5 to 7.5 sodium phosphate buffer for pH 8.0. Likewise to get optimum temperature and incubation time the master mix (buffers, lactate, and NAD^+) was incubated at different temperature (5°C to 70°C) and time duration (1-10s). The effect of lactate concentration on biosensor response was determined by varying the concentration of lactate in the range 0.001 μM-65 mM (Figure 2a). The limit of detection (LOD) was calculated using the equation: LOD = 3xσ/slope, where σ=standard deviation.

Evaluation of lactate biosensor

The evaluation of the working electrode was done by analyzing its analytical performance in terms of electroactive surface area, electron transfer coefficient, linearity, limit of detection, analytical recovery,

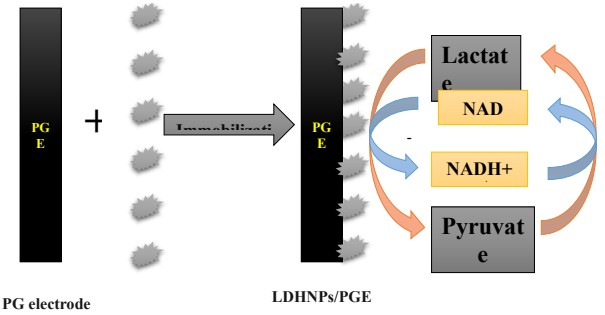

Figure 1: Schematic representation of chemical reaction involved in the fabrication of LDHNPs/PG electrode.

coefficient of variation and correlation coefficient. The electroactive surface area of electrode was calculated by Randles –Servcik equation ($I=2.69\times10^5AD^{1/2}n^{3/2}v^{1/2}C$), where I is the maximum current (A), A stands for electroactive surface area of electrode (cm^2), D represents the diffusion coefficient (cm^2/s), *n* is number of electrons transferred in the redox event (usually 1), *C* shows concentration (mol/cm^3) and *v* is scan rate (V/s). Heterogeneous electron transfer coefficient (k_s) was calculated by Laviron formula i.e., $E_p = E_0 + (RT/\alpha nF)\ [\ln (RTk_s/\alpha nF) - \ln\upsilon]$ where E_p is cathodic peak potential, E_0 is reference potential, n is number of electrons, T is temperature coefficient, R is gas constant, F and α stands for faraday constant and electron transfer constant. The average concentration (Γ) of electroactive surfaces onto surface of electrode was calculated by using the formula: Peak current (I_p) = $n^2F^2A\Gamma\upsilon/4RT$. The analytical recovery and coefficient of variation were calculated by the equations:

$$\%\ \text{Recovery} = \frac{\text{Sample absorbance} \times 100}{\text{Standard absorbance}}$$

$$\text{Coefficient of variation} = \frac{\text{standard deviation} \times 100}{\text{Mean of samples}}$$

The effect of various interferants found in the blood such as citric acid, glutamic acid, uric acid, ascorbic acid, and urea were measured at their physiological concentrations into the blood.

Application of lactate biosensor

Blood samples (1 mL each) of apparently healthy people's and patients suffering from cardiogenic shocks in different age groups (n=20 each) were collected from local Pt. BDS Post Graduate Institute of Medical Sciences (PGIMS) Rohtak Hospital. The samples were centrifuged at 5000×g for 5 min to get their supernatants (sera) and kept at 4°C until use. The quantity of lactate in sera was measured by the present biosensor in the same fashion as elucidated above for its response measurement, under optimized operational conditions except that serum was used instead of lactate. The current (mA) was recorded and the quantity of lactate in serum was interposed from the standard curve between lactate concentrations and current (in mA) under optimal operational conditions (Figure 2a).

Reusability and storage stability of enzyme electrode

The storage stability and long-term stability of the biosensor were analysed over a period of 180 days at its regular use, while its storage at 4°C.

Results and Discussion

Characterization of LDHNPs

The size of LDHNPs as measured by TEM was between 5 nm

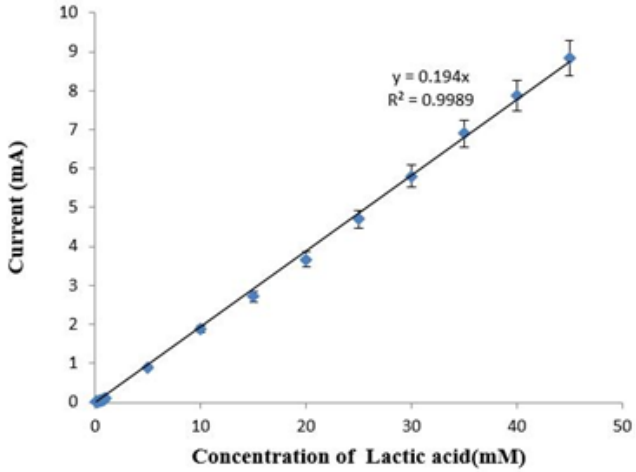

Figure 2a: Standard curve and effect of lactate concentration by lactate biosensor based on LDHNPs/PG electrode.

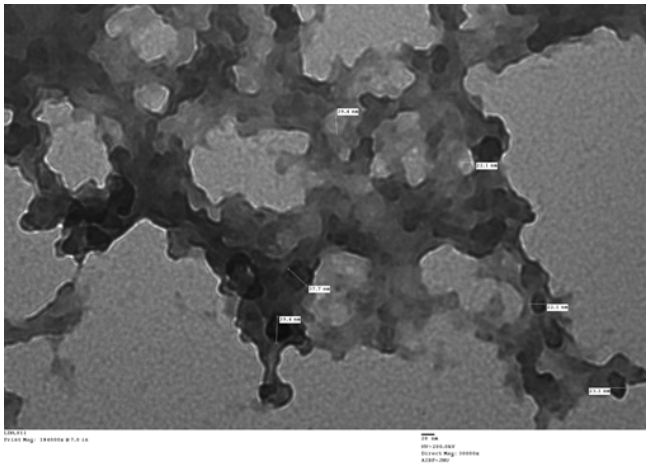

Figure 2b: Transmission electron microscopic (TEM) images of LDHNPs.

to 100 nm (Figure 2b) and showed an average size of 20 nm, which enhanced the surface area of the electrode as compared to the native enzyme. FTIR spectra of LDHNPs showed transmittance at 3299 cm^{-1}, 2191 cm^{-1}, 2170 cm^{-1}, 2122 cm^{-1}, 2089 cm^{-1}, 2050 cm^{-1}, 2034 cm^{-1}, 2001 cm^{-1}, 1989 cm^{-1}, 1959 cm^{-1}, 1948 cm^{-1}, 1928 cm^{-1}, 1636 cm^{-1}, 1077 cm^{-1}, 630 cm^{-1}, 594 cm^{-1}, 556 cm^{-1}, 534 cm^{-1}, and 520 cm^{-1}. The transmittance at 3299 cm^{-1} divulged the presence of free -NH_2 group inflicted by cysteamine dihydrochloride, the transmittance at 2191-1928 cm^{-1} betrayed the presence of amide I and amide II linkage and 1636-520 cm^{-1} peaks represent C=N stretching. These functional groups showed good coupling of LDHNPs with PG which helped in the enhancement of storage stability, detection limit and sensitivity (Figure 2c). UV and visible spectra exhibited strong absorbance peak at 250 nm, revealing a high degree of absorption of aromatic amino acids of peptide chain/free enzyme following their NPs formation (Figure 2d).

Characterization of enzyme electrode (LDHNPs/PG) at different stages of its construction

By scanning electron microscopy (SEM): Figure 2e showed the SEM images of bare PG electrode and LDHNPs modified PG electrode. Bare electrode depicted the smooth and flat morphology, whereas

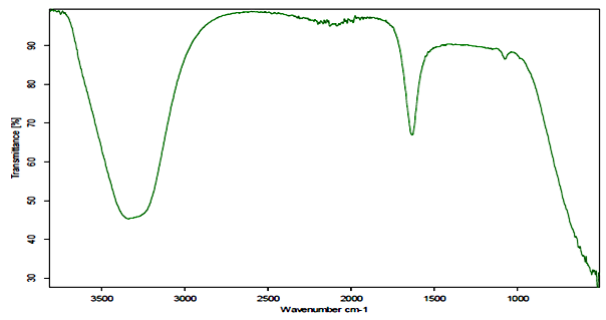

Figure 2c: UV spectrum of LDHNPs

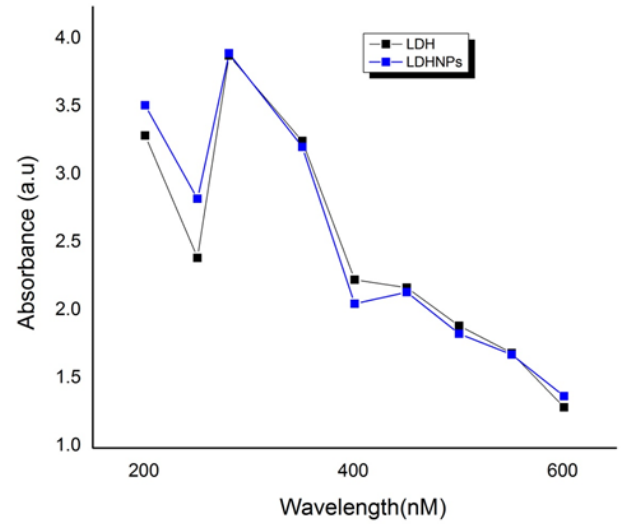

Figure 2d: FTIR spectrum of LDHNPs.

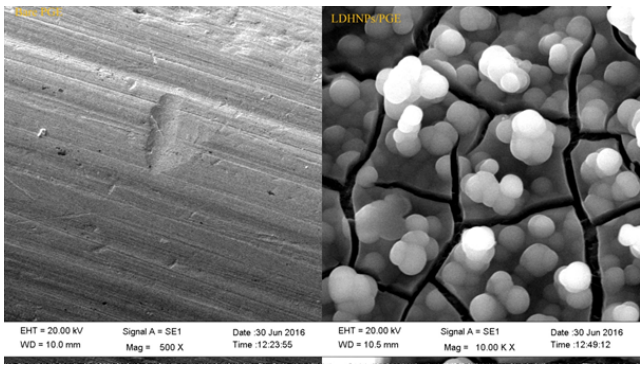

Figure 2e: Scanning electron microscopic (SEM) images of bare PG electrode and LDHNPs/PG electrode.

LDHNPs/PG electrode revealed globular structural morphology. The globular morphology was due to enzyme nanoparticles of LDH, which were clearly immobilized onto PG electrode.

By electrochemical impedance spectroscopy (EIS): The EIS provides the change in impedance of PG electrode before and after its modification with immobilization of LDHNPs. The semicircle diameter at higher frequencies contributed to the electron transfer resistance (R_{CT}), which directed the electron transfer kinetics of the redox probe at the surface of electrode, while at lower frequencies linear part corresponded to Warburg diffusion process. In Figure 2f, the Nyquist plot presented EIS of bare PG electrode and LDHNPs/PG electrode in 5 mM ($K_3Fe(CN)_6/K_4Fe(CN)_6$) respectively. The R_{CT} value for the LDHNPs/PG electrode was obtained as 160 Ω, which was higher compared to bare PG electrode (R_{CT}=20 Ω). The increased R_{CT} value of LDHNPs/PG electrode was due to high surface area and good electrical properties of enzyme nanoparticles. This increase in R_{CT} could be assigned to the reason that most biological molecules, including enzyme nanoparticles, have poor electrical conductivity at low frequencies (<10 kHz; applied voltage: 0.1V) and create obstacle to the transfer of electrons. However, LDHNPs offer high surface to volume ratio and increase the electrocatalytic property of electrode for electro-oxidation of lactate.

Voltammetric response of the LDHNPs/PG electrode

The amperometric response of electrodes bare PG, LDHNPs/PG without and with substrate was recorded (Figure 3a). The bare PG and LDHNPs/PG electrode without substrate showed no oxidation and reduction peak, while the LDHNPs/PG electrode in presence of lactate exhibited excellent oxidation and reduction peaks, thus also revealed the reliability of biosensor. The current increases linearly with increase in scan rate from 5 mV/s to 20 mV/s (Figure 3b) indicating that the electron transfer kinetics was diffusion controlled which was significant for electrochemical applications. The optimum oxidation and reduction peaks were observed at 20 mV/s and hence all electrochemical studies were done at this scan rate.

Current response measurement of LDHNPs/PG electrode

The amperometric response of the LDHNPs/PG electrode was analyzed as a function of lactate concentration (0.1 mM) by measuring CV at 20 mV/s scan rate in 0.1M PBS of pH 7.0 containing 5 mM $[Fe(CN)_6]3-/4-$ (the electrical conductivity of PBS = 25Ω). At a lactate concentration of 30 mM, the amperometric response of the PG electrode with native enzyme LDH was 0.21 mA, while the same electrode with mixture of ENPs (LDHNPs) was 2.18 mA.

Optimization of lactate biosensor

An amperometric lactate biosensor was constructed by decorating LDHNPs onto PG electrode. The optimum concentration of NAD^+ was determined by investigating the response of biosensor at different NAD^+ concentrations when applied for 0.1 mM lactate. The maximum current was predicted at 6.6 mM concentration of NAD^+. Thus, 6.6 mM NAD^+concentration was used for further work. The biosensor

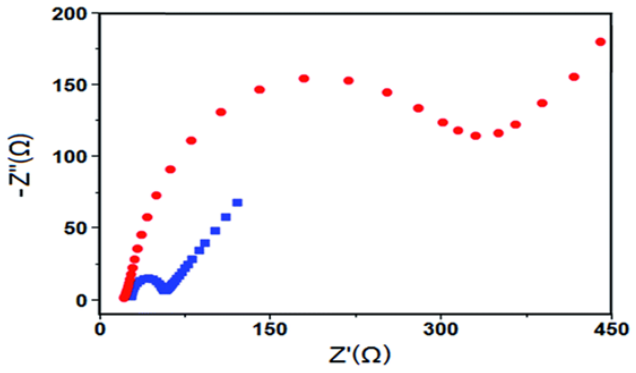

Figure 2f: Electrochemical Impedance Spectra (EIS) of bare PG electrode and LDHNPs/PGE.

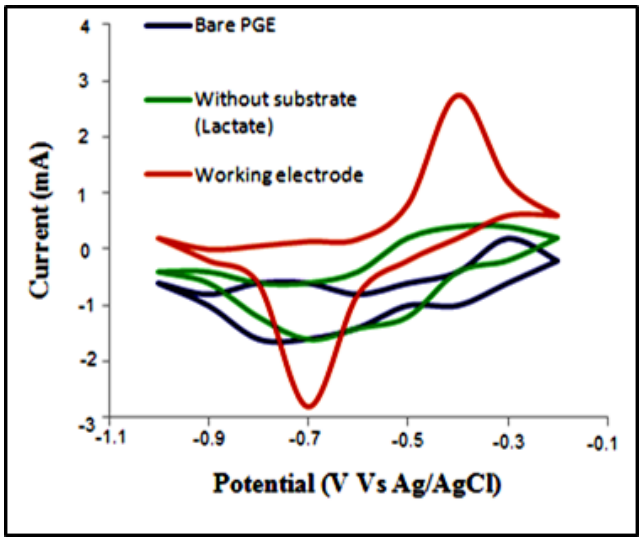

Figure 3a: Cyclic voltammogram for LDHNPs/PG electrode in 25 ml 0.1 M sodium phosphate buffer (pH=7.0) containing 30 mM L-lactic acid (0.1ml) at a scan rate of 20 mVs^{-1}.

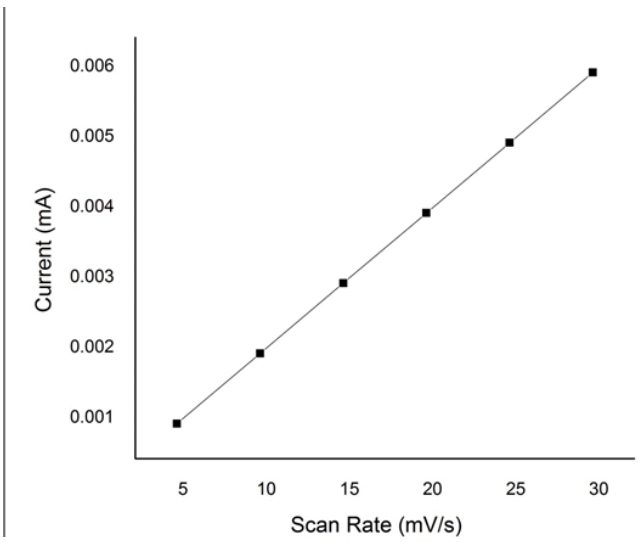

Figure 3b: Effect of scan rate on applied potential of 0.1 V.

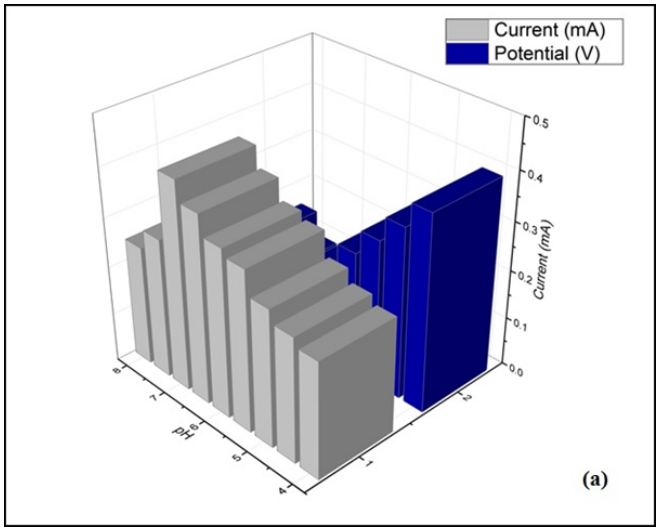

Figure 4a: Influence of pH on the current response of LDHNPs/PG electrode.

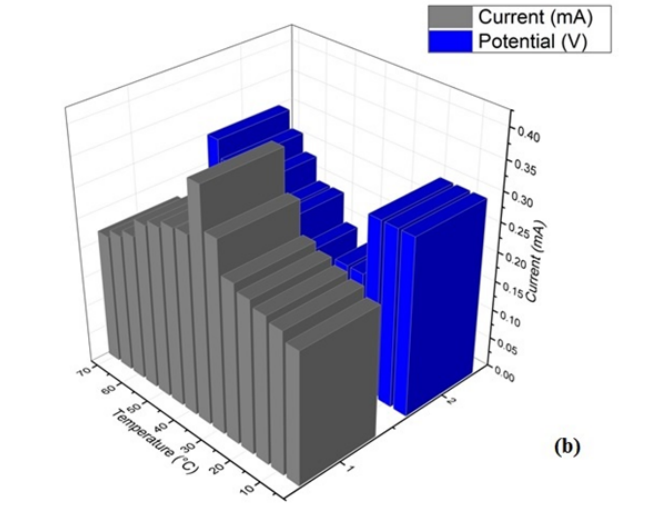

Figure 4b: Influence of temperature on the current response of LDHNPs/PG electrode.

expressed the optimum current at pH 7.0 (Figure 4a), incubation temperature 35°C (Figure 4b) in 0.1 M sodium phosphate buffer with in 2.5s (Figure 4c). While at other lower and higher pH and temperature there was gradual shift in the oxidation and reduction peaks with decline in the current range. Hence, the succeeding electrochemical studies for lactate detection were performed in 0.1 M sodium phosphate buffer of pH 7.0 and temperature 35°C at optimal potential of -0.4 V. The response of current for LDHNPs increases linearly with increase in lactate concentration. At high concentration of lactate, a plateau current is perceived, depicting the feature of Michaelis–Menten kinetic process. The apparent Michaelis–Menten constant (Km), provided the evidence of enzyme substrate kinetics, which can be attained through the Lineweaver–Burk equation

$$\frac{I}{Iss} = \frac{I}{Imax} + \frac{Km}{ImaxC}$$

Here, I_{ss} indicated steady-state current after addition of substrate, C represented the bulk concentration of the substrate, and I_{max} showed the maximum current sustained under saturated substrate conditions. The LDHNPs/PG electrode exhibited a V_{max} of 3.16 ± 0.11 mA cm^{-2} mA with app. K_m of 0.3 ± 0.1 mM and showed high sensitivity of 2.45 ± 2.0 µA cm^{-2} $µM^{-1}$at a potential of -0.4V. The small Km value means that the immobilized ENPs at PG electrode possess a high affinity to lactate.

Evaluation of lactate biosensor

The LDHNPs/PG electrode exhibited an excellent electroactive surface area of 4.862 mm^2 as compared to native enzyme LDH bound PG which had 3.456 mm^2. Hence, the synergistic effect of enzyme nanoparticles was significant in improvement of electroactive surface area. The heterogeneous electron transfer constant (k_s) for native enzyme electrode and enzyme nanoparticles electrode was calculated as 1.3 s^{-1} and 3.4 s^{-1} respectively. In case of LDHNPs modified electrode, high k_s value was attributed to the large surface area and high biocompatibility

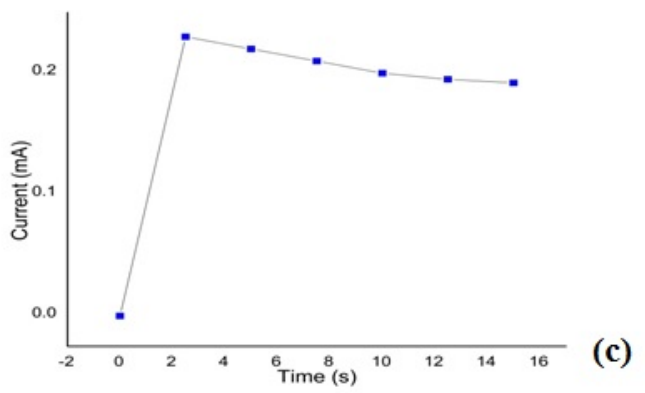

Figure 4c: Influence of time on the current response of LDHNPs/PG electrode.

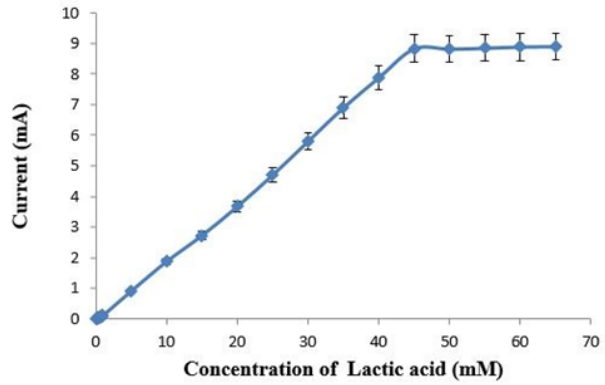

Figure 4d: Effect of lactate concentration on the current response of LDHNPs/PG electrode.

of enzyme nanoparticles. The average surface concentration (Γ) of immobilized LDHNPs was 3.86 $\times 10^{-8}$ molcm^{-2} which was higher than the concentration of native LDH i.e 1.11$\times 10^{-8}$ molcm^{-2}. This higher concentration is due to porous and biocompatible nature of PG electrode which can amplify the number of enzyme nanoparticles and further enhanced the specificity of biosensor. The biosensor offered a wide linear response between current (mA) and lactate concentration ranges of 0.001 μM-45 mM, under optimized operational conditions (Figure 4d), which was better than earlier reported biosensors (Table 5) [16]. The limit of detection limit (LOD) of the present biosensor was 0.001 μM, which was lower than already reported biosensors (Table 5) [16]. The analytical recoveries of added lactate in sera at concentration of 0.5 mM and 1.0 mM were 98.01% and 98.77% respectively (Table 1). In addition, within and between batches coefficients of variation for working electrode were detected as 0.03% and 0.04%, respectively (Table 2).

Correlation

The lactate level detected by the present biosensor was also compared with the standard enzymic colorimetric kit method. A good correlation coefficient (R^2 = 0.99) was obtained between the present method and standard enzymic colorimetric kit method (Figure 5a). The correlation coefficient of biosensor was better than earlier biosensors [16].

Application of lactate biosensor

The quantity of sera lactate in apparently healthy persons as detected by the current biosensor was in the range 0.3 ± 0.01-2.6 ± 0.02 (n=20) mM, which was in normal established range. The sera lactate level in persons suffering from cardiogenic shocks was in the range 14 ± 0.02-35 ± 0.03 (n=20), which was notably higher than the levels in apparently healthy persons (Table 3).

Interference study

Interfering components like citric acid, glucose, glutamic acid, and ascorbic acid had basically no effect on lactate biosensor response at their physiological concentrations, under the standard assay conditions. The interference obtained was less than 5%, which had no effect on the amperometric response of the present biosensor Table 4.

Storage stability of the LDHNPs/PG electrode

The storage stability of working electrode was investigated by measuring the response of lactate concentration (0.01 mM) in 0.1 M PBS for 6 weeks (Figure 5b). The biosensor showed no change in redox peaks, current and potential during 4 weeks, but in last 2 weeks it showed a drop in the value of the current without any change in redox peaks and potential. On that account, biosensor lost 10% of its initial activity during 6 weeks of its regular uses, while being stored at 4°C, which was better than earlier biosensors (Table 5). The biosensor fabrication reproducibility was checked for three electrodes by determining response of 0.01 mM lactate by cyclic voltammetry. The relative standard deviation (RSD) for the current response was 1.95%.

Lactate added (mM)	Lactate found (mM)	% Recovery
-	1.52	-
0.5	1.876	98.01 ± 0.1
1.0	1.479	98.77 ± 0.8

Table 1: Analytical recovery of added lactate in the serum samples, as measured by lactate biosensor based on LDHNPs/PG electrode.

N	Lactate(mM)	CV (%)
Within assay (5)		
62	62.8 ± 0.6	0.03
63		
65		
64		
60		
Between assay (5)		
59	61.0 ± 0.5	0.04
62		
63		
64		
57		

Table 2: Within and between assay coefficients of variation for determination of lactate in the serum samples, as measured by lactate biosensor based on LDHNPs/PG electrode.

Conclusion

In the present study, the better electroanalytical properties and improved analytical performance was achieved in view of enzyme nanoparticles as compared to native enzyme. ENPs exhibited high surface to volume ratio, which allow a large electroactive surface of PG electrode. The large electroactive surface area of LDHNPs/PG electrode has contributed in improved analytical performance of biosensor in terms of rapid response rate (2.5 s), low detection limit (0.001 μM), wide linear response (0.001 μM to 45 mM) and prolonged storage stability (180 days). The improved analytical performance of biosensor

Sex	Age	Healthy Persons (mM)	Sex	Age	Cardiac patients(mM)
M	32	0.4 ± .03	M	42	27 ± .04
M	41	0.6 ± .04	F	35	20 ± .05
F	27	2.1 ± .02	M	46	19 ± .02
M	29	0.9 ± .01	M	49	20 ± .01
F	14	1.5 ± .04	F	60	18 ± .04
F	12	1.0 ± .05	F	17	23 ± .03
F	24	2.2 ± .04	M	32	17 ± .02
M	26	1.7 ± .03	F	54	18 ± .06
M	55	1.9 ± .03	F	10	15 ± .04
F	25	1.0 ± .06	F	16	18 ± .01
M	18	1.8 ± .05	M	46	19 ± .03
M	33	1.6 ± .03	M	52	12 ± .04
F	38	1.7 ± .05	F	24	17 ± .02
F	12	0.6 ± .01	M	29	19 ± .04
M	22	1.2 ± .03	F	17	14 ± .02
F	09	0.8 ± .05	F	09	26 ± .06
M	17	1.4 ± .02	M	39	28 ± .02
M	54	1.5 ± .05	F	54	30 ± .03
M	15	0.9 ± .04	M	45	21 ± .02
F	33	1.7 ± .02	M	52	23 ± .05

Table 3: Serum lactate levels in apparently healthy persons and cardiac shock patients, as measured by lactate biosensor based on LDHNPs/PG electrode.

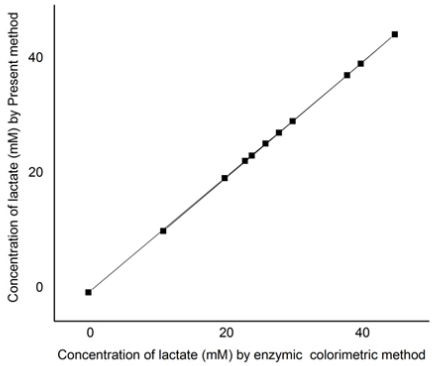

Figure 5a: Correlation between serum lactate values measured by Enzo kit method (x-axis) and the current method (y-axis) employing the lactate biosensor based on LDHNPs/PG electrode.

Interferents	Response before addition	Response after addition	Change %
Glucose (1.0 g dm^{-3})	7	7.27	4.2
Citric acid (0.5 g dm^{-3})	7	7.16	2.7
Glutamic acid (0.25 g dm^{-3})	7	7.24	3.8
Ascorbic acid (0.25 g dm^{-3})	7	7.09	1.5

Table 4: Effect of some metabolites on the lactate biosensor response.

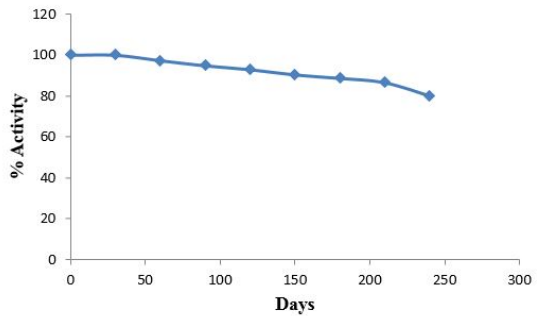

Figure 5b: Storage stability of LDHNPs/PGE biosensor at 4°C.

Enzyme used	Support of immobilization	Methods for immobilization	Type of biosensor	Working potential (V)	Optimum pH	Optimum temp. (°C)	Response time (s)	Linearity (µM)	Detecti-on limit (LOD) µM	Storage stability (Days)	Sensitivity (µAmp/ mM/cm^2	Reproducibility RSD %	Application in
LDH	MWCT/MB	Covalent Binding	Amperometric	NR	7.0	32	7	100-10,000	100	21	3.46	NR	Blood
LDH	MBRS/SPCE	Covalent Binding	Amperometric	NR	6.5	37	NR	55-10000	550	NR	NR	4.28	Serum
LDH	pTTCA/MWCNT/Au	Covalent Binding	Amperometric	NR	7.0	35	15	5000 -90000	1000	25	10.6	NR	Commercial milk and blood samples
LDH	Fe3O4/MWCNTs/GC	Covalent Binding	Amperometric	0.65	7.5	37	NR	0-300	0.3	NR		4.5	Human Serum Samples
LDH	Fe3O4/MWCNT/LDH/NAD$^+$	Covalent Binding	Amperometric	0.65	7.5	37	NR	50–500	5	NR	7.67	4.7	Human Serum Samples
LDH	NADH/LDH/NanoCeO2/GCE	Electrodeposition	Amperometric	0.3	7.4	35	4	200-2000	50	12	571.19	2.8	Clinical Diagnosis, Food quality analysis
LDH	ZnO/LDH/Au	Covalent Binding	Amperometric	NR	7	32	1	0.2-0.8	0.004	7	571.19	NR	Blood
LDH	NB/MSA / CDTe/QDS	Adsorption	Fluorescence based	NR	7.4	22	900	50-10000	50	NR	NR	10	Cancer Cells
LDH	LDH/ DP/ TTH	Adsorption	Amperometric	0.15	7.3	35	NR	1.4-55	NR	9	0.044	4.7	Beer Samples
LDH	LDH/ GrONPs/ PGE	Covalent Binding	Amperometric	0.7	7.3	35	5	5000-50000	0.1	60	NR	5.04	Wine, Beer, Curd, Milk and sera samples
LDH	LDH-MB-polysulfone- composite film/SPE	Adsorption	Amperometric	NR	7.3	35	30	1-120	0.87	NR	80	2	Blood
LDHNPs	LDHNPs/Au (Present Biosensor)	Covalent	Amperometric	0.01	7.0	30	16	100-55000	.000001	80	0.345	8.38	Cardiac Patients

Table 5: Comparison of different analytic parameters of present lactate biosensor with those of earlier biosensors (Common reference to all lactate biosensors except present biosensor) [15].

can be accredited to the large surface area, good biocompatibility and high electrical conductivity of PG electrode. PG electrode allows the uniform immobilization of ENPs retains their bioactivity and increases the direct electron kinetics. Accordingly, enzyme nanoparticles modified PG electrode could be used in analytical performance improvement of other biosensors also. The future research could be focused to design electronic chip and lab on paper chip to develop a fully automatic portable device, which can be used by the patients at his/her bedside.

References

1. Robergs RA, Ghiasvand F, Parker D (2004) Biochemistry of exercise-induced metabolic acidosis. Am J Physiol Regul Integr Comp Physiol 287: 502-516.
2. Essén B, Pernow B, Gollnick PD, Saltin B (1975) Muscle glycogen content and lactate uptake in exercising muscles. Metabol Adapt Prolonged Phys p.130–134.
3. Falk B, Bar-Or O, MacDougall JD, McGillis L, Calvert R, et al. (1991) Sweat lactate in exercising children and adolescents of varying physical maturity. J Appl Physiol 71: 1735–1740.
4. Green JM, Bishop PA, Muir IH, Lomax RG (2001) Lactate–sweat relationships in younger and middle-aged men. J Aging Phys Activity 9: 67–77.
5. Gladden LB (2008) Current trends in lactate metabolism. Med Sci Sport Ex 40: 475–476.
6. Svenmarker S, Häggmark S, Östman M (2006) What is a normal lactate level during cardiopulmonary bypass? Scandinavian Cardiovascular J 40: 305–311.
7. Amorini AM, Nociti V, Petzold A, Gasperini C, Quartuccio E, et al. (2014) Serum lactate as a novel potential biomarker in multiple sclerosis. Biochim Biophys Acta, Molecular basis of disease 1842: 1137–1143.
8. Li C (2002) Sol gel encapsulation of lactate dehydrogenase for optical sensing of lactate Biosens Bioelectron 17: 323–330.
9. Malhotra BD, Chaubey A (2003) Biosensors for clinical diagnostics industry. Sens Actuat B: Chem 91: 117–127.
10. Kost GJ, Nguyen TH, Tang Z (2000) Whole-blood glucose and lactate trilayer biosensors, drug interference, metabolism and practice guidelines. Arch Pathol Lab Med 124: 1128-1134.
11. Leverve XM (1999) Energy metabolism in critically ill patients: lactate is a major oxidizable substrate. Curr Opin Clin Nutri Metabol Care 2: 165–169.
12. Barker S (1957) Preparation and colorimetric determination of lactic acid. Meth in Enzymol: 241–246.
13. Rao G, Sastry G (1971) Titrimetric determination of ascorbic acid with cerium (IV) sulphate Anal Chim Acta 56: 325–328.
14. Yao T, Kobayashi N, Wasa T (1991) Highly sensitive determination ofL-lactate

and pyruvate by liquid chromatography and amperometric detection with lactate oxidase-lactate dehydrogenase coimmobilized reactor involving amplification by substrate recycling, Electroanal 3: 493–497.

15. Batra B, Narwal V, Pundir CS (2016) An amperometric lactate biosensor based on lactate dehydrogenase immobilized onto graphene oxide nanoparticles-modified pencil graphite electrode. Engg Life Sci16: 786–794.
16. Narwal V, Pundir CS (2017) Graphene based lactate biosensor. Lambert Academy Press, Germany.
17. Pundir CS, Narwal V, Batra B (2016) Determination of lactic acid with special emphasis on biosensing methods: A review. Biosens Bioelectron 86: 777–790.
18. Wang J, Chen Q (1994) Lactate biosensor based on a lactate dehydrogenase/nicotinamide adenine dinucleotide biocomposite. Electroanal 6: 850–854.
19. Narwal V, Yadav N, Thakur M, Pundir CS (2017) An amperometric H_2O_2 biosensor based on hemoglobin nanoparticles immobilized onto a gold electrode. Bioscience Reports 37: 1-10.
20. Narwal V, Pundir CS (2017) An improved amperometric triglyceride biosensor based on co-immobilization of nanoparticles of lipase, glycerol kinase and glycerol 3-phosphate oxidase onto pencil graphite electrode. Enz Microb Technol 100: 11–16.
21. Kariuki J, Ervin E, Olafson C (2015) Development of a novel, low-cost, disposable wooden pencil graphite electrode for use in the determination of antioxidants and other biological compounds. Sensors 15: 18887–18900.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
23. Liu G, Lin Y, Ostatna V, Wang J (2005) Enzyme nanoparticles-based electronic biosensor. Chem Commun 3481.