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<AT>An improved amperometric L-lactate biosensor based on covalent immobilization of microbial lactate oxidase onto carboxylated multiwalled carbon nanotubes/copper nanoparticles/ polyaniline modified pencil graphite electrode

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- <ABS-HEAD>Highlights► Constructed amperometric lactate biosensor based on microbial LOx/cMWCNT/CuNPs/PANI/PGE.► Biosensor showed improved analytic performance eg. 5s response time, LOD- 0.25 µM and broader linear range1µM – 2500 µM.► Biosensor measured lactic acid in plasma, milk products, alcoholic beverages and fruit juice.► Biosensor used 180 times over a period of 140 days, with only 40% loss in its initial activity.► Biosensor exhibited good correlation with standard method.

<ABS-HEAD>Abstract

<ABS-P>An improved amperometric L-lactate biosensor was constructed based on covalent immobilization of lactate oxidase (LOx) from Pediococcus species onto carboxylated multiwalled carbon nanotubes (cMWCNT)/copper nanoparticles (CuNPs)/polyaniline (PANI) hybrid film electrodeposited on the surface of a pencil graphite electrode (PGE). The enzyme electrode was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS), while CuNPs synthesized by chemical reduction method, were characterized by transmission electron microscopy (TEM), UV spectrascopy and X-ray diffraction (XRD). The biosensor showed maximum response within 5s at pH 8.0 in 0.05M sodium phosphate buffer and 37°C, when operated at $20mVs^{-1}$. The biosensor had a detection limit of 0.25 µM with a wide working range between 1 µM-2500 µM. The biosensor was employed for measurement of L-lactic acid level in plasma of apparently healthy and diseased persons. Analytical recovery of added lactic acid in plasma was 95.5 %. Within- and between-batch coefficients of variations were 6.24% and 4.19% respectively. There was a good correlation (R^2=0.97) between plasma lactate values as measured by standard colorimetric enzymatic method and the present biosensor. The working enzyme electrode was used 180 times over a period of 140 days, when stored at 4°C.

<KWD>Keywords: L-Lactate oxidase; Lactic acid; Copper nanoparticles; Carboxylated multiwalled carbon nanotubes; Polyaniline; Pencil graphite electrode,

<H1>1. Introduction

L-Lactate is generated form pyruvate in muscles and liver due to insufficient supply of O_2. The normal range of L-lactate in blood is 0.5-2.5 mM. The pattern of change or the trend towards an increase of blood lactate is sensitive indicator of survival [1]. Lactate level in blood plasma indicates the oxygenation state of tissues, warning of ischemic condition of heart, haematological disorders [2] and hypovolemia shock [3] Various methods are available for determination of lactate in blood such as titrimetric [4], gasometric[5], colorimetric [6], enzymatic colorimetric using lactate oxidase (LOx)[7], poly-o-phenylene-diamine multilayer flow injection analysis[8], enzymatic spectrophotometric using lactate dehydrogenase (LDH) [9], gas chromatographic mass spectrometry (GC/MS) [10], chemiluminiscence [11] HPLC [12], indirect UV detection by capillary electrophoresis [13], ion chromatography [14], and LDH cytotoxicity method [15]. However, these methods suffer from one or the other drawbacks such as non specificity and low sensitivity or requirement of time consuming sample preparation, expensive equipment and trained person to operate. Although enzymatic colorimetric method employing free LOx has been used in clinical laboratory, it becomes expensive due to high cost of enzyme. Further, this method suffers from poor stability of enzyme and laborious procedure. Biosensing methods employing immobilized LOx/LDH overcome these drawbacks, as these not only simple, highly sensitive, rapid and specific but also provides the reuse of enzyme and their higher stability. However, LDH based biosensors have complications due to reversibility of its reaction, involvement of two enzymes (LDH and NADH oxidase) and generation of low current, hence LDH based biosensors are ill favoured. Nevertheless, LOx based biosensors are preferred over LDH**,** due to its simple reaction and easy biosensor design, which involves aerobic oxidation of lactic acid into pyruvate and H_2O_2. This H_2O_2 can be measured directly electrochemically as follow:

$$\text{Lactic acid} + O_2 \xrightarrow{\text{LOx}} \text{Pyruvate} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{highvoltage}} 2H^+ + O_2 + 2e^-$$

LOx based L-lactate biosensor can be classified into various categories based on (i) non-conducting polymer e.g. polyvinyl alcohol (PVA) membrane and eggshell membrane on oxygen electrode, (ii) conducting polymer: polyaniline/fluoroaniline/indium tin oxide (ITO) coated glass plate, poly-5,2´-5´,2´´-terthiophene-3´-carboxylic acid (pTTCA)/MWCNT/AuE and PPY-PVS (polypyrrole-polyvinyl sulphonate), (iii) hydrogel: albumin mucin hydrogel/polycarbonate membrane, laponite - chitosan hydrogel modified GC electrodes, (iv) sol-gel: nano-TiO_2 (titanium nanoparticles)/silica-sol gel modified Au electrode and (v) screen printed electrode: Pt printed electrode, screen-printed carbon

electrode (SPCE)/ Meldola's blue-reinecke salt (MBRS). However all these enzymes electrodes are impaired from drawbacks such as poor electrical response, blockage of membrane pores (for non-conducting polymer), high cost, complicated fabrication process, poor mechanical stability, shorter life span (for conducting polymer), low mechanical strength hence difficulty in handling (for hydrogel), non-conductive diffusion limitation steps, low (for sol-gel) and expensive due to one time use (for screen printed) [16]. et al., 2014 N.D Miraida Pagan,et The use of nanomaterials in construction of enzyme electrodes has led to the improvement of biosensors such as, their stability, reusability, sensitivity, and anti-interference ability [17]. Among various nanoparticles, copper nanoparticles have attracted considerable attention because of their good biocompatibility and catalytic, optical, and electrical conducting properties, resulting in increased response current [18,19]. Carbon nanotubes (CNTs) have also been employed to improve the analytical performance of biosensors, because of their distinctive physical and chemical properties, which promote fast electron transfer. Composite materials based on integrations of CNT with some other nanomaterials possess properties of the individual components with a synergistic effect. Due to its facile preparation, high conductivity, and good environmental stability, a conducting polymer has become the most attractive one for the construction of CNT-based composites [20]. Polyaniline (PANI) is one of the most intensively investigated conducting polymer due to its excellent stability in different solutions, good electronic properties, and strong biomolecular interactions [21]. It is also unique due to its relatively facile synthesis, conductivity, and environmental stability [22-25]. Thus CuNPs/MWCNT/PANI composites synthesized chemically or electrochemically have attracted interest to improve the electrical conductivity, electrochemical capacitance, and electrocatalytic properties of enzyme electrode [26]. Earlier composites of ZnO Nanorods, MWCNT, Pt NP, SWCNT have been employed for improvement of LOx based biosensor but with limited success [27-30].
In the present work, we chose pencil graphite as the material for electrode, because the pencil graphite electrode (PGE) has a larger active electrode surface area and is therefore able to detect low concentrations and/or volumes of the analyte. This is significant, when only small amounts of analyte are available. Moreover, disposable PGEs have been used by virtue of their high electrochemical reactivity, good mechanical rigidity, low cost, low background current, wide potential window, chemical inertness and ease of modification, renewal, and miniaturization [31]. We describe herein the construction of an improved amperometric L-lactate biosensor by immobilizing lactate oxidase covalently onto carboxylated multiwalled carbon nanotubes (cMWCNT)/CuNPs/PANI composite electrodeposited onto pencil graphite (PG) electrode, its optimization, evaluation and application in measurement of lactic acid in various biological materials.

<H1>2. Experimental

<H2>2.1 Materials

L-Lactate oxidase (LOx from Pediococcus species), (L-0638, LOx 100 units/mg) from Sigma Aldrich USA and carboxylated multi-walled carbon nanotubes (cMWCNTS) from Intelligent Materials Pvt. Ltd. Panchkula, tetraethylorthosilicate (TEOS) from Fluka Mumbai were used. All other chemicals (AR grade) were from SRL Mumbai. Double distilled water (DW) was used during the experimental studies. Blood plasma samples were collected from hospital of local Pandit Bhagwat Dayal Sharma Postgraduate Institute of Medical Sciences. Commercially available milk products, various wines prepared from purple grapes with brand name as Sauvignon Blanc, Cabernet Blend, Merlot, Nine Hills Chenin

Blenc and Sula Chenin Blanc and beer (Brand name: Orangeboom, Hoegarden, Tsingtao, Heineken and Tuborg) were purchased from local market.

<H2>2.2 Assay of free LOx

The assay of free L-lactate oxidase was based on quantification of H_2O_2, generated from oxidation of lactic acid catalyzed by lactate oxidase, using a colour reaction consisting of 4- aminophenazone, phenol, and peroxidise as a chromogenic system [32]. The reaction mixture consisting of 1.8 ml of sodium phosphate buffer (pH 7.5, 0.1 M), 0.1 ml of lactic acid solution (10 mM) and 0.1 ml of L-lactate oxidase solution (1.0 mg/ml) was incubated at 37 ^oC for 10 min. Then 1 ml of color reagent (50 mg of 4- aminophenazone, 100 mg of phenol, and 1.0 mg of horseradish peroxidise in 100 ml of 0.4 M sodium phosphate buffer, pH 7.0 was added and incubated at 37 ^oC in dark for 30 min to develop the color. A_{520} was read and H_2O_2 concentration was interpolated from its standard curve (A_{520} vs. concentration of H_2O_2). One unit of L-lactate oxidase is defined as the amount of enzyme required to catalyze the formation of 1.0 µmol of H_2O_2 from oxidation of L-lactate per min/ml under the standard assay conditions.

<H2>2.3 Construction of L-lactate oxidase/c-MWCNT/CuNPs/PANI modified PG electrode

<H3>2.3.1 Preparation of CuNPs

CuNPs were prepared using chemical reduction method [22]. NaOH (0.1 M, 10 ml) was added to a mixture of aqueous solution of $CuSO_4$ (0.4M, 100ml) and ethylenediaminetetraacetic acid (EDTA, 0.1 M, 10 ml). $NaBH_4$ (0.3 M, 100 ml) was added to this mixture solution and heated to 60 °C. After stirring for 45 min, the product i.e. CuNPs were washed three times with ethanol and acetone separately by centrifugation and finally dried in a vacuum oven at 35 – 45 °C.

<H3>2.3.2. Characterization of CuNPs

CuNPs were characterized by taking their images in transmission electron microscope (TEM), at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi, India and recording their UV spectra in UV spectrophotometer (Dynamica HALO DB-20, UK) and X-ray diffraction (XRD) pattern in X-ray diffractometer at National Physical Laboratory (NPL), New Delhi.

<H3>2.3.3. Electrodeposition of c-MWCNT/CuNPs/PANI onto PG electrode

The wooden casing of one end of 6B pencil (purchased from local market) was carved upto 3cm of its length with a sharp blade to expose its graphite rod. The surface of graphite rod i.e. PG electrode (2cm x 2mm) was polished manually by alumina slurry (diameter of 0.05µm) with a cloth, followed by thorough washing with DW and then placed into ethanol, sonicated to discard adsorbed particles, and finally washed with DW 3 to 4 times. cMWCNTs (1.0 mg) were suspended in a 4 ml mixture of concentrated H_2SO_4 and HNO_3 in a 3:1 ratio and ultrasonicated for 3 to 4 hr to get its finely dispersed black-colored solution. The dispersed cMWCNT solution (0.1 ml) was added to a mixture of 0.5 ml of 0.2 M, N-

ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) HCl and 0.5 ml of 0.2 M N-hydroxysuccinimide (NHS) (pH adjusted to 6.0) and kept at room temperature for 1 hr. Aniline (400µl) was added to 25.0 ml 1 M KCl and electropolymerized onto pencil graphite electrode through cyclic voltammetry using a potentiostat/galvanostat (Autolab PGSTAT 30 electrochemical workstation, Eco Chemie The Netherlands with GPES 4.9 software) by applying 20 successive polymerization cycles at -0.2 + 0.6 V [21] CuNPs suspension (400µl) and 1.0 ml of cMWCNT suspension were added into 25 ml of 1M KCl to get a mixture of cMWCNT and CuNPs. This mixture was electrodeposited onto PANI modified PG electrode in a electrochemical cell using a potentiostat/galvanostat by applying 20 polymerization cycles from -0.2 to 0.6 V. During the electrochemical polymerization, the colour of the surface of graphite lead/rode became black gradually, indicating the deposition of cMWCNT/CuNPs/PANI film onto PG electrode. The resulting cMWCNT/CuNPs/PANI/PG electrode was washed thoroughly with DW to remove unbound matter and kept in a dry petri plate at 4°C.

<H3>2.3.4. Immobilization of LOx onto cMWCNT/CuNPs/PANI/PG electrode

cMWCNT/CuNPs/PANI/PG electrode was immersed into 2 ml of dissolved LOx (1 mg/ml 0.1 M sodium phosphate (PB), pH 7.0) and kept overnight at room temperature for immobilization. The resulting electrode (cMWCNT/CuNPs/PANI/PG) was washed 3 to 4 times with 0.1 M PB buffer (pH 7.0) and used as a working electrode and stored dry at 4 °C, when not in use. Fig.1 shows various steps and chemical reaction and depicts the step involved in the fabrication of LOx electrode.

<H3>2.3.5. Characterization of LOx electrode (LOx/cMWCNT/CuNPs/PANI/PGE)

The LOx electrode was characterized by scanning electron microscopy (SEM) at different stages of its construction. The SEM images of bare pencil graphite electrode, PANI/PG electrode, cMWCNT/CuNPs/PANI/PG electrode, and LOx/cMWCNT/CuNP/PANI/PG electrode were taken in a scanning electron microscope (Zeiss EV040) at AIRF J.N.University, New Delhi, on commercial basis.

<H2>2.4. Construction and testing of LOx electrode/L-lactate biosensor

An amperometric L-lactate biosensor was constructed by connecting LOx/cMWCNT/CuNPs/PGE as working electrode with Ag/AgCl as reference electrode and Pt wire as auxillary electrode through potentiostat-galvanostat (Eco-Chemie The Netherland). Cyclic voltammetry (CV) of c-MWCNT/CuNPs/PANI/PG electrode with and without LOx was recorded in a potentiostat-galvanostat from -0.2 to +0.6 V versus Ag/AgCl electrode in 25 ml of reaction buffer, 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1ml of 30mM lactic acid. The reaction buffer was saturated with O_2 bubbling before use. The current response (in µA) was recorded by applying different potentials ranging from -0.2 to +0.6 V.

<H2>2.5. Optimization of L-lactate biosensor

To optimize working conditions of the biosensor, effect of pH, incubation temperature, time, and substrate (lactic acid) concentration on biosensor response were measured. To determine the optimal pH, the pH was varied between 6.0 to 9.0 at an interval of 0.5 pH using the sodium phosphate buffer, at a final concentration of 0.05 M. Similarly to study the optimal temperature, the reaction mixture was incubated at different

temperature (25 – 45 ^{o}C) at an interval of 5 ^{o}C. The effect of lactic acid concentration on biosensor response was determined by varying the concentration of lactic acid in the range 1µM - 2500µM.

<H2>2.6. Application of L-lactate biosensor

L-lactate content was measured in plasma of healthy and diseased persons by the present biosensor in the same manner as described for its response measurement under its optimum conditions except that L-lactate was replaced by plasma. The content of L-lactate was extrapolated from standard curve between L-lactate concentrations vs. current response (µA) of biosensor. The lactic acid level was also measured by the present biosensor in various biological materials (milk products and wines) using the same procedure as for serum.

<H2>2.7. Evaluation of L-lactate Biosensor

The lactate biosensor was evaluated by studying its limit of detection (LOD), analytical recovery, precision and correlation with the standard enzymic colorimetric method. The LOD was calculated by using the following formula:

$$LOD = 3.3 \times SD/S$$

where SD is the Standard Deviation of the response, S is the slope of calibration curve.

To calculate analytical recovery, known amount of lactic acid in plasma at two levels, 10 µM and 20 µM and determined the lactate value in the plasma before and after addition of exogenous lactic acid. The analytical recovery (%) was calculated using the following formula:

$$\% \text{ Recovery} = \frac{\text{Conc. of plasma lactic acid measured after addition of exogenous lactic acid}}{\text{Conc.of lactic acid in plasma before addition} + \text{conc. of added exogenous lactic acid}} \times 100$$

To study the precision, lactic acid content was determined in five plasma samples six times on the same day (within batch) and in the same samples after their storage at -20 °C for one week (between batch) by the present biosensor. Both within and between batches coefficients of variation (CV) in plasma were calculated as follow:

$$CV = \frac{SD}{Mean} \times 100$$

To study the accuracy of present biosensor, lactic acid levels in 15 plasma samples was determined by the present lactate biosensor (y) and compared with those by standard enzymic spectrophotometric method using LDH [33] (x), which was based on measurement of NADH at 340 nm in an alkaline medium (responsible for displacing the equilibrium towards pyruvic acid)

The values obtained by both the methods were correlated using regression equation. A regression plot was drawn between the two methods and correlation coefficient (r) was calculated using the following formula:

$$r=\frac{n(\sum xy)-(\sum x)(\sum y)}{\sqrt{[n\sum x^2-(\sum x)^2][n\sum y^2-(\sum y)^2]}}$$

<H2>2.8. Storage stability of LOx/c-MWCNT/CuNPs/PANI/PG electrode

The storage stability of the enzyme electrode was studied for near about 5 months by measuring its current response weekly. The electrode was stored dry at 4 ^{o}C, when not in use.

<H1>3. Results and discussion

<H2>3.1. Characteristics of CuNPs

The TEM images of CuNPs (Fig. 1A) showed their spherical shape and size as 4.28, 6.35, 7.05, 8.07 nm i.e. in the range, 4.28 nm to 8.07 nm with an average size of 6.5 nm. The UV and visible spectra of CuNPs exhibited a maximum absorbance at 650nm, confirming their synthesis (Fig. 1B). The XRD patterns of the CuNPs clearly showed the characteristics peaks of CuNPs at 36.64°, 43.52° and 50.66° (Fig. 1C).

<H2>3.2. Characterization of enzyme (LOx) electrode

<H3>3.2.1. By SEM image

The SEM images of the surface of bare PG electrode, PANI/PG electrode, cMWCNT/CuNPs/PANI/PG electrode, and LOx/cMWCNT/CuNPs/PANI/PG electrode are shown in Fig. 2A(a-d) respectively. The stepwise modification of electrode can be seen clearly from these SEM images. The SEM image of the bare electrode showed a smooth and featureless morphology (Fig. 2A(a)). The SEM image of the PANI/PG electrode showed a fibrillar structure of PANI (Fig. 2A(b)). The cMWCNT/CuNPs/PANI/PG composite film exhibited a net structure, homogenous and cable- like morphology of the nano composite film, indicating that c-MWCNTs and CuNPs were well dispersed over the PANI layer in the hybrid film (Fig. 2A(c)). After immobilization of LOx enzyme on c-MWCNT/CuNPs/PANI hybrid film, the enzyme electrode showed the sporadic/random appearance of globular/beaded structure on the hybrid film (Fig. 2A(d)), indicating that enzyme was immobilized on the surface of c-MWCNT/CuNPs/PANI composite film.

<H3>3.2.2. By FTIR spectra

Fig.2B. shows FTIR spectra for PANI/PG electrode (panel i), cMWCNT/CuNPs/PANI/PG electrode (panel ii), and LOx/cMWCNT/CuNPs/PANI/PG electrode (panel iii). FTIR spectra of electrodeposited PANI/PG composite showed a band at 2922.39 cm^{-1} (Fig. 2B, curve i) due to N-H stretching of the benzenoid ring. The peak at 1633.87 cm^{-1} is assigned to the quinonoid ring, 1028.20 cm^{-1} is attributed to the benzoid ring, and 1056.90 cm^{-1} to the combination modes of benzoid and quinonoid units and 3435 cm^{-1}, to the N-H stretching mode of PANI. In the FTIR spectra of cMWCNT and CuNPs, the peak at 2922.18 cm^{-1} corresponds to the C-H stretching. When PANI and cMWCNT composite was formed, there is a slight shift in absorption peak from 1028.20 cm^{-1} to 1056.90 cm^{-1}, which might be due to the interaction between cMWCNT, PANI and CuNPs (Fig. 2B, curve ii). The interaction between the cMWCNT and PANI due to the charge transfer may result in an increase in N-H stretching intensity. There was a shift in peak from 3435 to 3427 cm^{-1} and from 1633.87 to 1583.52 cm^{-1}. The enzyme binding on cMWCNT/CuNPs/PANI/PG electrode were indicated by the appearance of additional absorption bands at 1583.52 and 1047.88 cm^{-1} (Fig. 2B, curve iii), which were assigned to –N-H bonding (amide II band) and C-O vibrations, respectively, indicating that LOx was immobilized covalently through -CONH- bond formed between free -COOH- group of cMWCNT and -NH_2- group at end of PANI chain in cMWCNT/CuNPs/PANI composite film. The FTIR spectrum of the LOx/cMWCNT/CuNPs/PANI/PG bioelectrode (curve iii) also showed the appearance of an additional band at 2361 cm^{-1} which is assisgned to the nitrile stretch (C=N).

<H2>3.3. Construction of L-lactate biosensor

Fig.3. provides the schematic representation of construction of enzyme working electrode based on covalent immobilization of LOx from Pediococcus species on CuNPs decorated cMWCNT and PANI composite film electrodeposited onto PG electrode. First, aniline was electropolymerized on the surface of PG electrode, because this method is easy and layer thickness could be controlled, and then cMWCNT and CuNPs were co-electrodeposited onto the PANI coated PG electrode. LOx was immobilized covalently onto cMWCNT/CuNPs/PANI/PG electrode through EDC/NHS activated c-MWCNTs. The CVs of LOx/cMWCNT/CuNPs/PANI/PGE exhibited enhanced currents response than cMWCNT/CuNPs/PANI/PG electrode (Fig.4), revealing that cMWCNT/CuNPs/PANI/PG composite film has large effective surface area and could provide composite/matrix for faster kinetics. Hence, the c-MWCNT/CuNPs/PANI acting as electron transfer mediator help in enhancing the biosensor response and thus increase its sensitivity. After immobilization of LOx onto the modified electrode, the current response enhanced, due to electrochemical breaking down of H_2O_2, generated from LOx reaction.

<H2>3.4. Response measurements of L-lactate biosensor

The maximum response, as measured in μA of the present working lactate biosensor was obtained within 5s, at the applied potential 0.28V (Fig.4) and hence subsequent studies were carried out at this voltage. This working potential is lower than that of earlier lactate biosensor based on LOx cross-linked with polymeric matrixes (0.65V) [34], RGo (reduced graphene oxide)-AuNPs/LDH (0.48V) [35] and but close to hydrogen titanate nanotube/LOx based biosensor, (0.27V) [36]. The response time of present biosensor 5s is equal to that based on hydrogen titanate nanotube bound LOx (5s) [36], but lower than that of based on polyaniline-co-fluoroaniline film bound LOx (50s) [37], tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ)/LOx (80s) [38], LOx immobilized membrane on a micro planar Pt. electrode (10s) [39].

<H2>3.5. Optimization of LOx biosensor

The biosensor showed optimal current at pH 8.0 (Fig. 5A), which is higher than that of biosensor based on LOx- MWCNT/polysulfone (PS) onto carbon screen printed electrodes (pH 7.5) [40], LOx/dimethyl-ferrocine modified linear (ethylenimine) ($FcMe_2$-LPEI) hydrogel/GC electrode (pH 7.5) [41], but similar to that based on LOx/MWCNT/chitosan onto screen-printed electrodes (pH 8) [42] and slightly lower to that based on Sol-Gel Silica matrix/LOx (pH 8.4) [43]. The optimum incubation temperature of the biosensor was 37 °C (Fig. 5B), which is lower to that based on LOx/Purssian blue/Screen printed chip electrode/LOx (45 °C) [44] and similar to those based on tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ)/LOx (37 °C) [38] and glassy carbon electrode with a hydrogel film composed of laponite (37 °C)[45]. The current response decreased rapidly after 37 °C, due to the inactivation of the enzyme at high temperature. The current response increased as the lactic acid concentration was increased upto 2500 µM after which it became constant upto 3500 µM. (Fig. 6).

<H2>3.6. By electrochemical impedance spectra (EIS)

Fig.7(a-c) shows electrochemical impedance spectra of bare PG electrode (curve a), cMWCNT/CuNPs/PANI/PG electrode (curve b), and LOx/cMWCNT/CuNPs/PANI/PG electrode (curve c) in a solution 0.05 M sodium phosphate buffer (pH 7.0) containing 5mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) as a redox probe. The R_{CT} values for the PANI/PG, cMWCNT/CuNPs/PANI/PG and LOx/cMWCNT/CuNPs/PANI/PG electrodes were obtained as 480 Ω, 245 Ω, 320 Ω respectively (the average of three replications). The decrease in R_{CT} value of the cMWCNT/CuNPs/PANI/PG electrode (curve b) compared to bare PG electrode was because of the attachment of nanomaterials onto the electrode resulting into high electron transfer efficiency. However, the R_{CT} of cMWCNT/CuNPs/PANI/PGE after immobilization of LOx was increased due to binding of enzymes (LOx) molecules, which are poor electrical conductors at low frequencies and cause hindrance to electron transfer. These results also confirmed the immobilization of LOx onto the cMWCNT/CuNPs/PANI/PG electrode.

<H2>3.7. Evaluation of biosensor

<H3>3.7.1. Linearity

There was a linear relationship between the current (in µA) and the L-lactic acid concentration in the range, 1 µM -2500 µM (Fig. 6 Inset) in the reaction buffer, which is a better than those of earlier biosensors based on GC electrode with a hydrogel film composed of laponite (0.01–1.0 mM) [45], amucin/albumin hydrogel matrix/Nafion Polymer/Pt electrode (2–1000µM) [46], fabrication of ZnO nanorods on Au coated glass substrate (0.01 mM to 1 mM) [27] and positively charged nanoscaled cobalt phthalocyanine (NanoCoPc) modified GC electrode (0.020 mM–4.0 mM) [47], MWCNTs/FcMe/CS/HRP/BSA/LOx/Screen printed graphite electrode (SPBGE) (30.4 µm-243.9 µm) [48] and Pt printed electrode (0.008−1 mM) [49] but comparable to that of based on ZnO nanowired-gated A/GaAs/GaAs (0.3 nM- 300 mM) [50] (Table 1).

<H3>3.7.2. Detection limit

The current biosensor exhibited a low detection limit of 0.25µM (S/N=3), which is lower than those of earlier biosensors based on sol-gel silica matrix/LOx (1.5 µM) [43], mucin/albumin hydrogel matrix naffion membrane (0.8 µM) [46] eggshell membrane on oxygen electrode (8.6 µM) [51], $FcMe_2$-LPEI Hydrogel GC electrode (3 µM) [41] conducting polymer/MWCNT modified gold (Au) electrode (1µM) [28] and slightly higher than that of earlier biosensor based on RGo-AuNPs (0.13µM) [35] (Table 1).

3.7.3. Analytical recover<remove picture pageno 18>y

Analytical recoveries of lactic acid added to serum (at levels of 10 µM and 20 µM) varied from 97% and 94% respectively (Table 2), showing the high reliability of the present biosensor. The present analytic recovery is better than those of based on mucin/albumin hydrogel matrix on naffion membrane modified Pt electrode (89 ± 6%) [46].

<H3>3.7.4. Precision

Within –sample and between-sample coefficients of variations (CVs) for the determination of lactic acid in plasma on the same day and after 1 week of storage at -20°C were 4.5% and 6.5% respectively (Table 3). These high precisions shows the good reproducibility and consistancy of the present method, which can be attributed to the excellent immobilization of LOx onto the cMWCNT/PANI/CuNPs/PG electrode.

<H3>3.7.5. Accuracy

The accuracy of the present method was tested by comparing lactate values in 20 plasma blood samples by the present method (*y*) with those obtained by standard enzymatic spectrophotometry method (*x*). The values obtained by both methods showed a very good correlation with R^2= 0.975 with the following regression equation 1.085x-32.56 (Fig. 8A), which is higher than that of biosensor based on LOx immobilized onto nylon net/Pt electrode (R^2=0.9547) [52] and slightly lower than that of based on eggshell membrane on oxygen electrode (R^2 = 0.9998).

<H2>3.8. Applications of L-lactate biosensor

L- Lactate level in plasma samples as measured by the present biosensor was 474±15 to 654±25 µmol/L in apparently healthy persons and 2309±31 to 908±64 µmol/L in persons suffering from lactoacidosis (Table 4), 928±11 µmol/L in curd, 943±4 µmol/L in buffalo milk, 895±49 µmol/L in yogurt, 896±1 µmol/L in cheese, 910±14 to 1035±64 µmol/L in white wine's, 1052±66 to 1152±66 µmol/L in beers (Table.5). Our results of plasma lactate level in apparent healthy are in normal established range (500 µmol/L-2500 µmol/L) [37].

<H2>3.9. Interference study

The interference study of the current LOx biosensor was carried out by comparing the amperometric response before and after adding interferent such as citric acid, glutamic acid, glucose, L-ascorbic acid, urea, uric acid, glycine, succinic acid and pyruvic acid along with 30mM lactic acid in 0.05 M sodium phosphate buffer (pH 8). The results showed that uric acid and glycine showed only 2.13% and 7.20% inhibition

respectively, while rest compounds had no interfering effect. Earlier an inhibition (20% and 38%) for lactate biosensor by uric acid has been reported based on polyaniline/fluoroaniline/indium tin oxide (ITO) coated glass plate and CA membrane, respectively [37,53].

3.10. Stability and reusability of LOx electrode

The enzyme electrode maintained 60% of its initial activity even after 180 regular uses over a period of 140 days (Fig. 8B), which is better than earlier biosensors based on LDN/MWCNT/CHIT (65% after 7 days) [42] but lower than that of dissolved oxygen (DO) metric biosensor based on LOx/CHIT/eggshell membrane (80% after 1 year), which has its own limitations [51].

<H1>4. Conclusion

An improved amperometric lactate biosensor was constructed based on covalent immobilization of LOx from Pediococcus species onto hybrid of cMWCNT/CuNPs/PANI electrodeposited onto PG electrode. The biosensor had a very rapid response (5s), with a lower detection limit (0.25µM) and broader linear range (1 µM -2500 µM), good reproducibility and a higher storage stability (140 days). Thus the use of cMWCNT/CuNPs/PANI hybrid film has improved the analytical performance of a lactate biosensor and could also be employed for the improvement of other biosensors.

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<Figure>Fig. **1A** Transmission electron microscopic (TEM) images of CuNPs, Fig. 1**B** UV spectrum of CuNPs and **Fig.1C** X -ray diffraction (XRD) pattern of CuNPs.

<Figure>Fig. **2A(a-d)** SEM images of **(a)** bare PG electrode **(b)** PANI/PG electrode **(c)** PANI/cMWCNT/CuNPs/PG electrode, and **(d)** LOx/cMWCNT/CuNPs/PANI/PG electrode and **Fig.2B(i-iii)** FTIR spectra of **(i)** PANI/PG, **(ii)** cMWCNT/CuNPs/PANI/PG and, **(iii)** LOx/cMWCNT/CuNPs/PANI/PG electrode.

<Figure>Fig. **3.** Schematic representation of steps and chemical reaction involved in the fabrication of LOx/cMWCNT/CuNPs/PANI/PG electrode.

<Figure>Fig. **4.** Cyclic voltammogram for electrode of (a) LOx/cMWCNT/CuNPs/PANI/PG electrode and (b) LOx/cMWCNT/CuNPs/PANI/PG electrode.

<Figure>Fig. **5A** Effect of pH on LOx/cMWCNT/CuNPs/PANI/PG electrode, **Fig.5B** Effect of incubation temperature on LOx/cMWCNT/CuNPs/PANI/PG electrode.

<Figure>Fig. **6.** Effect of lactate concentration on L-lactate biosensor based on LOx/ cMWCNT/CuNPs/PANI/PG electrode. **Inset:** Standard curve for lactic acid determination.

<Figure>Fig. **7.** Impedance spectra of bare PG electrode, cMWCNT/CuNPs/PANI/PG electrode and LOx/cMWCNT/CuNPs/PANI/PG electrode.

<Figure>Fig. **8A** Correlation between plasma lactate values measured by the standard colorimetric method (*x*-axis) and present biosensor (*y*-axis) based on LOx/cMWCNT/CuNPs/PANI/PG electrode and **Fig.8B** Effect of storage at 4°C on the response of LOx/cMWCNT/CuNPs/PANI/PG electrode.

Legend to Tables:

Table. **1.** A comparison of analytic properties of present lactate oxidase biosensor based on LOx/cMWCNT/CuNPs/PANI//PG electrode with earlier biosensors.

Table. **2.** Analytical recovery of added lactic acid in plasma, as measured by LOx/cMWCNT/CuNP/PANI/PG electrode.

Table. **3.** Within- and between-assay coefficients of variations for determination of lactic acid in plasma as measured by LOx/cMWCNT/CuNPs/PANI/PG electrode.

Table. **4.** Plasma lactate levels in apparently healthy persons and lactoacidosis patients, as measured by lactate biosensor based on LOx/cMWCNT/CuNPs/PG electrode.

Table. **5.** Lactate level in various biological materials as measured by present lactate biosensor based on LOx/cMWCNT/CuNPs/PANI/PG electrode.

Table. **1**

Matrix	**Enyme**	**Method for Immobilization**	**Linearity**	**Detection Limit**	**Response time**	**References**

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Polyaniline-co-Fluoroaniline/ITO	LOD from *Pediococcus* sp.	Glutaraldehyde coupling	0.1-5.5mM/l	0.1mM/l	50s	Suman et al.(2005)
LOx/albumin-Mucin Hydrogel/polycarbonate Membrane/PtE	LOx	GA crosslinking	< 1.5mM	0.7 µM	90 s	Romero et al.(2008)
pTTCA/MWCNT/Au	LDH	Covalent immobilization	5mM -90mM	5mM	7 sec	Rahman et al.(2009)
ZnO nanorods/Au Coated glass substrate	LOx	GA crosslinking/ Physical adsorption	0.01mM-1mM	1.0×10^{-4} - 1×10^{0} mM	10 s	Ibupoto et al.(2010)
MBRS/SPCE	LDH	Electrode deposition	0.55mM-10mM	0.55mM	10 s	Piano et al.(2010)
CeO2/GCE	LDH	Adsorption	0.2mM-2mM	0.2mM	4 s	Nesakumar et al.(2014)
PtNp-CNF-PDDA/ SPCES	LOx	Electrode deposition	25µM-1500µM	11µM	60 s	Ardisana et al.(2014)
SWCNT/PtE modified With (4-ATP)	LOx	Covalent immobilization	upto 0.12 mM	4.0 µM	NR	Pagan et al.(2015)
RGo-AuNPs	LDH	Sol-Gel Entrapment	10µM-5000µM	0.13µM	8s	Azzouzi et al.,(2015)
NB/MSA/CDTe/QDS	LDH	Adsorption	50µM-10000 µM	50µM	900s	Zhang et al.(2016)
Pb/SPCE	LOx	Adsorption	20µM-150µM	NR	NR	Jiang et al.(2016)

$FcMe_2$-LPEI Hydrogel GCE	LOx	Cross linking	0-5000µM	3µM	5s	Hickey et al.(2016)
DNPs/Sol-Gel Matrix/AuE	LOx	Sol-Gel Entrapment	53µM-1600µM	16µM	NR	Briones et al.(2016)
CS/MWCNTs/FcMe-HRP/BSA/LOx-SPBGE	LOx	Covalent Linkage	30.4µM-243.9µM	22.6µM	NR	Ibanez et al.(2016)
PPY_{3DOM}/HRP/ LOx/AuE	LOx	Entrapment	1µM-100µM	0.52µM	100s	Gomez et al.(2016)
Albumin/AuE	LOx	Entrapment	5µM-800µM	5µM	5s	Shkotova et al.(2016)
cMWCNT/CuNPs/ PANI/PGE	LOx	Electrode deposition	1 µM -2500µM	0.25µM	5 s	Present work

<Table>Table 2**.**

LA added (µM)	LA found (µM)	(%) Recovery
-	590	100
10	599.7	97.0
20	608.8	94.0

LA = Lactic acid

Table. **3.**

n	Lactate (mM)	CV (%)
Within assay (6)		
760	783.33	6.24
765		
850		
735		
750		
840		
Between assay (6)		
860	913.33	4.19
930		
940		
870		
930		
950		

CV = coefficient of variation

<Figure>Fig. **4.**

Age group in years (n = 08)	Sex		Plasma lactate level µmol/L(mean± SD)	
	Healthy	Diseased	Healthy	Diseased
< 10	F	F	474±15	908±64
	M	M	512±13	983±27
11-20	F	F	651±19	1254±63
	M	M	588±11	1069±39

21-30	F	F	583±34	1366±81		
	M	M	608±12	1875±66		
31-40		F	F		617±10	2140±121
		M	M		757±32	2309±31
41-50		F	F	654±25	2524±57	
		M	M	716±27	1641±44	
51-60		F	F	549±22	1561±24	
		M	M	558±23	1543±46	
61 & above		F	F	524±23	2174±45	
		M	M	531±17	2045±128	

<Figure>Fig. **5.**

Milk products Mean ± SD (*n*=2)	Lactate level(µM)	White wines brands	Lactate level(µM) Mean ± SD (*n*=2)	Beer brands	Lactate level(µM) Mean ± SD (*n*=2)
Diluted curd (from buffalo milk)	927.5±10.6	Sauvignon Blanc	910±14.14	Orangeboom	1073±31.11
Cheese(Amul)	896.0±1.4	Cabernet Blend	1035±63.63	Hoegarden	1147±67.88
Milk(buffalo)	942.5±3.5	Merlot	1005±35.35	Tsingtao	1107±11.31
Yogurt (Amul)	895.0±49.4	Nine Hills Chenin Blenc	995±35.35	Heineken	1052±66.46
Orange juice	950.0±56.5	Sula Chenin Blanc	1000±14.14	Tuborg	1152±66.46

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