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Fabrication of Biosensor for Determination of L-lactate using Elite Nanomaterials based LDH-cMWCNT-MB/Chitosan/SWCNT-Au Electrode

Kavita Rathee,[1] Rekha Dhull,[1] Sandeep Singh[1] and Vikas Dhull [2,*]

[1]*Department of Biochemistry, Maharshi Dayanand University Rohtak-124001, Haryana, India*
[2]*Department of Biotechnology, University Institute of Engineering & Technology, Maharshi Dayanand University, Rohtak-124001, Haryana, India*

*Corresponding Author, Tel.: +91-9813488148
E-Mail: v.nano87@gmail.com; vikasbtdhull.gf.uiet@mdurohtak.ac.in

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Abstract- Lactate level being an important parameter is to be monitored in body regularly. During acute conditions it elevation may result in heart failure, hepatic disease, sepsis, tissue hypoxia and many more. A simple and sensitive biosensor has been fabricated by immobilizing lactate dehydrogenase enzyme. It was covalently immobilized on the nanocomposite based working electrode. The developed biosensor showed optimal response at pH 7.5, temperature 30℃, response time was 8s with the substrate concentration of 600 µM, the oxidation peak was obtained at 0.66 V which is at a low potential, K_m and I_{max} values for newly fabricated biosensor were 66.7 µM and 153.8 µA respectively. It possessed lower detection limit of 0.015 mM with storage stability of 3 months. A good correlation has been observed when developed method is compared with standard enzyme kit method proving reliability of the method. Blood samples were successfully screened for lactate concentration using working electrode. The newly developed biosensor is less time consuming, accurate, reliable, less costly, requires no sample pre-treatment, screen large number of samples, suitable for on field determination of lactate concentration and finally reusable due to covalently immobilized enzyme.

Keywords- L-lactate, *Lactate dehydrogenase*, Biosensor, Nanomaterial, Covalent immobilization

1. INTRODUCTION

Anaerobic respiration of glucose results in formation of byproduct L-Lactate (2-hydroxy propanoic acid). Lactate concentration in body remains constant till when concentration of lactate generated overcomes its removal rate from the body. Lactate level in blood is found in the range of 0.5-1.5 mmol/l at normal resting condition, but in excretion elevation in lactate concentration has been noticed to 25 mmol/L [1,2]. Concentration of lactic acid can elevate during strenuous exercise and heart stroke [3], hemorrhage [4], respiratory failure [5], hepatic disease [6], a severe infection [7] and tissue hypoxia [8]. As lactic acid breakdown is also a function of liver, concentration of lactic acid may also increase in the condition when liver is severely damaged [9].

So, monitoring L-lactate concentration during medical emergency such as surgery and diagnosis is very crucial. Hartmann's and Lactated Ringer's solution also comprises of lactate which are in regular use in medicine [10]. Many conventional analytical methods for lactate estimation and determination are present which include: colorimetry [11], chemiluminescence [12], high performance liquid chromatography (HPLC) [13] with fluorometry [14] and magnetic resonance spectroscopy [15]. But above methods suffer from one or more drawbacks related to sample pre-treatment, time consuming, less accurate, skilled personals. So, situation demands development of a fast, simple, sensitive, reliable and accurate method for onsite estimation of lactate level.

Biosensors fulfil such requirements, as most attractive candidate. Biosensing method for determination of lactate has many advantages over these routine techniques in terms of reducing time, sensitivity and cost of analysis, inherent miniaturization, not affected by turbidity and length of optical path of sample, requires less power and are highly compatible with present fabrication strategies [16]. Electrochemical and optical lactate biosensors have gained the most significant attention. Several amperometric sensors have been reported based on *lactate dehydrogenase* (LDH) and *lactate oxidase* (LOD) [17,18]. Also, biosensor based on lactate dehydrogenase was developed using potentiometric enzyme electrode [19]. Similarly lactate sensor based on optical property have also been developed using- either fluorometry or (electro) chemiluminescence methods [20].

The present research is focused on the fabrication of L-lactate biosensor using nanomaterial based composite material including LDH, carboxylated multiwalled carbon nanotubes (cMWCNT), methylene blue (MB), Chitosan (CHIT) and single walled carbon nanotubes (SWCNT) deposited on the gold wire (Au-wire). The resulting electrode LDH-cMWCNT-MB/Chitosan/SWCNT-Au was characterized using different techniques, evaluated and further optimized for determining the concentration of L-lactate in blood samples.

2. EXPERIMENTAL METHODS

2.1. Reagents

L-Lactate dehydrogenase (LDH, EC 1.1.1.27, activity 500 U mg^{-1}) from rabbit's muscle, NAD^+, NADH and L-Lactate and sodium dihydrogen phosphate were purchased from SRL Chemicals, Mumbai, India. Chitosan, Glutaraldehyde, Hydrogen tetrachlorourate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate and Methylene blue (MB), Single walled carbon nanotubes (SWCNT) and multi walled carbon nanotubes (cMWCNT) were purchased from Sigma Aldrich Chemicals, India. Graphite powder and paraffin oil were purchased from Qualigens, Thermo Electron LLS India Private ltd. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) were obtained from Sisco Research laboratories, Mumbai, India. Au wire (2-inch length; 1 mm diameter) (23 carat) was purchased from Local market, Rohtak, India. Uric acid, glucose, ascorbic acid, dopamine and acetaminophen were used as a possible interferents and purchased from SRL Chemicals, Mumbai, India. Throughout the experiment all aqueous solutions were prepared using double distilled water (DDW). Phosphate buffer solution (PBS) with different pH was made by using sodium dihydrogen phosphate to prepare different stock solutions of lactate and NADH.

2.2. Instrumentation

Cyclic voltammetry analysis was done using potentiostat (Model PGSTAT 302 N, ECO Chemie, Netherland). The assembly comprised of newly fabricated working electrode, platinum (Pt) wire auxiliary electrode and Ag/AgCl electrode (3 M/KCl) reference electrode. All potentials were measured with respect to the reference electrode. The morphological characterization of AuNPs was carried by transmission electron microscopy (Model JEM-2100F) at Jawaharlal Nehru University, New Delhi, India. Spectrometric measurements were carried out on Shimadzu UV 2450 spectrophotometer at Centre for Biotechnology, Maharshi Dayanand University, Rohtak. X-Ray diffraction (XRD) (Mini Flex Desk Top X-Ray Diffractometer) studies of AuNPs, were carried out at Department of Physics, Maharshi Dayanand University, Rohtak.

The morphological characterization of cMWCNT-SWCNT/AuNPs-Au electrode and LDH-cMWCNT-SWCNT/AuNPs-Au electrodes were carried by Scanning Electron Microscopy (*Zeiss* EVO 40) at Jawaharlal Nehru University, New Delhi, India. FTIR Spectra of working cMWCNT-MB/ Chitosan/SWCNTs-AuNPs-Au in presence of LDH was recorded by Fourier transform infrared (FTIR) spectroscopy (Bruker) at Department of Biotechnology Engineering, University Institute of Engineering &Technology, Maharshi Dayanand University, Rohtak. Ultra-sonication was done on Ultrasonic Liquid Processors of Misonix make (Model: XL-2000). Digital water bath shaker (N.S.W. New Delhi), Digital pH meter

(EUTECH), Centrifuge, Refrigerator (LG), Magnetic stirrer (HICON), Deep freezer (Voltas) and Microwave oven (LG) were also used at CBT (Maharshi Dayanand University, Rohtak).

2.3. Fabrication of LDH-cMWCNT-MB/Chitosan/SWCNT-Au electrode

AuNPs were synthesized in laboratory using chemical reduction method with some modifications [21]. First, 100ml of chlorauric acid ($HAuCl_4.3H_2O$) solution (0.01 wt. %) was heated at 100ºC & stirred continuously. Then, 200 µL of trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$) solution (1 wt. %) was added. Gradual change in color of solution was noticed from yellow to black and then to purple or wine red within 3 min. The solution was then heated for 4-5 mins and allowed to cool at room temperature. Finally, solution was centrifuged, pellets were collected and dried. For fabricating working electrode single-walled carbon nanotube (SWCNT) along with AuNPs and graphite powder in the ratio of 3:3:4 were mixed with paraffin oil to obtain consistent paste. Above mixture was crushed properly for 15 min. Resulting nanomaterial was casted in the plastic tube (1 cm long and 0.4 cm wide) with a fine Au wire for achieving electrical contact. After solidification of paste, plastic tube was carefully removed; this formed core of working electrode. Then, SWCNT/AuNPs-Au core electrode was washed with DDW in order to remove unbound material and stored at 4 ºC.

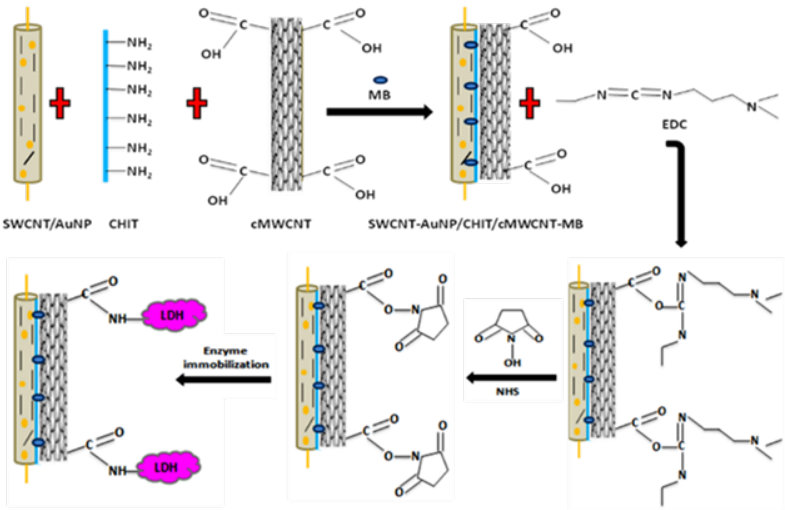

Fig. 1. Schematic illustration of the stepwise fabrication of amperometric Lactate electrode based on LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au system

Aqueous solution of methylene blue (MB) as a mediator was used for adsorption on cMWCNT which resulted in formation of cMWCNT-MB. For this, concentration of 5×10^{-4} mol L^{-1} of MB solution was prepared. To 20 mL of MB solution, 6 mg of cMWCNT was mixed and vortexed for 2 h with intervals. Then, mixture was subjected to filtration followed by washing with DDW (5 times). Further, it was left for drying at 60 ºC for 3 h. Solution of

CHIT with composition 0.25 g in 20 ml of acetate buffer 0.05 M was prepared by stirring for around 3 h at room temperature until completely dissolved. To the above solution CHIT solution cMWCNT-MB was mixed and stirred on a magnetic stirrer at room temperature for 30 min and then sonicated for about 3 h. This resulted in uniform dispersion of CHIT solution on cMWCNT-MB to form cMWCNT-MB/Chitosan solution. Fabricated SWCNT/AuNPs-Au core electrode was immersed into cMWCNT-MB/Chitosan solution while stirring for electrodeposition. After electrodeposition, cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode was obtained. LDH was crosslinked using EDC-NHS chemistry on cMWCNT. For this, fabricated nanomaterial electrode was put in 0.1 M PBS (pH 7.0) having solution of EDC (10 mM) and NHS (10 mM) for 4 h. Excess of EDC and NHS was removed by washing with 0.1 M PBS (pH 6.8). The schematic representation is shown in Fig. 1.

The above electrode was first kept in 1 mg/ml LDH solution in 0.1 M PBS for overnight. This working/LDH was attached to potentiostat for current estimation from LDH reaction. Working electrode was stored in same PBS at 4 $^{\circ}$C until use.

2.4. Characterization of Nano structures and enzyme electrodes

UV-Vis spectroscopy was performed for examining successful synthesis of AuNPs. Absorption spectra were obtained for 1 ml of AuNPs suspension. TEM was used for morphological and structural characterization of synthesized AuNPs. The suspension of AuNPs was prepared in ethanol and homogenized properly for 15 min. Drop from above prepared AuNPs suspension was put on copper grid coated with carbon and left for drying at room temperature. An XRD diffractometer was used to investigate crystal structure of AuNPs. First, sample was crushed properly and pressed into sample holder to get a smooth plane surface and diffraction pattern was recorded over a 2θ range of 30^{o}-120^{o}. The diffractogram obtained was compared to the standard database of International Centre for Diffraction Data (ICDD).

SEM was used for investigating morphology of bare gold (Au) wire, SWCNTs-AuNPs and cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode with and without immobilized LDH enzyme. The electrode was cut into small pieces transferred on specimen stub, and surface morphology examined by SEM. Fabricated electrode LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs was also subjected to FTIR. FTIR Spectra of working electrode was recorded at different stages of fabrication of working electrodes. It was performed for analyzing banding pattern after immobilization of LDH on working electrodes.

2.5. Assembly and electrochemical measurements of LDH biosensors

Amperometric LDH biosensor was constructed by using three electrode systems. This comprised of newly fabricated nanomaterials electrode as working electrode, Pt wire as

auxiliary electrode, Ag/AgCl (3 M KCl saturated) electrode as reference. The electrodes were connected via electrical connections through potentiostat/galvanostat as shown in Fig. 2.

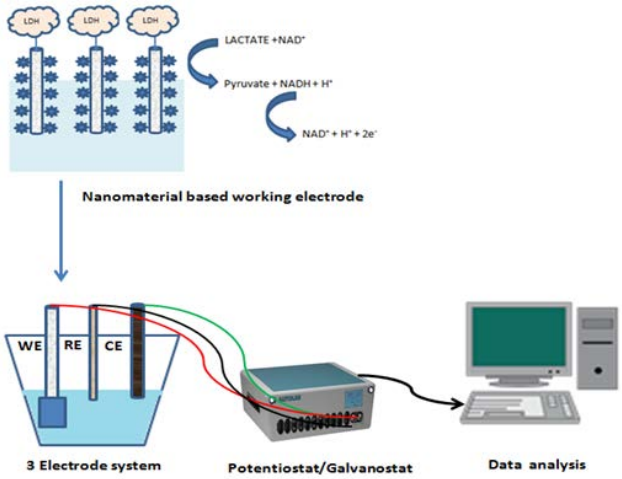

Fig. 2. Assembly of lactate biosensor

For analysis of electrochemical behavior of newly fabricated biosensor, working LDH/cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode was tested amperometrically by cyclic voltammetry at a potential ranging from 0 to 1.2 V versus Ag/AgCl at a scan rate of 30 mV s^{-1}. For testing of activity, 20 ml of 0.1 M phosphate buffer (pH 7) containing 0.2 ml of 5 mM NAD^+ and 0.2 ml of 50 µM of lactate was used. LDH converts L-lactate into pyruvate and NAD. Further NAD was converted into NADH.

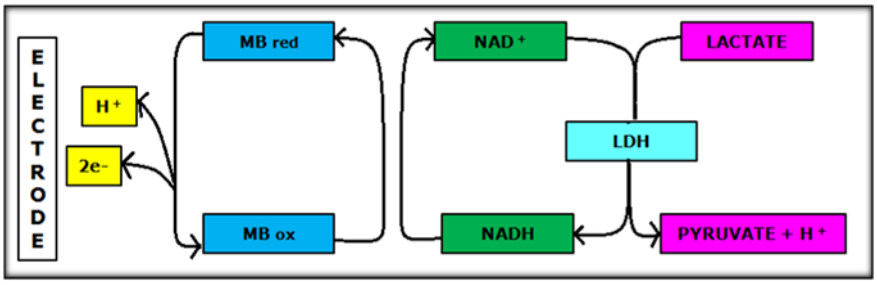

Fig. 3. Strategy for the electrochemical measurement of lactate by immobilized LDH based cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode

Then, complex of NADH and MB+ was formed and its further dissociation resulted to MB in reduced state. The oxidation of MB under applied voltage generates a signal which is proportional to concentration of lactate present in the sample. The strategy is shown in Fig. 3.

2.6. Lactate determination in serum

The blood samples (1ml from each) were collected from healthy (15) and the patients (15) suffering from lactate acidosis. The samples were from Pt. B.D. Sharma, PGIMS, Rohtak and stored at low temperature until used. Lactate concentration was determined by plotting a graph using standard plot between lactate concentration at particular current response. It was determined from the range of 0.09 and 0.54 mg/dl *vs*. current (mA) when the optimal conditions were provided to the biosensor.

2.7. Optimizing working parameters for fabricated biosensor

Optimization of L-Lactate measurements with LDH/cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode was done by analyzing its response with different pH ranging from 6.0 to 10.0 using buffers: sodium phosphate (pH 6.0, 6.5, 7.0 and 7.5) and borate buffer (pH 8.0, 8.5, 9.0, 9.5 and 10) each at 0.1 M final concentration containing 0.1 M KCl. For determination of optimum temperature, reaction mixture was subjected to temperature ranging from 20 ºC to 60 ºC with regular interval of 5 ºC. For analyzing effect of substrate concentration on working of biosensor L-Lactate concentrations from 10 to 120 µM were used. The reaction mixture was subjected to cyclic voltammetry from 0.0 V to 1.2 V with 20 mVs^{-1} scan rates. Effect of incubation time on the response of biosensor was also investigated from 2 to 12 secs with an interval of 2 s. The linearity of biosensor was also determined using lactate concentrations (0 to 120 µM) and NAD^+ cofactor (5 mM).

Lineweaver Burk plot was plotted between reciprocal of lactate concentration (1/S) *vs* reciprocal of current (*1/I*) of enzyme reaction for both electrodes. K_m and I_{max} were calculated from plot using following Michaelis-Menten equation adapted for amperometric biosensor:

$$\frac{1}{1} = \frac{K_m}{I_{max}}\left(\frac{1}{S}\right) + \frac{1}{I_{max}}$$

Whereslope $= \frac{K_m}{I_{max}}; intercept = \frac{1}{I_{max}}$, K_m= Apparent Michaelis-Menten constant I_{max} = Maximum current of enzyme electrode

2.8. Evaluating L-lactate biosensor

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined using linear calibration curves [22]. Analytical recovery was also determined by using newly developed biosensor. Two concentrations of lactic acid 0.5 mg/dl and 1 mg/dl were added to serum samples and lactate in serum was quantified before and by spiking blood samples with lactate. Reproducibility of method was also determined. For this, L-Lactate concentration was

estimated 5 times on same day (within batch) and in same blood samples after gap of a week (between batch). Coefficients of variation (CVs) were determined using present method from means and standard deviations of the readings. In order to determine accuracy of present methods, lactate concentration in 25 blood samples were determined by standard enzymic kit (x) as well as by the present method (y), values obtained by both methods were correlated using regression plot. Electrode was tested for effect of different electroactive species such as ascorbic acid, glucose, glutamate, uric acid, acetaminophen, dopamine, glycine and citric acid. For this, each compound was added at its respective physiological concentration along with lactate in assay mixture. Storage stability of newly developed electrode was determined by storing in PBS and further it was stored in refrigerator when not in use but was in operation for 3 months.

3. RESULTS AND DISCUSSION

3.1. Characterization of Au nanoparticles

The absorbance peak of AuNP was obtained at 519 nm as shown in Fig. 4 which confirmed the successful synthesis of AuNPs. TEM image of gold nanoparticles showed regular perfect shape having diameter of 20 nm which is in nanoscale as shown in Fig. 5. The nanoparticles were homogenous due to minimal difference in electron penetration efficiency on gold. XRD graph for Au nanoparticles had diffraction peaks at 38.4°, 44.6° and 64.7° which were designated to crystal facets of (111), (200) and (220) of gold as shown in Fig. 6. These peaks are specific diffraction peaks of fcc gold crystalline plane and were in agreement with standard XRD data (JCPDS Card Number 00-001-1172).

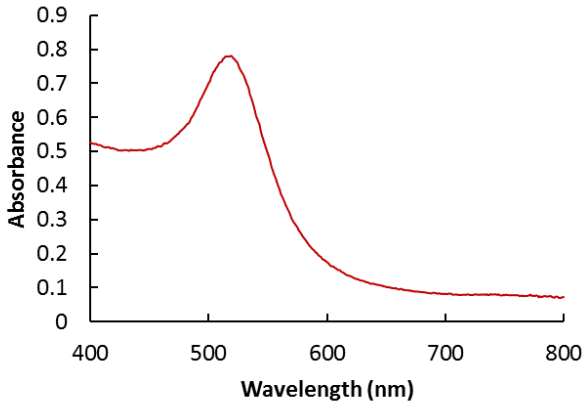

Fig. 4. The UV-Vis spectrum of gold nanoparticles

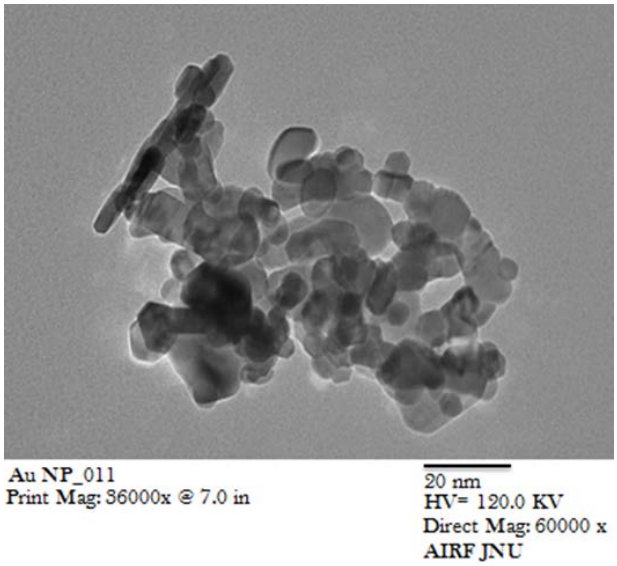

Fig. 5. The TEM image of synthesized gold nanoparticles

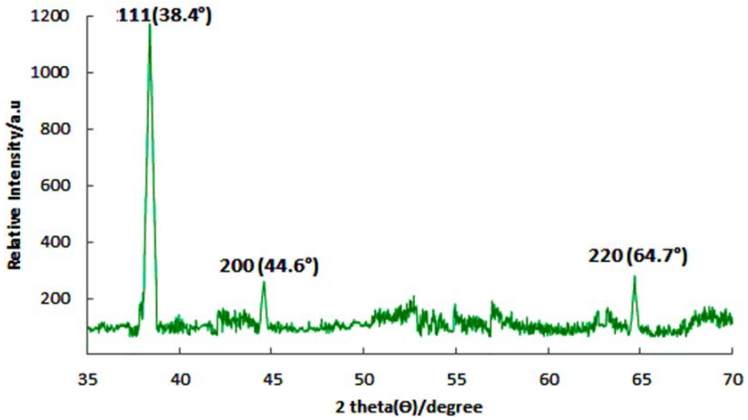

Fig. 6. The XRD spectrum of gold nanoparticles

3.1.1. Fabrication and characterization of core electrode

The morphological characterizations of fabricated working electrode with bare Au wire, SWCNT/Au, with complete electrode without LDH enzyme and working electrode with LDH were done by SEM and are shown in Fig. **7**(a), Fig. **7**(b), Fig. **7**(c) and Fig. **7**(d) respectively. A change in morphology has been observed when the SWCNT/AuNP paste was applied on Au wire (Fig. **7**b). Electrode had clouded morphology due to mixture of SWCNT and AuNP. Fig. **7**c displays SEM image of final electrode without enzyme and cMWCNT-MB was apparently visible, specially rod-like surface morphology due to cMWCNT was clear. On immobilization of LDH (Fig. **7**d), surface appearance changed to a globular structural morphology. This was then used as final working electrode.

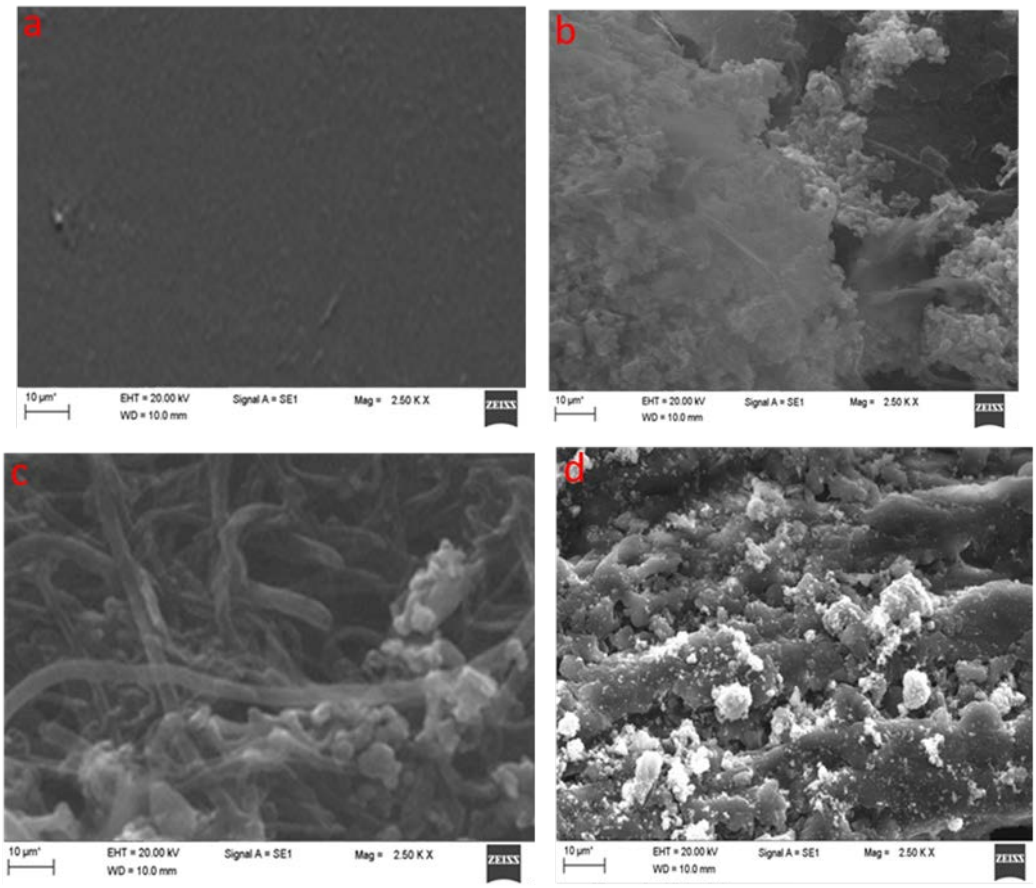

Fig. 7. SEM images of (*a*) Au wire; (*b*) SWCNT/AuNP layered Au wire; (*c*) cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au electrode, and (*d*) final electrode with immobilized LDH.

3.2. FTIR and CV analysis of newly fabricated electrode

FTIR Spectra of working electrode was recorded during fabrication at room temperature in the range of 1000-4000 cm^{-1} as shown in the Fig. 8. The deposited surface complex i.e. LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs was scraped off from working electrode. Dry potassium bromide (KBr) was mixed with scraped material, grinded properly and mixture was pressed with mechanical press. Then, resulting powder was kept in sample holding socket of Bruker FT-IR spectrophotometer and spectrum was recorded. Graph represents FTIR spectra of cMWCNT-MB/Chitosan/SWCNTs-AuNPs complex in presence of LDH. Peak at 1250 to 2000 cm^{-1} in graph corresponds to vibration band on (-C-H) stretching vibrations peak. Further, additional peaks at 1450 cm^{-1} (aromatic C-C stretch), 1650 cm^{-1} (N-H stretch) and 2970 cm^{-1} (aromatic C-H bending) appeared due to coupling of LDH on cMWCNT/SWCNT-Au electrode.

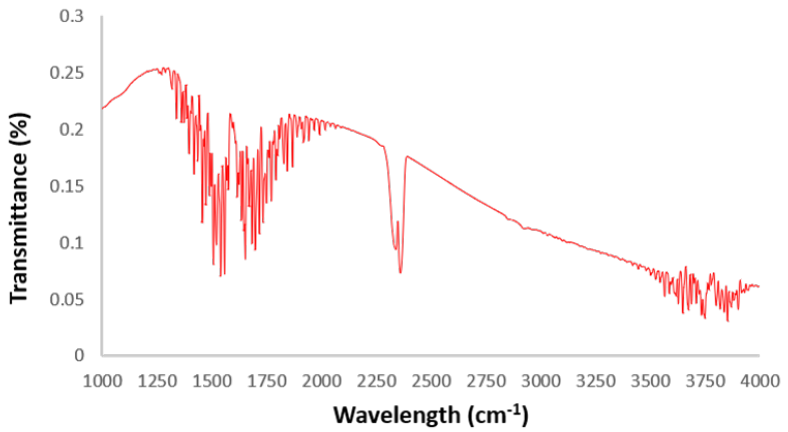

Fig. 8. The infra-red spectra of cMWCNT-MB/Chitosan/SWCNTs-AuNPs modified Au with immobilized LDH

Cyclic voltammogram technique was also used to evaluate charge transfer properties of prepared electrode. The reaction mixture for cyclic voltammetry comprised of phosphate buffer (20 ml of 0.1 M, pH 7) with NAD^+ (0.2 ml of 5mM) and spiked with of lactate (0.2 ml of 100 µM). No voltammetric response was observed for base electrode (Fig. 9a). Voltammetric response of cMWCNT-MB/Chitosan/ SWCNTs-AuNPs modified Au electrode (Fig. 9c) and final electrode (Fig. 9b) are displayed. An oxidation peak was displayed at 0.66V. Immobilization of enzyme lead to a slight decrease in current here also due to decline in redox process at interface of fabricated electrode resulting as a result of slow electron transfer rate, this proves that LDH is successfully immobilized.

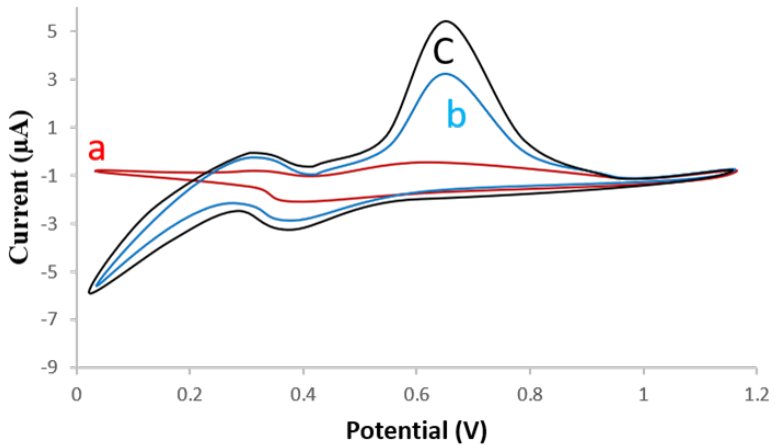

Fig. 9. The cyclic voltammogram of (a) base electrode, (b) without enzyme and (c) LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

3.3. Optimization of the working electrode

For this study, current response was measured at different pH from 6.0 to 10.0 using the following buffers: sodium phosphate (at pH ranging from 6.0 to 7.5 with increment of pH 0.5) and borate buffer (pH ranging from 8.0 to 10.0 with same increment) each at 0.1 M final concentration containing 0.1 M KCl. Deviation in response current has been observed from the pH ranging from pH 6.0 to pH 8.0 with maximum current at pH 7.5 as shown in Fig. 10. The biosensor was not found active at pH higher or lower than reported pH. Further, this pH was selected for experiment.

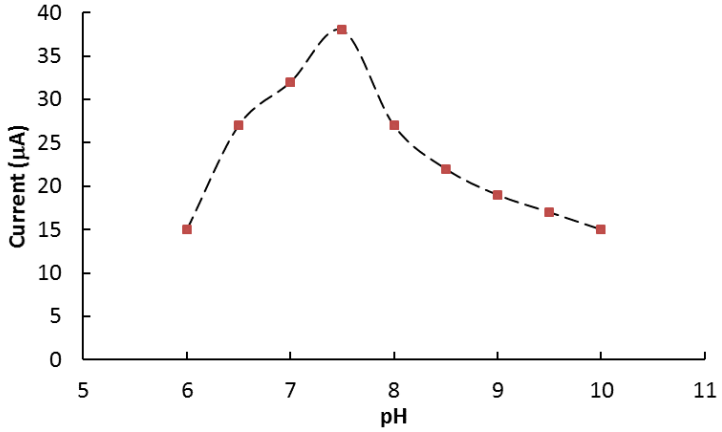

Fig. 10. Effect of pH on LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

The optimum temperature was 30^{o}C for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode as shown in Fig. 11. LDH activity decreased strongly on both sides of these optimum temperatures were selected for subsequent assays.

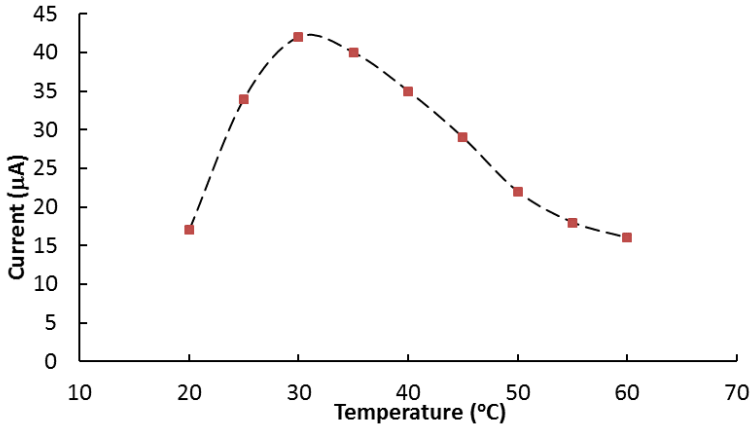

Fig. 11. Effect of temperature on LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

Response time was studied from 2 to 12 s with interval of 2 s. Optimal activity was recorded at 8 seconds for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode after which a decrease in response was observed as shown in Fig. 12.

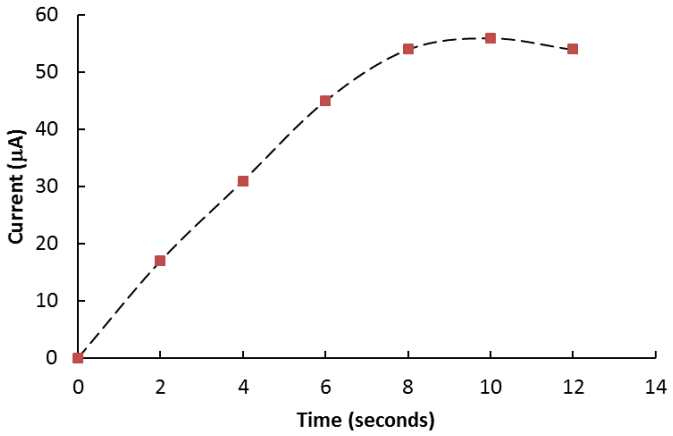

Fig. 12. Effect of time of incubation on LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

Eelectrochemical responses of the biosensor and oxidation current increased with increase in lactate concentrations was shown in Fig. 13.

The apparent Michaelis–Menten constant for immobilized LDH were calculated from LB plot as shown in Fig. 14. The values were determined by extrapolating graph to negative x-axis in plots of reciprocals of current *vs*. triolein concentration. App K_m value was found to be 66.7 µM for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode, which showed electrode had higher affinity for substrate. At the same time, app I_{max} of electrode was 153.8 µA.

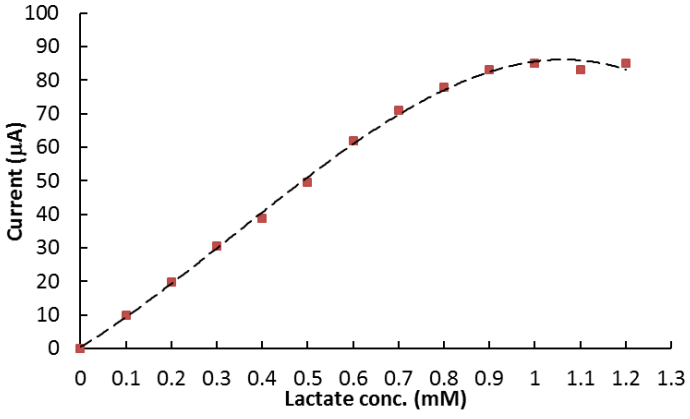

Fig. 13. Effect of substrate concentration on LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

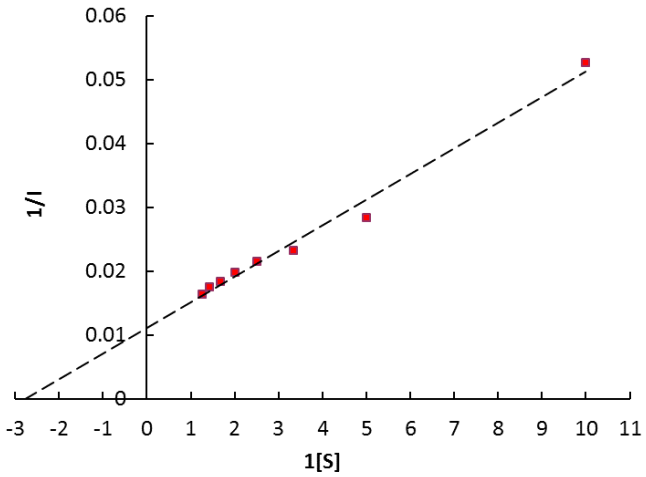

Fig. 14. Lineweaver-Burk plots of effect of substrate concentration on LDH- cMWCNT-MB/Chitosan/SWCNTs-AuNPs

3.4. Evaluation of the working electrode

The LOD and LOQ values were calculated for LDH-cMWCNT-MB/Chitosan/ SWCNTs-AuNPs working electrode, LOD and LOQ were 0.015 mM and 0.05mM respectively.

The accuracy of present method was determined using the spiked s era samples by L-lactate and their analytical recoveries were monitored. The mean of analytical recoveries was 97.57% and 94.92% for the spiked concentrations of 0.5 mg/dl and 1.0 mg/dl respectively. The results obtained using LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode are presented in Table 1.

Table 1. Analytical recoveries of added lactate in serum samples determined by lactate biosensor

Lactate Spiked (mg/dl)	**(LDH-cMWCNT-MB/ Chitosan/SWCNTs-AuNPs)-Au wire**	
	Lactate Recovered (mg/dL)	**% Recovery**
-	1.56	-
0.5	2.01	97.57±0.47
1.0	2.43	94.92±0.3

Mean and standard deviation were used for determining the CVs which represents precision of developed method. CVs for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode were 2.23% (within batch) and 2.99% (between batch) as presented in Table 2. Accuracy of the method was evaluated by determining serum lactate determined in 22 serum samples using both standard enzymic kit and present method. There was a good

correlation of enzymic kit with r=0.9989 for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode (y=0.9732x+0.0223) as shown in Fig. 15.

Table 2. Within and between batch assay for determining serum lactate level

N	Lactate (mg/dl)	CV (%)
Within assay (5) 1.85 1.80 1.78 1.74 1.84	1.802±0.04	2.23
Between assay* (5) 1.87 1.91 1.98 2.04 1.96	1.952±0.058	2.99

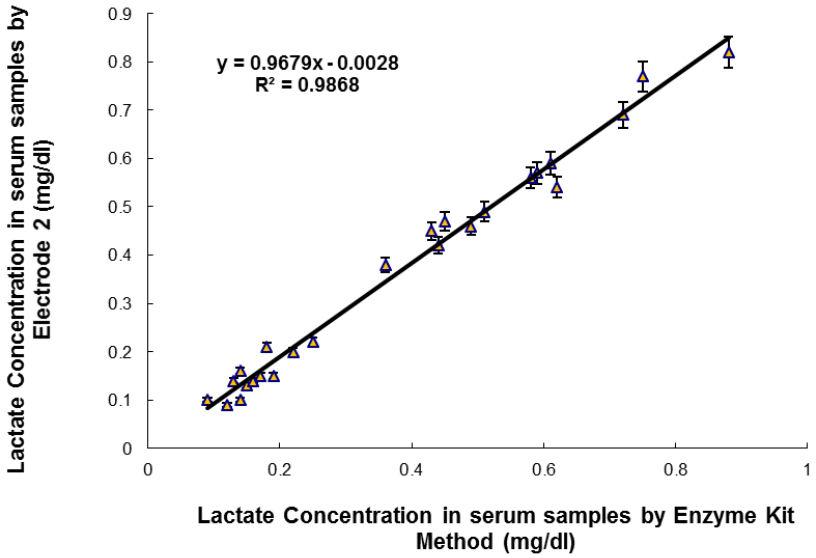

Fig. 15. Regression plot of serum lactate estimation by enzyme kit method (x) and LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs (y)

Effect of different electroactive species including glucose, acetaminophen, ascorbic acid, uric acid, glutamate, dopamine, glycine and citric acid on the working of nanomaterial-based working of electrodes. A minor decrease had been observed in activity of working electrode with addition of electroactive species. An Interference of 7-10% had been observed using LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs as shown in Fig. 16.

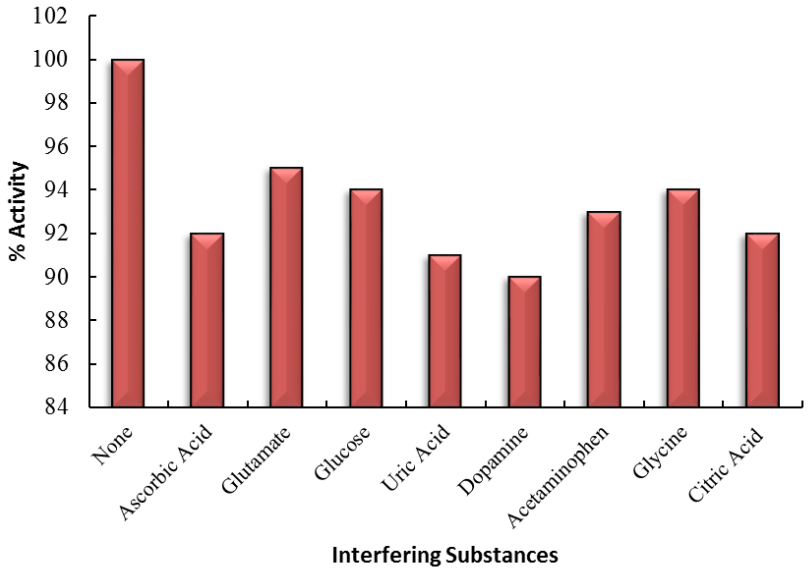

Fig. 16. Interference studies on estimation of lactate by LDH-cMWCNT-MB/Chitosan/ SWCNTs-AuNPs

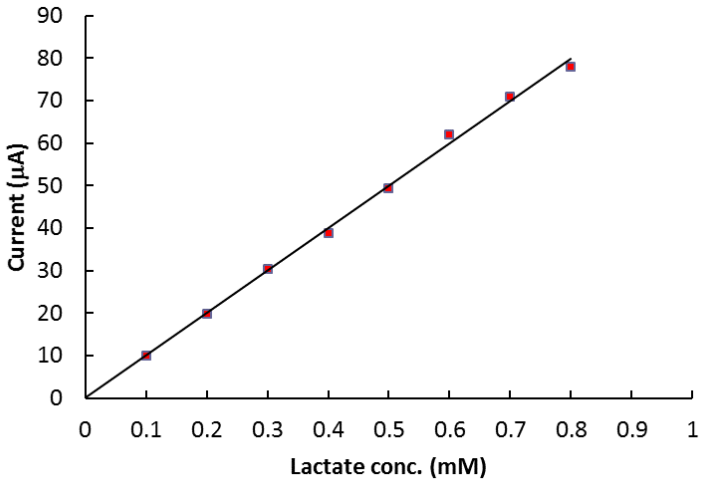

Fig. 17. Standard curve of lactate by LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs

3.5. Determination of serum lactate

For estimation of serum lactate, standard graph was prepared for electrode as shown in Fig. 17. Serum lactate levels in healthy persons as analyzed by present method from the range of 0.5±.09 mM to 1.07±.05 mM for LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs working electrode are presented in Table 2, which was in established normal range (0.5 to 1.0 mM). The lactate levels in sera of lactic acidosis sufferers were from 5.8±.04 mM to 10.5±.02 mM for LDH-cMWCNT-MB/Chitosan/ SWCNTs/AuNPs working electrode are presented in Table 3. These levels matched with known concept of >5 mM blood lactate in lactic acidosis condition.

Table 3. Serum lactate level of healthy and lactic acidosis persons measured using LDH-cMWCNT-MB/ Chitosan/SWCNTs-AuNPs-Au electrode

Sex (n=15)	**Lactate acidosis patients (mM) (Mean ± S.D.)**	**Sex (n=15)**	**Healthy persons (mM) (Mean ± S.D.)**
M	10.5± .02	M	0.7±.01
M	8.5±.07	F	1.06±.03
F	7.2±.02	F	0.67±.03
M	8.1±.01	M	0.47±.06
F	7.6±.08	F	0.91±.08
M	8.5±.07	F	0.5±.09
M	9.1±.03	M	0.75±.05
F	9.8±.05	M	1.0±.05
M	6.3±.03	M	0.72±.05
F	5.8±.04	F	0.87±.05
M	6.8±.05	M	0.93±.02
F	7.3±.04	M	0.85±.06
F	8.1±.07	F	1.07±.05
M	9.2±.05	F	0.77±.01
M	8.4±.06	M	0.69±.03

3.6. Storage stability

The storage stability was investigated by using the newly fabricated electrode for determination of electrochemical response to detect L-lactate analyte in the sera. This analysis was done for 3 months at regular intervals of 10 days. The LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs nanocomposite-based electrode retained its 50% activity during this period as shown in Fig. 18. The developed biosensor was also compared with the earlier reported methods in Table 4.

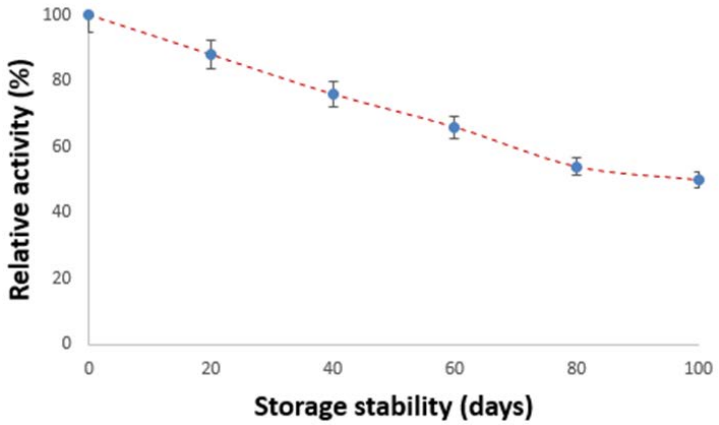

Fig. 18. Storage stability of LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs used for serum lactate estimation

Table 4. Comparison of LDH-cMWCNT-MB/ Chitosan/SWCNTs-AuNPs-Au electrode-based biosensor with the earlier reported methods

Electrode	**pH**	**Temperature**	**Response Time**	**Km value**	**LOD**	**Storage Stability**	**Ref.**
MWCNT/Chitosan	7.4	NR	3s	NR	0.76 µM	7 days	[17]
Ferrocene methanol/laponite/chitosan hydrogel	7.0	20 °C	5s	3.8 mM	3.8 µM	30 days	[18]
Carbon paste electrode	6.8	35°C	NR		7.5 µM	21 days	[23]
Au/NanoZnO	7.4	NR	1s	0.38 µM	4.73 nM	NR	[24]
NanoCeO2/GCE	7.4	35°C	4s	1.536 mM	NR	12 days	[25]
Epoxy-graphite composite	7.3	35°C	NR		0.87 µM	7 days	[26]
E-GrCE modified ZnO/Au		35°C					[27]
ZnO/Au	7.3	35°C	5s	32 mM	0.1 µM	NR	[28]
PPY-PVS	7.2	40ºC	NR	1.9 mM	NR	NR	[29]
Sol-gel derived from MPTS (merceptopropyl trimethoxysilane)/ AuNPs	7.2	NR	2-3 s	NR	100 nM	NR	[30]
Fe3O4/MWCNT/GC	7.5	37 °C	5 s	95µM	5 µM	2 weeks	[31]
AuNPs anchored on reduced graphene oxide (RGO)-modified SP electrode	7.5	35 °C	8 s	NR	0.13 µM	NR	[32]
PAA/Si_4N_3 nano-structured	7.5	37 °C	NR	NR	0.2 µM	25 days	[33]

surface							
pTTCA/MWCNT/Au		37 ^{o}C	10s		550 µM	17 days	[34]
PCS hydrogel on teflon/Pt	7.5	23 ^{o}C	2s	NR	4.3 µM	11 days	[35]
MWCNT/ P3MT polymer/GCE	7.4	NR	5s		0.56µM	15 days	[36]
PPY-PVS	7.2	40 ^{o}C	40 s	4.5 mM	100 µM	2 weeks	[37]
pTTCA/ MWCNT based conducting polymer modified gold working electrode	6.8	30 ^{o}C	15 s	NR	1 µM	25 days	[38]
Pt-black nanoparticles and Ferricyanide mediator	NR	37 ^{o}C	50 s	NR	0.4 U µL^{-1}	7 days	[39]
Sol-gel silica material of TEOS, APTES and TASPGA	8.4	21-25 ^{o}C	NR	0.77 mM	1.5µM	7 days	[40]
SWCNTs/Variamine blue/SPE	7.5	NR	NR	NR	1 µM	NR	[41]
PEI/NAD^{2+}/ carbon paste	7.0	25 ^{o}C	NR	5.6 mM	30 mM	31 days	[42]
CA membrane/Meldola Blue	8.0	32 ^{o}C	NR	NR	8.0×10^{-3} mol dm^{-3}	3 days	[43]
Dialysis membrane trapped enzymes	7.4	20 ^{o}C	NR	NR	3.1×10^{-9} gL^{-1}	9 days	[44]
LDH-cMWCNT-MB/Chitosan/SWCNTs-AuNPs	7.5	30 ^{o}C	8 s	66.7 µM	0.015 mM	3 months	Present Method

*NR (Not Reported)

4. CONCLUSION

In present research L-lactate biosensor has been successfully developed for determining concentration of lactate in blood samples. Combination of nanomaterial used has enhanced the electrocatalytic activity due to synergistic action of MWCNTs, SWCNTs and Au nanoparticles. This resulted in breakdown of substrate by LDH enzyme at very low potential. The developed biosensor is also reusable due to covalent immobilization of enzyme with electrode and has enhanced storage stability of electrode. There is almost negligible effect of the interfering compounds and other electroactive species. The method showed a good correlation when compared with standard enzyme kit method. The biosensor works to a wide

range of linearity and also possess low detection limit which can even determine very low concentration of analyte lactate in the blood samples. The response time is also very low. The newly fabricated biosensor does not require any sample preparation for determining the serum concentration. The developed sensor can be used for on-site screening of the samples after its miniaturization. This combination of nanomaterials can also be used to immobilize other enzymes for development of analytical electrochemical biosensors for human welfare.

Conflict of interest

The authors declare no Conflict of Interest in publication of this article.

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