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Covalent Immobilization of Lactate Oxidase onto Zirconia Coated Silica Nanoparticles/Chitosan Hybrid Film for Amperometric Determination of Lactate

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Abstract

An improved amperometric L-lactate biosensor was constructed based on covalent immobilization of lactate oxidase (LOx) onto zirconia coated silica nanoparticles (SiO_2@ZrONPs)/chitosan (CHIT) hybrid film electrodeposited on the surface of a gold electrode (AuE). The enzyme electrode was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS), while SiO_2@ZrONPs were synthesized by chemical reduction method and characterized by transmission electron microscopy (TEM), UV spectroscopy and X-ray diffraction (XRD). The biosensor showed an optimal response within 3s at pH 7.5 in 0.05M sodium phosphate buffer and 30°C, when operated at 20 mVs^{-1}. The biosensor had a low detection limit of 0.2 nM with a wide working / linear range between 0.1 – 4000 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 (5.0 mM and 10.0 mM)) in plasma was 99% and 96.6% respectively. Within- and between-batch coefficients of variations were 1.79% and 2.89% respectively. There was a good correlation (R^2=0.99) between plasma lactate values as measured by standard enzymatic spectrophotometric method and the present biosensor. The enzyme electrode was used 160 times over a period of 120 days, when stored dry at 4°C.

Keywords: Lactate biosensor; Lactic acid; Lactate oxidase; Chitosan; Zirconia coated silica nanoparticles; Gold electrode

Abbreviations: LOx: Lactate Oxidase; SiO2@ZrONPs: Zirconia Coated Silica Nanoparticles; CHIT: Chitosan; AuE: Gold electrode; CV: Cyclic Voltammetry; SEM: Scanning Electron Microscopy; FTIR: Fourier Transform Infrared Spectroscopy; EIS: Electrochemical Impedance Spectroscopy; TEM: Transmission Electron Microscopy; UV: Ultra Violet Spectroscopy; XRD: X-ray Diffraction; CVs: Coefficient of Variation

Introduction

L-lactate, a biochemical compound produced from pyruvate in muscles, liver and kidney due to insufficient supply of oxygen [1]. The normal range of L-lactate in blood 0.5-2.5 mM. Reliable determination of L-lactate is important in food technology, fermentation and wine industries as well as clinical chemistry and sport medicine [2]. L-lactate concentration in blood is essential for the diagnosis of patient conditions in intensive care and during surgery [3]. An elevated lactate level in blood is a major indicator of ischemic conditions of the respective tissue. The ischemic situation can be caused by all types of shock, suffocation and respiratory insufficiency. Another reason for an altered lactate level is a disturbed lactate metabolism, which may be caused by diabetes. In sports medicine, blood lactate levels during exercise are an indicator for training status and fitness [4-6]. During the demand of high intensity exercise, the cell utilizes a substantial amount of glycogen and glucose. The product of the anaerobic glucose breakdown is lactate. The increase of lactate level coincides with an increase in blood and muscle acidosis. Therefore, lactate is an excellent indirect marker of cellular fatigue. Lactate determination is considered to be the single most important determinant of success in endurance related activities [3].

Various chromatographic and spectrophotometric techniques have been reported for the determination of L-lactate. However, these methods are time-consuming, costly, and non-selective or need large sample quantities. Electrochemical enzyme bio-sensing is an alternative candidate, which is inexpensive, rapid, well selective and reliable for L-lactate detection. Biosensor employing methods employing lactate oxidase (LOx) or lactate dehydrogenases (LDH) have been reported [7]. However, LDH based biosensors have problems such as reversibility of its reaction, which interfere in the accurate measurement of lactic acid and involvement of two enzymes (LDH and NADH oxidase), difficulty in oxidation of coenzyme (NADH or NADPH) and generation of low current, hence LOx based biosensors are preferred. Further LOx has been 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 follows:

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

$$H_2O_2 \xrightarrow{high\ voltage} 2H^+ + O_2 + 2e-$$

A number of LOx based amperometric biosensors for lactate have been reported [7]. However, these biosensors showed poor electrical response, low sensitivity, narrow working range and low storage stability. Recently, nanomaterials have been used to improve the analytic performance of biosensors. Coated nanoparticles are the particles containing a core and a shell, with the dimensions in nanometer scale. Composite nanoparticles show the improved physical

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and chemical properties over their single-component counterparts, and therefore, are potentially useful in a wide range of applications. These nano-composite show a number of novel properties e.g., optical, electrical, catalytic, magnetic, and mechanical, besides providing large effective surface area. Zirconia coated silica nanoparticles (SiO_2@ ZrONPs)have been employed as an efficient catalyst and electron transfer mediator in enhancing the current response and thus increasing the sensitivity of bilirubin biosensor. The use of SiO_2@ ZrONPs has also lowered the working potential of this biosensor, as it also provided an environment for the enhanced electrocatalytic effect and fast electron-transfer rate [8]. Chitosan (CHIT) is an important biopolymer for immobilization of biomolecules, due to its excellent film forming ability, high permeability, mechanical strength, non-toxicity, biocompatibility, low cost and easy availability. Further, -NH_2 groups of CHIT provides hydrophilic environment for the biomolecules [9]. The use of SiO_2@ZrONPs and CHIT provides a synergetic effect on electocatalytic oxidation of H_2O_2, which contributed to the excellent performance of the biosensor [8]. Recently, we have reviewed a number of nanocomposites based lactate biosensors [7], but none of them has employed SiO_2@ZrONPs. We describe herein a novel approach of immobilizing lactate oxidase onto SiO_2@ZrONPs/CHIT modified Au electrode, for improved amperometric determination of lactate in plasma.

Material and Methods

L-Lactate oxidase (LOx from *Pediococcus* species), (L-0638, LOx 100 units/mg) from Sigma Aldrich USA, tetraethylorthosiicate (TEOS) from Fluka, Mumbai, India and chitosan, zirconia oxide nanoparticles (ZrONPs) from SISCO Research Laboratory, Mumbai, India were used. Au wire (2 mm × 2 cm) from local market. 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 Pt. BDS Postgraduate Institute of Medical Sciences.

Assay of free LOx

The assay of free L-lactate oxidase was carried out described by Satyapal and pundir (1993) with modifications [10]. The assay 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 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°C 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°C in dark for 30 min to develop the color. A520 was read and H_2O_2 concentration was interpolated from its standard curve (A520 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.

Preparation of SiO_2@ZrONPs

SiO_2@ZrONPs was prepared by the precipitation method [11]. To a mixture of TEOS (2.0 ml) and ethanol (20 ml) in a 50 ml beaker, NH_4OH (4.0 ml) was added dropwise. The mixture was stirred for 8 h and centrifuged at 4000 rpm for 5 min. The white colored silica oxide nanoparticles (SiO_2NPs) generated were dispersed into DW (10 ml). ZrONPs (200 μL) suspension was added to this SiO_2NP suspension and its pH was brought to pH 10 - 10.5, by adding NH_4OH. The zirconia coated silica nanoparticles (SiO_2@ZrONPs) that were formed, were kept at 40°C for drying.

Characterization of SiO_2@ZrONPs

The characterization of zirconia coated silica nanoparticles were characterized by taking their images in transmission electron microscope (TEM), at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU), New Delhi, India on commercial basis, recording their UV spectra in UV-visible spectrophotometer (Dynamica HALO DB-20, UK) and X-ray diffraction (XRD) pattern in X-ray diffractometer at National Physical Laboratory (NPL), New Delhi.

Electro-deposition of SiO_2@ZrONPs/CHIT hybrid film onto Au electrode

The surface of Au electrode was polished with alumina slurry (diameter 0.05 μM), followed by regular washing with DW and sonication in ethanol to remove adsorbed particles and finally washing with DW to remove ethanol. The cleaned electrode was dipped into 25 ml 1 M KCl containing chitosan (0.2%, 200 μL) and SiO_2@ZrONPs suspension (200 $_{\mu L}$) and subjected to 20 successive deposition cycles at -0.2 to 0.4 V using a potentiostat galvanostat. The resulting SiO_2@ZrONPs/CHIT modified Au electrode was washed thoroughly with DW to remove unbound matter and kept in a dry Petri-plate at 4°C.

Immobilization of LOx onto SiO_2@ZrONPs/CHIT modified Au electrode

SiO_2@ZrONPs/CHIT modified Au electrode was immersed into 1 ml of 2.5% glutaraldehyde in 0.1 M Tris–HCl buffer (pH 8.5), kept at room temperature for 7 h, washed thoroughly with DW until the pH of washing discard was 7.0, dipped into 1.5 ml of LOx solution and kept overnight at room temperature (30°C) for immobilization. The resulting electrode with immobilized LOx was washed 3-4 times with 0.1 M Tris–HCl buffer (pH 8.5) to remove residual unbound protein. The resulting LOx/SiO_2@ZrONPs/CHIT/Au electrode was used as working electrode and stored at 4°C, when not in use. This working electrode was characterized by SEM at different stages of its construction.

Scanning electron microscopy of enzyme electrode

The SEM images of bare Au electrode and SiO_2@ZrONPs/CHIT/ Au electrodes were taken in a scanning electron microscope (Zeiss EV040) at JNU, New Delhi, on commercial basis.

Construction and testing of L-lactate biosensor

Cyclic voltammetry (CV) of LOx/SiO_2@ZrONPs/CHIT/Au electrode was recorded in a potentiostat-galvanostat from -0.2 to 0.4 V versus Ag/AgCl as reference and Pt wire as counter electrode in 25 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1 ml of 10 mM lactic acid.

Optimization of L-lactate biosensor

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time and substrate (lactic acid) concentration on biosensor response were studied. To determine optimum pH, the pH of reaction buffer was varied from pH 6.0 to 9.0 at an interval of pH 0.5 using the following buffer, each at a final concentration of 0.1 M: pH 6.0–7.5, sodium phosphate buffers and pH 8.0–9.0, Tris–HCl buffer. Similarly to determine optimum temperature, the reaction mixture was incubated at different temperatures (15°C to 45°C). The effect

of substrate concentration on biosensor response was determined by varying the concentration of L-lactate in the range 0.1 μM – 6000 μM.

Application of L-lactate biosensor in plasma

Blood samples (1 ml each) were drawn from apparently healthy males and females and persons suffering from lactoacidosis at Pt. BDS PGIMS, Rohtak hospital and centrifuged at 1500 rpm for 15 min and their supernatants (plasma) were collected. L-lactate level in plasma was determined by the present biosensor method, as described for its response measurement, under its optimal working conditions except that L-lactate was replaced by plasma. The L-lactate content in plasma was interpolated from standard curve between L-lactate concentration vs. current in microampere prepared under optimal assay conditions of LOx/SiO_2@ZrONPs/CHIT/Au enzyme electrode.

Evaluation of L-lactate biosensor

Evaluation of the lactate biosensor was done by calculating its limit of detection (LOD), % analytical recovery, precision and correlation with the standard enzymatic spectrophotometric method. LOD was calculated using the following formula:

LOB = 3.3 × *SD*/*S*

Where SD = Standard deviation of the response, S = slope of calibration curve [12].

To study analytical recovery, known amount of lactic acid was added into plasma at two levels, 10 μM and 20 μM and measured the lactate level in the plasma before and after addition of exogenous lactic acid. Percent analytical recovery of added lactate was calculated To study the precision, lactic acid level in 5 plasma samples was measured six times on the same day (within batch) and in the same samples after their storage at -20°C for a week (between batch) by the present biosensor. Both within and between batches coefficients of variation (CVs) in plasma lactate levels were calculated as follow:

$$CVs = \frac{SD \times 100}{mean}$$

To study the accuracy of present biosensor, lactic acid content in 15 plasma samples was determined by the present lactate biosensor (y) and compared with those by standard enzymic spectrophotometric method using LDH based on measurement of NADH at 340 nm in an alkaline medium. Plasma lactate values obtained by both the methods were correlated by regression equation and correlation coefficient (R^2) was calculated using the following formula [13,14].

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

Storage stability of LOx/SiO_2@ZrONPs/CHIT/Au electrode

To study the storage stability of the enzyme electrode, its activity was tested weekly for 4 months during its storage at 4°C in dry condition.

Results and Discussion

Characterization of SiO_2@ZrONPs

The typical TEM images of SiO_2@ZrONPs nanoparticles (Figure 1a) showed the spherical shape of SiO_2@ZrONPs nanoparticles with an average size of 20 nm. Figure 1b shows UV and visible spectra of SiO_2@ZrONPs hybrid film with a strong absorbance peak at 230 nm, confirming the synthesis of SiO_2@ZrONPs. The XRD patterns of the

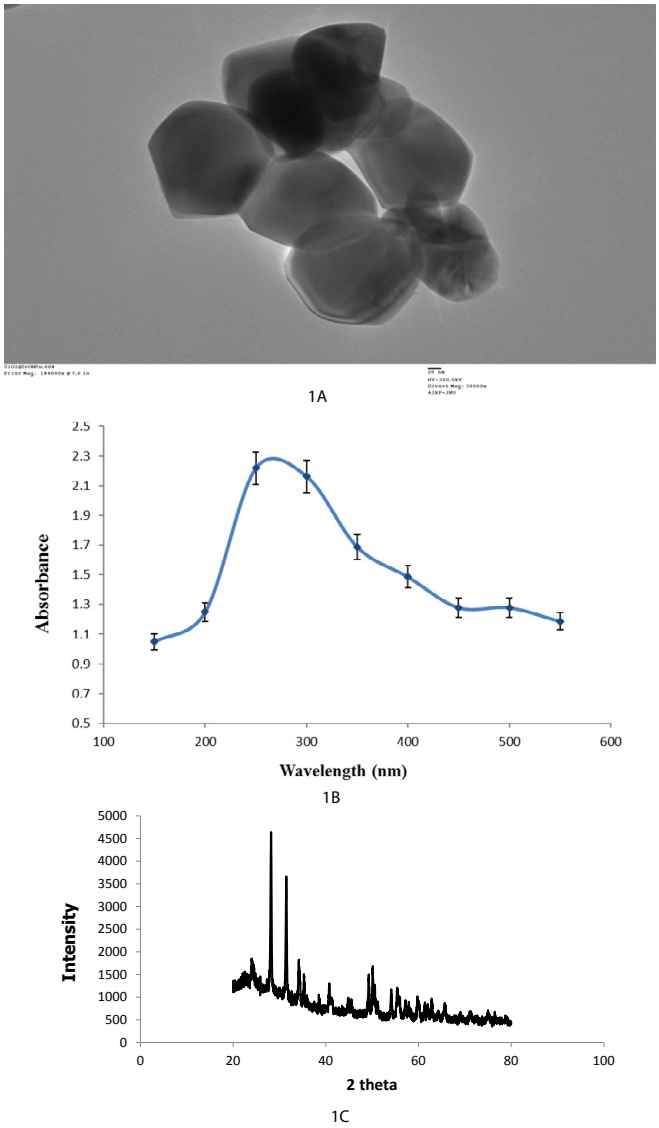

Figure 1: (a) Transmission electron microscopic (TEM) images of SiO_2@ZrONPs, **(b)** UV spectrum of SiO_2@ZrONPs and **(c)** X -ray diffraction (XRD) pattern of SiO_2@ZrONPs.

prepared SiO_2@ZrONPs (Figure 1c) exhibited the characteristic peak of SiO_2@ZrONPs corresponding to the presence of monoclinic phase. The XRD patterns of the SiO_2@ZrONPs clearly showed the characteristics peaks of SiO_2@ZrONPs at 28.2°, 31.4° and 34.1°, corresponding to Miller Indexes. These observations confirmed resultant particles were pure SiO_2@ZrONPs. No characteristic peaks of impurities were observed, revealing the high purity of SiO_2@ZrONPs.

Characterization of the enzyme electrode at various stages of its fabrication

By SEM: The SEM images of the surface of bare Au electrode, SiO_2@ZrONPs/CHIT/Au electrode and LOx/SiO_2@ZrONPs/CHIT/Au electrode are shown in Figures 2a, 2b and 2c respectively. The stepwise modification of the electrode could be seen clearly from these SEM images. The SEM image of the bare Au electrode showed a smooth and featureless morphology (Figure 2a). The SiO_2@ZrONPs/CHIT/Au composite film exhibited a net structure. The film is more uniform

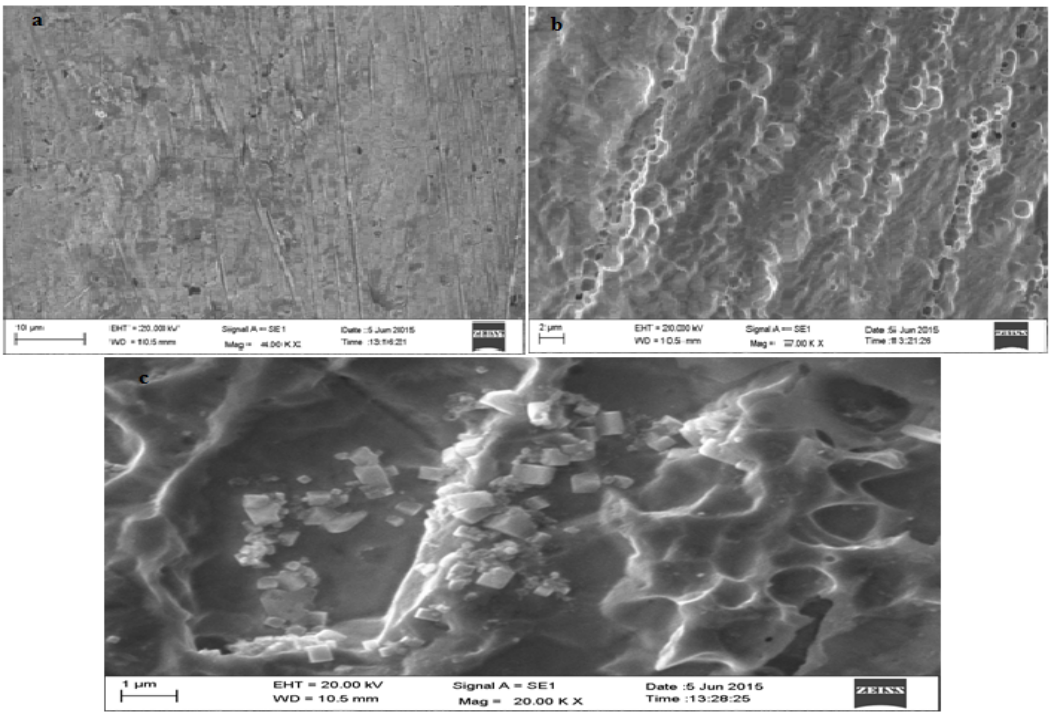

Figure 2: SEM images of **(a)** bare Au electrode **(b)** CHIT/SiO_2@ZrONPs electrode and **(c)** LOx/CHIT/SiO_2@ZrONPs electrode

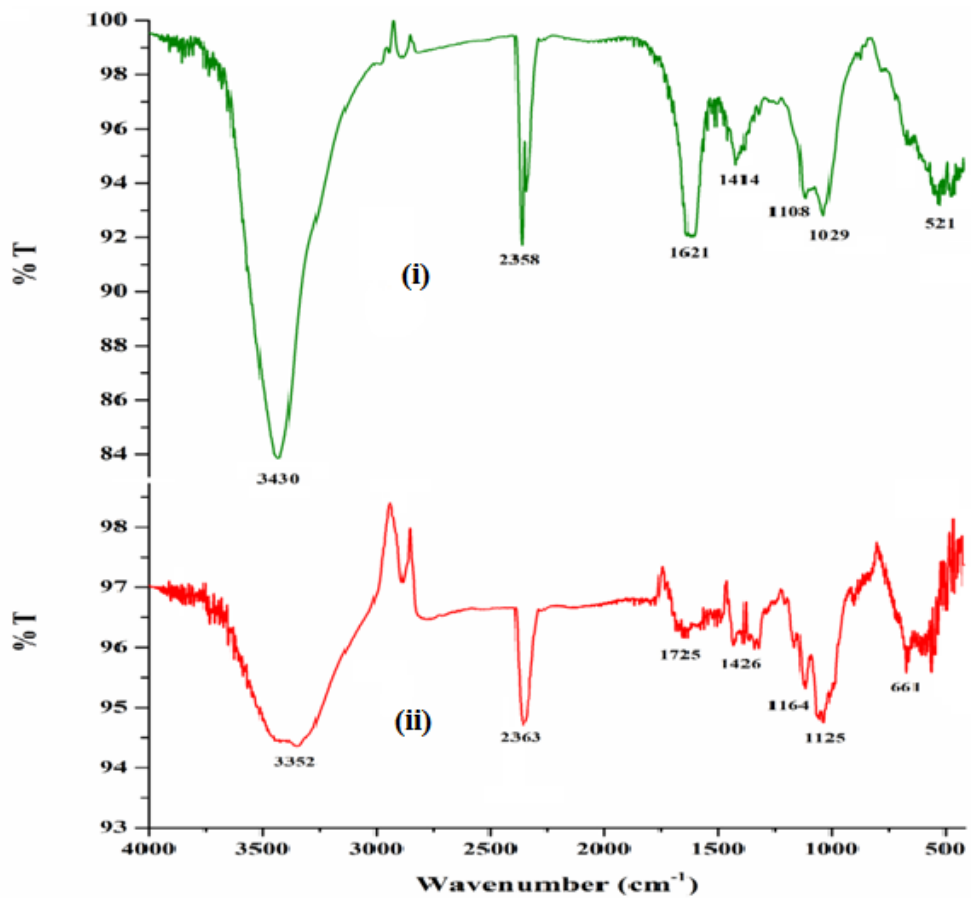

Figure 3: FTIR spectra of **(i)** CHIT/SiO_2@ZrONPs/Au electrode, **(ii)** LOx/CHIT/SiO_2@ZrONPs/Au electrode.

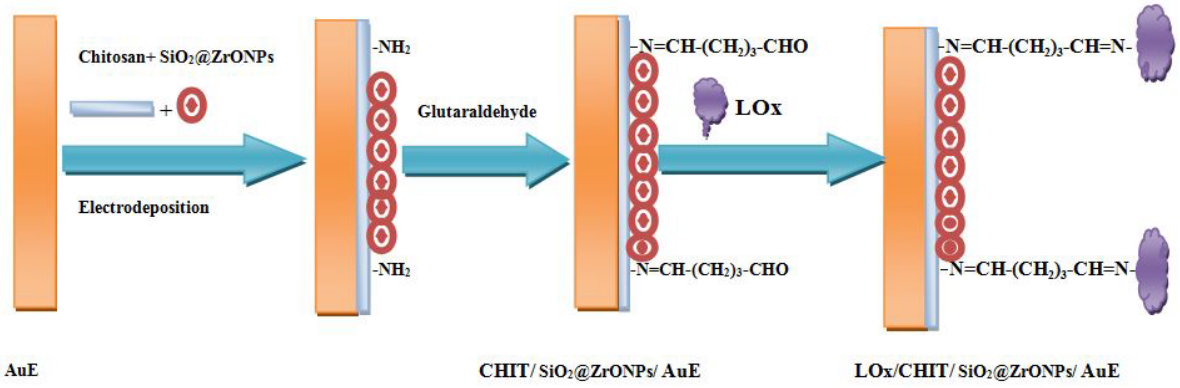

Figure 4: Schematic representation of steps and chemical reaction involved in the fabrication of LOx/CHIT/SiO_2@ZrONPs/Au electrode.

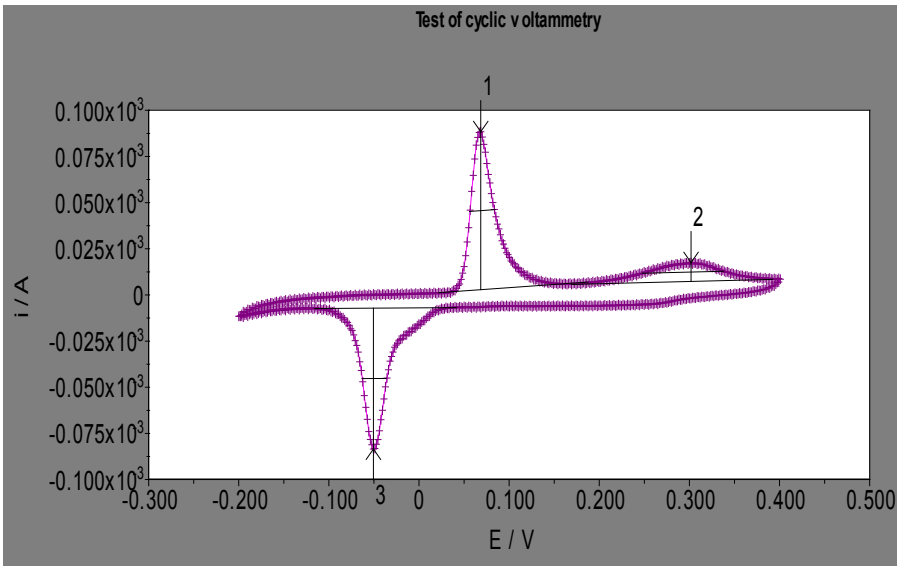

Figure 5: Cyclic voltammogram for electrode of LOx/CHIT/SiO_2@ZrONPs/Au.

and porous (Figure 2b) hence effective surface area is larger. On immobilization of LOx, the globular structural morphology appeared due to the covalent interaction between SiO_2@ZrONPs/CHIT/Au electrodes with LOx (Figure 2c).

By FTIR spectroscopy: Figure 3 (i-ii) shows FTIR spectra of SiO_2@ZrONPs/CHIT/Au electrode (curve i) and LOx/SiO_2@ZrONPs/CHIT/Au electrode (curve ii). FTIR spectra of electrodeposited SiO_2@ZrONPs/CHIT/Au composite showed bands at 2358 cm^{-1} and 1414 cm^{-1} (curve i) due to the stretching vibration mode of –OH and NH_2 group. The peak at 1725 cm^{-1} is assigned to C–O stretching, while the peak at 1426 cm^{-1} is attributed to amide I group (C–O stretching along with the N–H deformation mode) and 1125 cm^{-1} to the stretching vibration mode of the hydroxyl group as shown in curve ii. This change indicates that the enzyme was attached to the SiO_2@ZrONPs/CHIT/Au composite film. The successful covalent immobilization of LOx onto the SiO_2@ZrONPs/CHIT/Au composite film was indicated by the appearance of the IR absorption of amides I and II.

Construction of the enzyme electrode

Figure 4 summarizes the construction of enzyme electrode showing the covalent immobilization of LOx on SiO_2@ZrONPs decorated CHIT film electrodeposited onto the surface of Au electrode. CHIT and SiO_2@ZrONPs were co-electropolymerised on the surface of Au electrode, as this method is easy and the layer thickness could be controlled. To construct the enzyme electrode, LOx was immobilized covalently onto the SiO_2@ZrONPs/CHIT/Au electrode through glutaraldehyde coupling. One –CHO group of glutaraldehyde was bound to -NH_2 group of chitosan on the ZrONPs–CHIT composite film, while the other –CHO group gets linked to the free –NH_2 group of enzyme surface through C=N bond and thus the enzyme is linked covalently, which provided a physically more stable complex. The CV of SiO_2@ZrONPs/CHIT/Au exhibited higher currents than CHIT/Au, revealing that the SiO_2@ZrONPs/CHIT/Au composite film have large effective surface area than CHIT/Au composite film (Figure 5) and SiO_2@ZrONPs/ CHIT/Au composite film could provide a conducting path through the composite matrix for faster kinetics. Hence, the SiO_2@ZrONPs acting as electron transfer mediator help in enhancing the biosensor response and thus increases its sensitivity.

Cyclic voltammeric (CV) response measurement of L-lactate biosensor

In Figure 5, the maximum response (current in mA) was observed at 0.1 V and hence subsequent studies were carried out at this voltage.

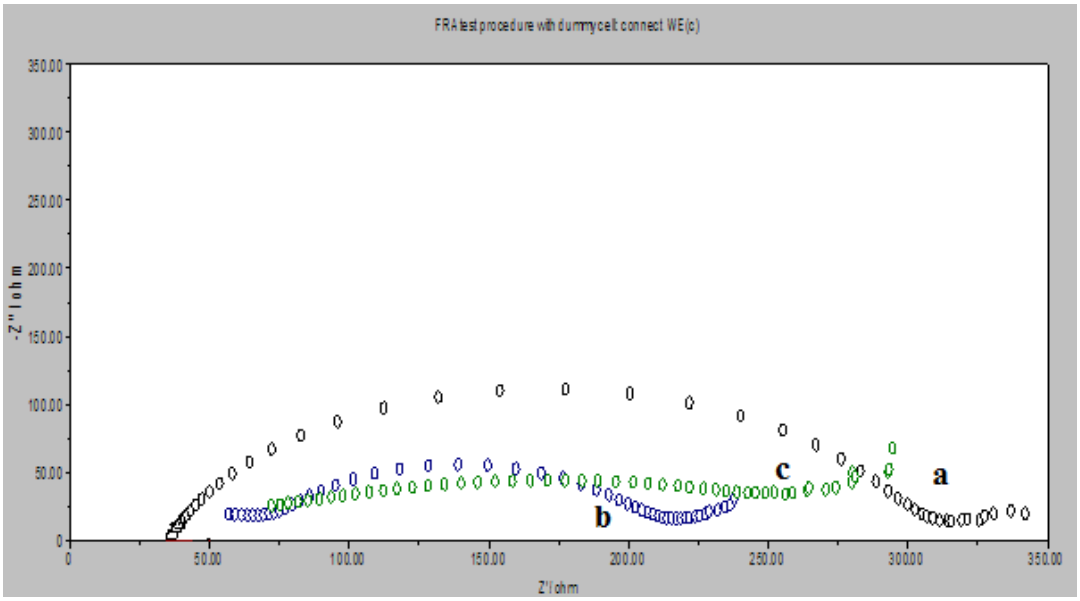

Figure 6: (a) Impedance spectra of bare PG electrode, **(b)** CHIT/SiO_2@ZrONPs/Au electrode and **(c)** LOx/CHIT/SiO_2@ZrONPs/Au electrode.

Amperometric response of LOx/SiO_2@ZrONPs/CHIT/Au increase by the addition of 0.1 ml (10 mM) L-lactate at the applied potential of 0.1 V. The optimum working potential of present L-lactate biosensor was lower than earlier reported amperometric L-lactate biosensor based on a hydrogen titanate nanotube/LOx based biosensor (0.27 V) [15], Sol-Gel Film and Multi-walled Carbon Nanotubes/Platinum Nanoparticles Enhancement (0.5 V) [16]. The lowering of the working potential in the present biosensor might be due to the presence of SiO_2@ZrONPs/CHIT/Au, which provides an environment for the enhanced electrocatalytic effect and a fast electron-transfer rate. The SiO_2@ZrONPs and CHIT had a synergistic electrocatalytic effect toward the oxidation of H_2O_2. The synergistic influence of SiO_2@ZrONPs and CHIT contributes to the excellent performance of the sensor. The existence of SiO_2@ZrONPs and CHIT provides a favourable potential window and electrocatalytic behaviour for the H_2O_2 electron transfer to the electrode. The ability of SiO_2@ZrONPs to promote the electron transfer of H_2O_2, suggests that SiO_2@ZrONPs have great promise as oxidase based biosensors. When L-lactate was added into the buffer solution, the oxidation current rose steeply to reach a stable value.

Response measurements of L-lactate biosensor

The maximum response, as measured in μA of the present lactate biosensor was obtained within 3 s, at the applied potential 0.1 V. This response time is lower than earlier reported L-lactate biosensor based on a tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ)/LOx (80s) [17], LOx immobilized membrane on a micro planar Pt. electrode (10s) [18] but similar to that of MWCNT/CHIT/LDH based biosensor (3s) [19].

Electrochemical impedance measurements

Figure 6 shows electrochemical impedance spectra (EIS) of (i) bare Au electrode (ii) SiO_2@ZrONPs/CHIT/Au electrode and (iii) LOx/SiO_2@ZrONPs/CHIT/Au electrode in 0.05 M sodium phosphate (pH 7.5) containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) as a redox probe. The diameter of the semicircle portion at higher frequencies of the Nyquist plot was equal to the charge transfer resistance (RCT), which controls the electron transfer kinetics of the redox probe at the electrode interface. The charge transfer process in LOx/SiO_2@ZrONPs/CHIT/Au electrode was studied by monitoring charge transfer resistance (RCT) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (RCT) depends on the dielectric and insulating features at the electrode/electrolyte interface. The RCT values for bare Au electrode, SiO_2@ZrONPs/CHIT/Au electrode and LOx/SiO_2@ZrONPs/CHIT/Au electrodes were 330 Ω, 225 Ω and 270 Ω, respectively. However the RCT of LOx/SiO_2@ZrONPs/CHIT/Au (iii) bioelectrode was higher compared to that of SiO_2@ZrONPs/CHIT/Au (ii) electrode. This increase in RCT can be attributed to the fact that most biological molecules, including enzymes, which are poor electrical conductors at low frequencies and cause hindrance to electron transfer. These results also indicate the binding of enzymes onto the SiO_2@ZrONPs/CHIT/Au composite. The lower value of Rct for SiO_2@ZrONPs/CHIT/Au (225 Ω) than for bare Au electrode (330 Ω) indicate the less resistance or more flow in current transfer due to presence of SiO_2@ZrONPs.

Optimization of biosensor

The experimental conditions affecting the biosensor response were studied in terms of effect of pH, incubation temperature, time and substrate (L-lactate) concentration. Figure 7a shows the optimum current was obtained at pH 7.5, which is slightly higher than that of biosensor based on Polypyrrole (PPY) 3DOM/HRP/LOx/Au (pH 7.3) [20], but similar to that based on Dimethyl ferrocene modified linear poly (ethylenimine) (FcMe2-LPEI) Hydrogel GCE (pH 7.5) [21] and slightly lower than that based on Cell debris/Osmium complex/Graphite electrode/LOx (pH 7.8) [22]. This optimum pH of the LOx electrode indicates the types of chemical groups involved at the active centre of the enzyme. The pH in the immediate vicinity of the immobilized enzyme may change, but in the present study, pH of immobilized enzyme did not change, due to the biocompatible microenvironment provided by the SiO_2@ZrONPs/CHIT/Au composite. Further, this optimum pH has the advantage of being close to physiological pH. Figure 7b showed the optimum temperature of the biosensor at 30°C, after which the activity of biosensor decreased due to thermal denaturation of the enzyme, which is higher than that of biosensor based on Cell debris/Osmium complex/Graphite electrode/LOx (24°C)

[22], similar to that based on Polypyrrole (PPY)3DOM/HRP/LOx/Au (30°C) [20] and lower than that of Diamond Nanoparticles (DNPs)/Sol-Gel matrix/Au (35°C) [23]. The working of the present biosensor at low temperature (30°C) could avoid the denaturation of enzyme and thus enhance the life the enzyme electrode.

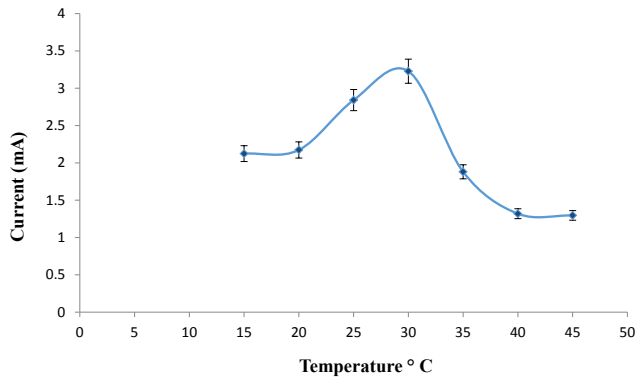

Figure 7A: Effect of pH on LOx/CHIT/SiO_2@ZrONPs/Au electrode.

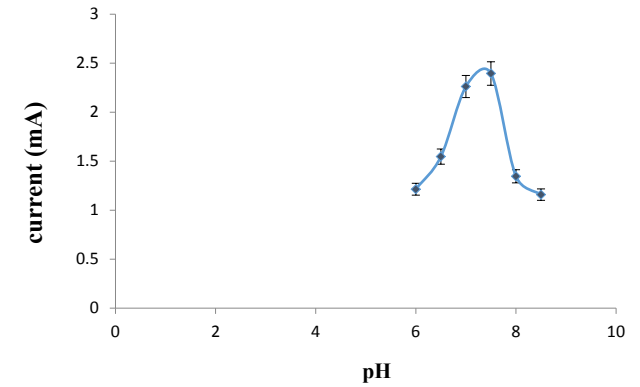

Figure 7B: Effect of incubation temperature on LOx/CHIT/SiO_2@ZrONPs/Au electrode.

Evaluation of biosensor

There was a linear relationship between the current (in μA) and the L-lactate concentration in the range 0.1–4000 μM [Figure 8 (inset)], which is better than those of earlier biosensors based on platinum nanoparticle decorated carbon nanofibers (PtNps)/GCNF–SPCEs/LOx (10–2000 μM) [24] and LOx/PtNp-CNF-PDDA /SPCEs (25-1500 μM) [25]. The detection limit of the present sensor was 0.1 nM (S/N=3), which is lower than that of earlier biosensors based on graphene oxide nanoparticles- modified onto pencil graphite electrode (0.1 μM) [26] and polyacrylic acid (PAA)/Si_4N_3 (ND) surface/LOx (0.2 μM) [27], due to zirconia coated silica nanoparticles/chitosan (SiO_2@ZrONPs/CHIT) which provided high biocompatibility and fast electron transfer rate between the enzyme and electrode.

Analytical recovery

The average recoveries of L-lactate added to blood serum (at levels of 5 mM and 10 mM) were 96.6% and 99% demonstrating the high reliability of the present biosensor (Table 1).

Precision

Within-sample and between-sample coefficient of variations (CVs) for the determination of L-lactate in plasma on the same day and after 1 week of storage were 1.79% and 2.89%, respectively (Table 2). These high precisions revealed good reproducibility and consistency of the present method, which can be attributed to the covalent immobilization of L-lactate oxidase onto the SiO_2@ZrONPs/CHIT/Au electrode.

Application of L-lactate biosensor

Table 3 shows the L-lactate level in apparently healthy persons and lacto acidosis patients, as measured by the present biosensor. L- Lactate level in plasma samples were 101 ± 1.15 to 423.66 ± 2.64 μmol/L in apparently healthy persons, which are in the normal established range (100-2000 μM). Mean plasma lactate values in the lacto acidosis patients were 2547 ± 44.09 to 7917 ± 62.16 μmol/L in persons suffering from lactoacidosis, which are significantly higher than those in apparently healthy persons.

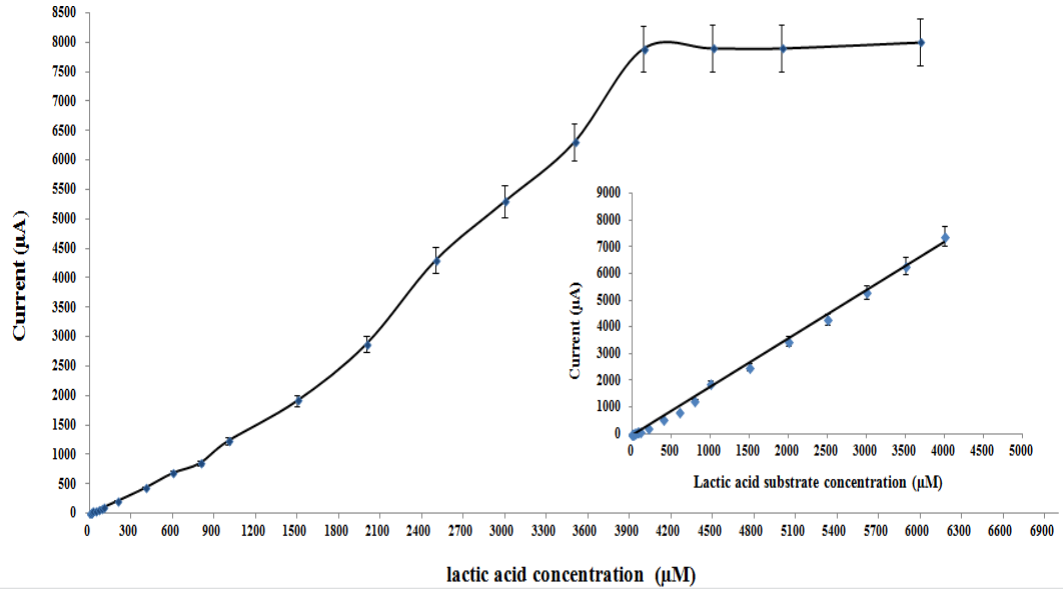

Figure 8: Effect of lactate concentration on L-lactate biosensor based LOx/CHIT/SiO_2@ZrONPs/Au electrode. **Inset:** Standard curve for lactic acid determination.

LA added (μM)	LA found (μM)	% Recovery
--	10.24	100
5	15.19	99
10	19.9	96.6
LA = Lactic acid		

Table 1: Analytical recovery of added lactic acid in plasma, as measured by LOx/SiO_2@ZrONPs/CHIT/Au electrode.

n	Lactate (mM)	CV (%)
Within assay (6)		
64	52.66	1.79
61		
52		
38		
54		
47		
Between assay (6)		
66	51.33	2.89
35		
45		
72		
39		
51		

Table 2: Within and between-assay coefficients of variations for determination of lactic acid in plasma as measured by LOx/SiO_2@ZrONPs/CHIT/Au electrode.

Sex	Age	Healthy persons (µM) Mean ± SD	Sex	Age	Lacto acidosis patients (µM) Mean ± SD
F	9	102 ± 2.64	M	10	2547 ± 44.09
M	10	107 ± 1.52	F	14	2648 ± 43.55
F	11	105 ± 5.03	M	16	3234 ± 20.54
M	14	270 ± 25.11	F	22	2958 ± 61.40
F	19	139 ± 17.15	M	26	6515 ± 66.89
M	22	344 ± 11.50	F	29	5455 ± 48.52
F	27	423 ± 2.64	M	30	2992 ± 6.08
M	31	274 ± 43.03	F	32	4795 ± 98.65
F	37	275 ± 40.70	M	34	5249 ± 42.67
M	42	328 ± 11.84	F	36	7917 ± 62.16
F	46	194 ± 5.13	M	40	3618 ± 69.78
M	50	101 ± 1.15	F	44	5282 ± 36.82
F	54	283 ± 42.01	M	52	6933 ± 66.56
M	61	221 ± 20.00	F	59	4147 ± 50.26
F	67	173 ± 24.19	M	62	4147 ± 50.26

Table 3: Plasma lactate levels in apparently healthy persons and lactoacidosis patients, as measured by lactate biosensor based on LOx/SiO_2@ZrONPs/CHIT/Au electrode.

Correlation

In order to determine the accuracy of the present method, L-lactate values in 20 plasma samples were determined by the present biosensor (y) and compared with those obtained by the standard enzymic spectrophotometric method (x), the values obtained by both the methods were correlated using regression equation. The regression plots between the two methods were drawn and the correlation coefficient was determined showing a good correlation (R^2=0.99) (Figure 9) between lactate values measured by standard method and present biosensor. The correlation coefficient is higher than that of Polypyrrole/carbon nanotubes/lactate oxidase nano-biocomposite film based modified stainless steel electrode lactate biosensor (R^2=0.95) [28].

Interference study and selectivity

The effect of addition of some possible interferents such as urea, uric acid, ascorbic acid, glycine, glucose, L-ascorbic acid, succinic acid and creatinine on the current response of the present biosensor was studied at their physiological concentrations using 10 mM L-lactate in 0.05 M Tris–HCl (pH 8.5) as reaction mixture. The results showed that none had practically any interference. At comparatively lower working potential (0.1 V), a number of serum substances are unable to undergo electro oxidation and thus do not interfere in the biosensor response.

Long-term stability of LOx electrode

The long-term stability of the enzyme electrode was investigated by measuring current response of the biosensor every week under its storage at 4°C. The enzyme electrode lost only 30% of its initial activity after 160 regular uses, over a period of 120 days (Figure 10) which is quite higher than those of majority of earlier reported amperometric biosensors (9-40 days) but lower than those of only two amperometric biosensors (192-216 days) [7, 29]. This higher stability of present electrode could be due to the covalent coupling of enzyme.

Conclusion

An improved amperometric lactate biosensor was constructed based on covalent immobilization of Lactate oxidase onto SiO_2@ZrONPs hybrid electrodeposited onto Au electrode through chitosan film. The biosensor had a very rapid response (3s), with a lower detection limit (0.2 nM) and broader linear range (0.1 μM – 4000 μM), good

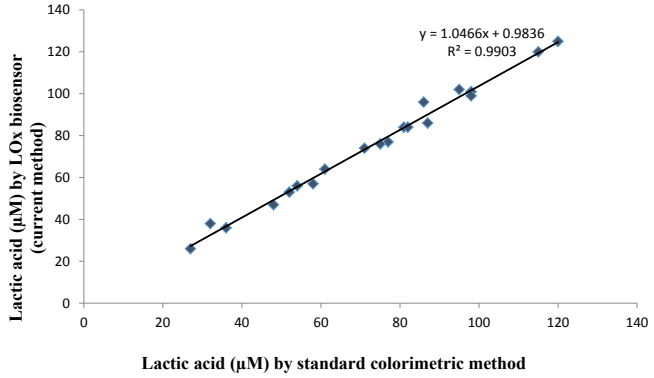

Figure 9: Correlation between plasma lactate values measured by the standard colorimetric method (*x*-axis) and present biosensor (*y*-axis) based on LOx/CHIT/SiO_2@ZrONPs/Au electrode.

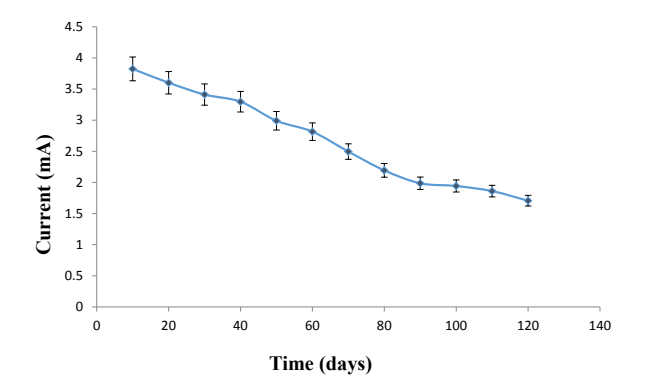

Figure 10: Effect of storage at 4°C on the response of LOx/CHIT/SiO_2@ZrONPs/Au electrode.

reproducibility and a higher storage stability (120 days). This improved the analytical performance of present lactate biosensor was due to the use of SiO_2@ZrONPs/CHIT hybrid film. The nanocomposite of SiO_2@ ZrONPs along with different polymers could also be employed for the improvement of other amperometric biosensors.

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