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Research Article

Preparation and Immobilization of Oxalate Oxidase Nanoparticles onto Au Electrode for Amperometric Determination of Oxalate in Urine and Plasma

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Abstract

The determination of oxalate in the biological fluids of great importance in the diagnosis and medical management of urinary stone disease and various intestinal diseases. We describe herein preparation of oxalate oxidase (OxOx) nanoparticles (NPs) and their electrodeposition / imobilization onto Au electrode for improved amperometric determination oxalate in serum and urine. An oxalate biosensor was fabricated by connecting OxOxNPsAu electrode, AgCl as standard electrode and Pt wire as auxillary electrode through potentiostat/Galvanostat. OxOxNPsAu electrode were characterized by transmission electron microscopy (TEM). The enzyme electrode was characterized by scanning electron microscopy (SEM) and cyclic voltammetry (CV). The biosensor showed optimum response within 3s at pH5.5 and 35°C, when operated at 0.345V. The biosensor exhibited excellent sensitivity with a detection limit (LOD) as low as 1 µM, fast response time and wider linear range (from1 to 400 µM). Michaelis Menten constant (Km) for OxOxNPs was 15.19 µM and Imax 0.001 mA. Analytic recoveries of exogenously added oxalate in urine (20 µM, 40 µM) were 97.5% and 99.1% respectively. Within and between batch coefficients of variation for urinary oxalate determination were <5.53% and <6.16% respectively. The biosensor showed good correlation of 0.98 with standard enzymic colourimetric method. Biosensor measured oxalate in urine and plasma of healthy persons and urinary stone formers.

Keywords: Oxalate; Oxalate oxidase Nanoparticles; Oxalate Biosensor; Urine; Plasma.

Introduction

Oxalate is the metabolic end product of amino acid and protein cataboilsm. It is not required in any process of the human body. Under normal conditions, oxalate deriving from endogenous production and intestinal absorption is fully excreted by the kidneys (Hoppe and Leumann, 2004). Oxalate is a divalent organic anion that affects many biological and commercial processes. It is derived from plant sources, such as spinach, rhubarb, tea, cacao, nuts, and beans, and therefore is commonly found in raw or processed food products (Constable *et al*., 1979; Petraunulo *et al*., 2009). The determination of oxalate in the biological fluids is of great importance in the diagnosis and medical management of primary hyperoxaluria, secondary hyperoxaluria and chronic renal failure (Hoppe *et al.,* 2003), calcium oxalate nephrolithiasis (Pinto *et al.,* 1974; Nath *et al.,* 1984) and nephrocalcinosis and various intestinal diseases (Leuman & Hoppe, 2004). Blood oxalate derives from erythrocytes, diet, the liver, and the metabolism of ascorbate. The plasma oxalate level is elevated in patients

with extreme hyperoxaluria but is generally normal (1-5 micromol/l) in patients with ideopathic calcium oxalate nephrolithia. Normal range of oxalate in human body is: Urine – 10-30mg/24hr and Plasma – 0.8-2.50µM (Baggio *et al.,* 1984; Narula *et al.,*1989).

There are number of methods to determine the oxalate level in biological materials such as titrimetric colorimetric, enzymic colorimetric, gas liquid chromatography, isotachophoresis, isotope dilution and mass spectrometry, ion chromatography, HPLC, atomic absorption spectro-photometry, continuous flow method are available for oxalate determination (Sharma *et al.,*1993).

Biosensor is a device which consists of biorecognition elements specific to the analyte of interest and a physiochemical transducer to relay the resultant signal from this biorecognition event (Narang & Pundir, 2017). Among these various kinds of biosensors, electrochemical biosensors are a class of the most widespread, numerous and successfully commercialized devices of biomolecular electronics (Pundir & Sharma, 2010; Crapnel *et al.*, 2023). Enzyme-based amperometric biosensors have been used widely in our life, such as health care, food safety and environmental monitoring (Hosseinparet *et al.,* 2024). Fabrication of a new sol gel-based sensor for detection of oxalate in some foods. Recently advance technologies for detection and quantification of oxalate in food system were reviewed (Gaur *et al.,* 2025). Nanomaterials especially nanoparticles (NPs), provide a promising way to increase the bio-recognition area (Chen *et al.,* 2004), because the high surface to volume ratio of nanoparticles provides a large number of sites available for molecular interactions. NPs are frequently defined as solid, colloidal particles in the range 10–1000 nm, which have some special physicochemical characteristics resulting from their "small" size structures.

In recent years, a wide variety of NPs with different properties have found broad application in biosensors. Because of their small physical size, nanoparticles present unique chemical, physical and electronic properties that are different from those of bulk materials, improved biosensors have been designed benefiting from these novel attributes. Nowadays, nanoparticles-enhanced biosensors show significant development (Narang & Pundir, 2018). We have reported the improved amperometric biosensors by using the enzyme nanoparticles (ENPs) such as glucose oxidase for glucose (Kundu *et al.,* 2012), cholesterol esterase & cholesterol oxidase for total cholesterol (Chawla *et al.,* 2013), uricase for uric acid (Chauhan *et al.,* 2013), lipase, glycerol kinase, glycerol-3phosphate oxidase for triglyceride (Pundir CS *et al.,* 2015; Pundir *et al.,* 2025), pyruvate oxidase for pyruvate, (Malik *et al.,* 2020), xanthine oxidase for xanthine (Ahlawat *et al.,* 2021), Beta galactosidase and glucose oxidase for lactose (Ahlawat *et al.,* 2022). Acetyl choline esterase and choline oxidase for Acetylcholine (Ahlawat *et al.,* 2023). ENPs show unique optical, electrical, thermal, chemical properties beside providing the increased surface area for catalytic reactions that enhance electron transfer between the reaction mixture in solution and electrode. Present work describes the preparation & chacterization of nanoparticles of oxalate oxidase (OxOxNPs) and their electro-deposition/ immobilization onto Au electrode for improved ampermetric determination of oxalate in urine and plasma (Pundir, 2015).

Materials and Methods

Oxalic acid, Sephadex G-100, DEAE-Sephacel, 4-aminophenazone, and solid phenol were from Sigma Aldrich, St. Louis USA. Horse radish peroxidase (RZ 3.0), succinic acid, ammonium sulphate (enzyme grade), sodium carbonate, sodium hydroxide, sodium-potassium tartarate, sucrose, sodium nitrate, potassium chloride, copper sulphate, Folin Ciocalteau's (FC) reagent, potassium hydrogen phosphate, and dipotassium hydrogen phosphate were purchased from SISCO Research laboratory. Pvt. Ltd, Mumbai. All other chemicals were of analytical reagent (AR) grade. Triple distilled water (DW) was used throughout the study.

Key Equipment Used

Autolab potentiostat/galvanostat (Eco Chemie, The Netherlands).

Assay of OxOx

Assay of oxalate oxidase was carried out according to the protocol of Satyapal & Pundir (1993). One unit of oxalate oxidase is defined as the amount of enzyme required to produce 1 nmoles of H_2O_2/min/mL under standard conditions of assay. The content of H_2O_2 generated in the assay was interpolated from the standard curve of H_2O_2 vs. A_{520}.

Purification of OxOx

Purification of OxOx from 10-0ld leaves of grain sorghum (*Sorghum vulgare* L.) was done as described in Pundir (2015). An overall 73-fold purification was achieved with 6% yield. The purified enzyme showed an apparently single band in poly-acrylamide gel, using coomassie blue as stain.

Preparation of OxOx Nanoparticles (OxOxNPs)

OxOxNPs were prepared by desolvation metod using ethanol as dehydrating agent and subsequent crosslinking with glutaraldehyde as described in Pundir (2015).

Briefly, OxOx ions in 1 ml 0.02M sodium phosphate buffer pH 6.8 were transformed into nanoparticles by continuously adding 4 ml of desolvating agent, ethanol at a rate of 0.5 ml/min under constant stirring at 500 rpm at room temperature. After the desolvation process, 2.5% glutaraldehyde in DW was added, and the suspension was stirred for the next 4 hours. The resulting nanoparticles were purified by three cycles of differential centrifugation (14

000xg, 10 min) at 4ºC and pellet was redispersed to the original volume in DW. Each redispersed step was performed in an ultrasonication bath over 5 min. ─SH groups were introduced on OxOxNPs by addition of 0.12 g of cysteine with constant stirring for 5-6 h. At last OxOxNPs were separated from free enzyme solution by centrifugation at 1200 rpm at 4ºC for 10 min followed by dispersion in 0.1M sodium phosphate buffer (pH 7.0) and stored at 4ºC.

Electro-Deposition of OxOxNPs onto Gold (Au) Electrode

Prior to surface modification, Au electrode was cleaned with piranha solution (H_2SO_4:H_2O_2 in 3:1ratio) for 20 min, then rinsed thoroughly with DW. The gold electrode was then polished with alumina slurry. OxOxNPs were electrochemically deposited onto polished Au electrode by cyclic voltammetry (CV) by immersing it into a mixture of 23 mL 2.5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) and 2 mL of OxOxNPs solution and then applying potential between -0.2 to +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 Vs^{-1}. OxOxNPs/ Au electrode was rinsed with DW and then dried at room temperature (Fig. 1).

Fig. 1: Cyclic voltammogram for electrochemical deposition of OxOx nanomaterials on Au electrode.

Construction and Optimization of Oxalate Biosensor

An oxalate biosensor was constructed connecting OxOxNPs / Au electrode, as working electrode, Ag/AgCl as standard electrode and Pt wire as auxillary electrode were linked to potentiostat/autolab. Various kinetic properties of OxOxNPs immobilized onto Au electrode were studied such as effect of pH, incubation temperature for maximum activity, time incubation, and effect of substrate (oxalic acid) concentration to study the optimum working conditions of the biosensor.

To determine optimum pH, the pH of the sodium succinate buffer (0.05M) was varied in the pH range 3.5-5.5 and 0.05 M sodium phosphate buffer in the pH range- 6.0 to 8.0. The current in terms of miliampere (mA) was measured using potentiostat. To determine the incubation temperature for maximum activity, the reaction mixture was incubated at different temperatures ranging from 20°C to 55°C at an interval of 5°C. The responses of enzyme electrode were measured at these temperatures as described above. To study time course, by recording the current response from 1 to 10s at an interval of 2s. The effect of oxalate concentration on the initial velocity of enzyme reaction was studied by varying the final concentration of oxalic acid in the range from 1µM to 1000µM. The immobilized OxOxNPs responses were measured by potentiostat.

Application Of Amperometric Oxalate Biosensor

The oxalate oxidase biosensor was used to determine the oxalate level in urine and plasma of apparently healthy and hyperoxaluric or urinary stone former patients.

Determination Of Urinary Oxalate by Amperometric Oxalate Biosensor

Collection of urine:

The first morning urine samples from apparently healthy and stone patients (both male and female of various age groups) were collected in plastic bottles. The concentrated HCl was added to urine samples to adjust its pH 2.5 and stored at 4ºC until use.

Pretreatment of urine samples:

1 mL of urine sample was diluted with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.01 M EDTA in 1:1 ratio and pH was adjusted to 5.7. To the urine samples, 0.1 mL of sodium nitrite solution (35 mg/10 mL of sodium phosphate buffer pH 6.0) was added to remove possible ascorbate interference.

Measurement of urinary oxalate:

The assay of urinary oxalate was carried out in as described for response measurement of oxalate biosensor under its optimal conditions except that pretreated urine samples was used. 0.1 mL pretreated urine in place of oxalate. The amount of oxalate concentration was calculated from standard curve between oxalate concentration and current (mA)(Fig. 9).

Determination Of Plasma Oxalate by Amperometric Oxalate Biosensor

Collection of plasma:

The blood samples (2 mL each) from apparently healthy individuals and stone formers (male and female) of different age group admitted to local PGIMS hospital were collected into vials containing 30 U heparin in each vial.

Pretreatment of blood samples:

To separate the plasma, heparinized blood samples were centrifuged at 2,000×g for 10 min and plasma (supernatant) was collected and set to pH 3.0 by adding 11 µL 3.5 N HCl. A possible ascorbate interference was removed by adding 10 µL of 5 mM sodium nitrite (prepared in 0.1 M sodium phosphate buffer, p H 7.0) to 0.1 mL of acidified plasma and vortexed vigorously.

Measurement of plasma oxalate

It was carried out in a similar manner as described for urinary oxalate except that plasma was used in place of pretreated urine. The oxalate concentration in plasma was calculated from standard curve for oxalate (Fig 9).

Evaluation Of Amperometric Method of Urinary and Plasma Oxalate Determination

The following parameters were studied in order to evaluate present oxalate biosensor.

Detection limit (LOD):

To study minimum detection limit (LOD) of the biosensor, the amperometric responses of enzyme electrode were studied at varying oxalic acid concentration and current was measured through potentiostat.

Analytic recovery:

To determine the reliability of the methods, two concentrations of oxalic acid (20 and 40 µM) were added to the urine samples and their oxalate content was determined in the samples before and after addition of oxalic acid by the present method. The % recovery of added oxalic was calculated.

Correlation:

In order to determine accuracy of present methods, the values in 12 urine samples were determined by standard enzymic colorimetric method (x) as well as by the present method (y), the values obtained by both the methods were co-related using regression equation.

Reuse And Storage of Enzyme Electrode

To reuse the enzymes electrode, it was washed 3-4 times in 0.1 M sodium succinate buffer pH 5.5. It was stored in reaction buffer at 4-8°C, when not in use.

Results and discussion

Preparation and Characterization of OxOxNPs

The nanoparticles of grain sorghum Oxalate oxidase were prepared by desolvation method, using ethanol as desolvating agent. OxOxNPs were characterized by transmission electron microscopy (TEM). The TEM images of OxOxNPs showed their size in the range of 20 – 100 nm with an average of 50.6 nm.

Optimization of the Biosensor

To optimize the working conditions of oxalate biosensor, effect of pH, incubation temperature, response time, and substrate (oxalate) were studied.

Effect of pH

The optimum response of biosensor was achieved at the pH 5.5 (Fig 3), which is slightly higher than that of free oxalate oxidase immobilized onto affixed alkylamine glass beads (pH 5.0) (Kumari *et al*., 2004), PVC membrane (pH5.0)(Pundir CS & Phaugat, 2009),carbon paste electrode (CPE)(pH4.5) (Mishra *et al.,* 20090), interdigitated AuE (pH3.6) (Milardovic *et al.*, 2008). Hence pH 5.5 (0.05M sodium succinate buffer) was used in all subsequent experiments.

Characterization of Enzyme Electrode (OxOxNPs/Au) at Different Stages of Its Construction

By Scanning Electron Microscopy (SEM): The surface morphologies of bare Au electrode and OxOxNPs/Au electrode were investigated by scanning electron microscopy (SEM). Bare Au electrode showed homogenous surface which became granular after deposition of OxOxNPs showing the electrodeposition of OxOxNPs.

By Cyclic voltammetry (CV): Cyclic voltammogram (CV) of OxOxNPs/Au electrode was recorded in Potentiostat–Galvanostat from −0.20 to 0.80V vs Ag/AgCl as reference and Pt wire as counter electrode in a 0.05M sodium succinate (pH 5.5) containing 10mM oxalate. In cyclic voltammogram of OxOxNPs/ Au electrode (Fig.1) OxOxNPs provided a large surface area and catalytic activity to enhance the flow of current to Au electrode.A peak in CV of OxOxNPs/ Au electrode (Fig 1) showed maximum current at 0.345 V Vs. Ag/AgCl due to oxidation of oxalate, hence subsequent electrochemical measurements were carried out at this potential.

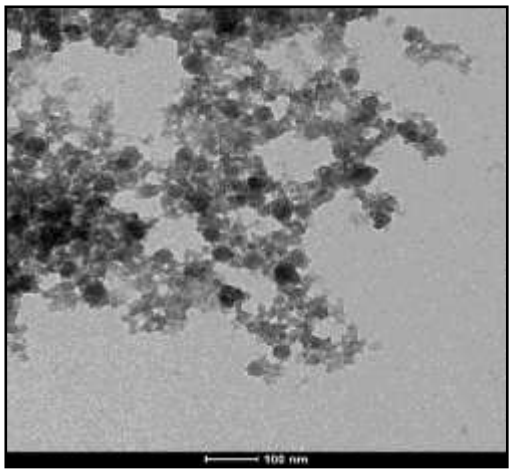

Fig. 2: Transmission electron micrograph (TEM) image of oxalate oxidase nanoparticles (OxOxNPs)

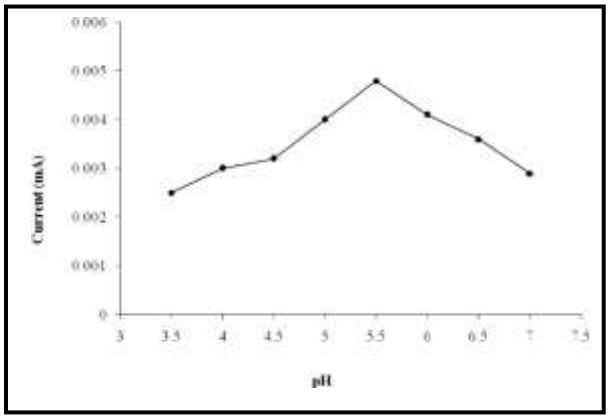

Fig.3. Effect of pH on the current response of OxOxNPs/Au electrode.

Effect of Incubation Temperature

As the incubation temperature increased, the current response of biosensor increased up to an optimum temperature, after which it declines rapidly. The optimum response was achieved at temperature, 35 °C (Fig.4), which is similar to free OxOx immobilized onto mucin-chitosan gel(Benediverz *et al.,* 2009) Hence in subsequent studies, reaction assay of oxalate was carried out at 35 °C.

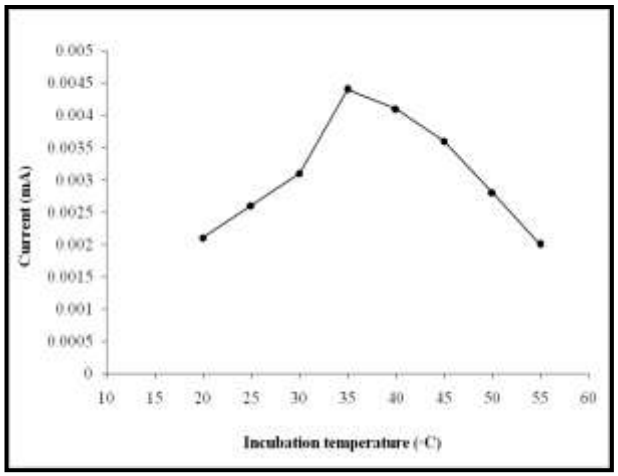

Fig. 4: Effect of incubation temperature on the current response of OxOxNPs/Au electrode.

Effect of Response Time

The biosensor /electrode showed maximum response at 3s (Fig.5), which is lower than that for free OxOx immobilized onto AuE (172s) (Kumari & Pundir, 2004), CPE (45s) (mishra *et al.,* 2009), PVC membrane (12s) (Pundir & Phaugat, 2009).

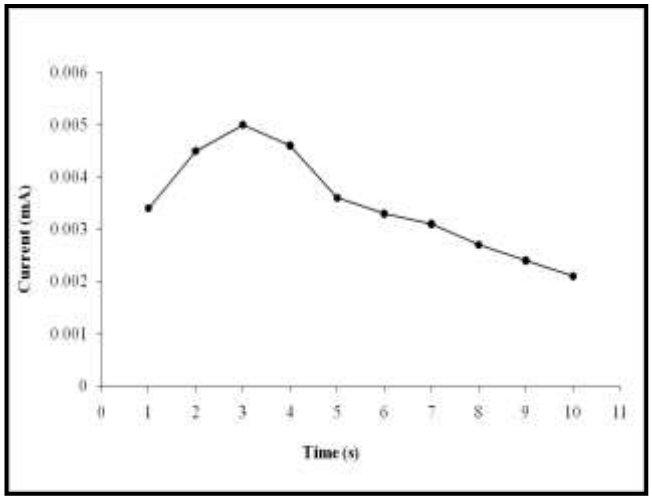

Fig. 5.: Effect of incubation time on the current response of OxOxNPs/Au electrode.

Effect Of Substrate/Oxalate Concentration

Fig.6 exhibited the electrochemical responses of OxOxNPs/Au electrode at different concentration of oxalic acid which showed that as oxalic acid concentration increased, oxidation current was also increased. There was a hyperbolic relationship between biosensor response and oxalic acid concentration from 1µM to 400µM, after which it was constant. The Lineweaver–Burk plot between 1/I (mA) and 1/[oxalic acid] (Fig.7) yielded an apparent K_m 15.19µM and I_{max} 0.001mA, indicating increased affinity of enzyme towards substrate (oxalic acid) after electrodeposition. The K_m for free oxalate oxidase immobilized onto alkylamine glass beads affied on wall of a glass beaker was $0.78x10^{-4}$mol/L

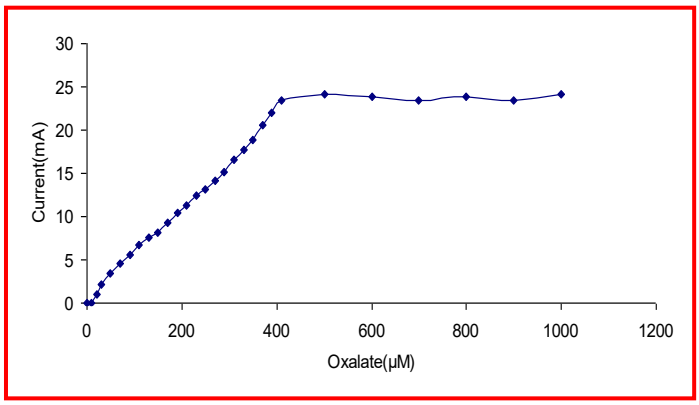

Fig. 6: Effect of substrate (oxalate) concentration on the current response of OxOxNPs/Au electrode.

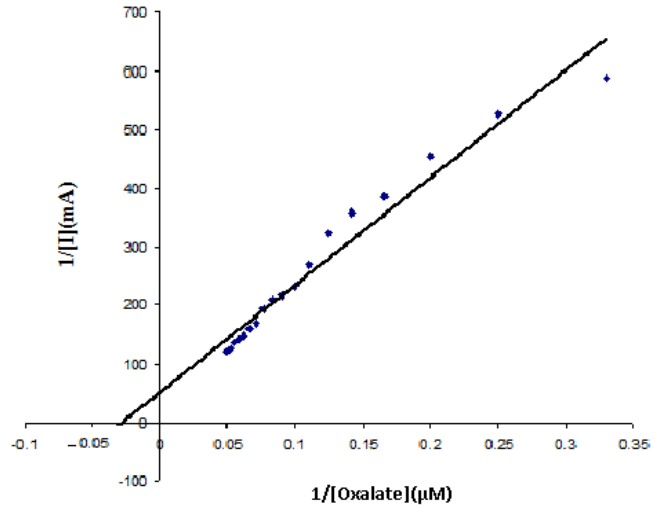

Fig. 7: Lineweaver-Burk plot for effect of oxalate concentration vs current response of OxOxNPs/Au electrode.

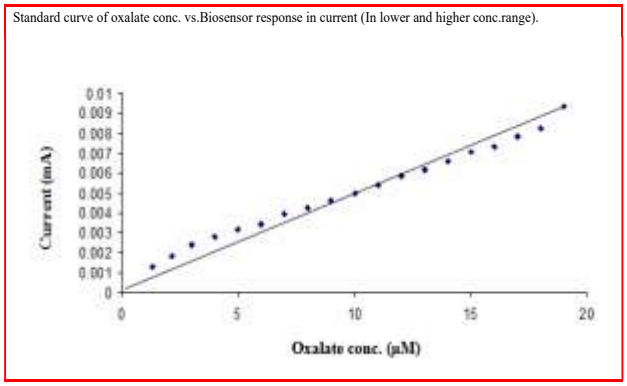

Fig. 8: Standard Curve for oxalate (Lower concentration range)

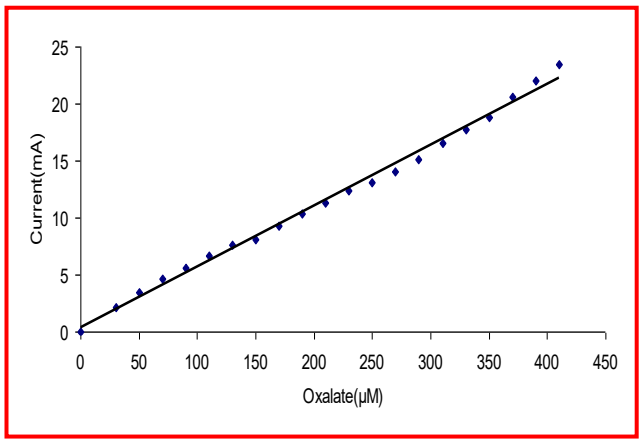

Fig. 9. Standard curve for oxalate (higher concentration range)

Evaluation Of Oxalate Biosensor

Linearity:

There was a linear relationship between oxalic acid concentrations ranging from 1µM to 400µM and current (mA)(Fig.7,9,10).

Minimum Detection limit (LOD):

The minimum detection limit of the biosensor was 1µM, which is lower than that of interdigitated gold electrode (19.04 µM)[39] and carbon paste electrode (27.77uM).

Analytical Recovery:

Analytical recoveries of exogenously added oxalate (20 µM, 40 µM) in urine sample as measured by present biosensor method were 97.5% and 99.1%, respectively, showing the reliability of the method (Table 1).

Table 1: Analytical recovery of added oxalate in urine samples as determined by oxalate oxidase

Added oxalate (µM)	**Oxalate found (µM)**	**% Recovery**
-	20	-
20	39	97.5±1.2
40	79.3	99.1±1.4

Correlation with the standard enzymic colourimetrc method:

The oxalated values for 20 urine samples obtained by the present method (y) were correlated with those by the enzyme colorimetric method (x), using the "regression equation". The experiment was repeated three times. The results showed a direct correlation with the regression equation, y =0.9807x + 0.7075 (Fig. 8).

Determination of Urinary and Plasma Oxalate

Urinary oxalate determination in healthy individuals:

The oxalate level in first morning urine samples of healthy individuals (both male and female) of different age groups were determined by the present biosensor. The results are given Table 2. The urinary oxalate content in healthy individuals was found to be in the range 7.2 to 20.6 mg /L in males and from 6.75 mg to 18.7 mg/L in females. This urinary oxalate concentration was different in different age groups. The urinary oxalate content in children of less than 10 years was 7.2 mg/L in males, which is higher than those in females (6.75 mg/L). Similarly, in the age group 31-40 year, the urinary oxalate level was 14.4 mg/L which is higher than those in female (13.5 mg/L). These observations indicate that urinary oxalate content was higher in males than in females.

Urinary oxalate determination in urinary stone formers

The oxalate values in first morning urine samples of stone formers (both male and female) of different age groups were determined by the present biosensor. The results are given in Table 4. The urinary oxalate content in stone formers was significantly higher than those from healthy individuals in different age group. The oxalate content in children of less than 10 years with urinary stones was in the range of 35.5 mg/L in males, which is significantly higher than healthy children of less than 10 years. Similarly, the oxalate content of male stone formers of age group 31-40 was found in the range of 48.2-52.3 mg/L , which is significantly higher than those in healthy individuals (Table 2).

Determination Of Oxalate Content in Plasma of Healthy Individuals and Stone Formers

The oxalate values in plasma of healthy individuals (both male and female) of different age groups were determined by the present method. The results are given in Table 3. The plasma oxalate content in healthy individuals ranged from 1.6-5.1 µmol/L for males and from 1.2-4.8 µmol/L in females. The oxalate values in plasma of urinary stone formers (both male and female) of different age groups were also determined by the present method. The results are given in Table 3. The plasma oxalate content in male stone formers of less than 10 years was found to be 7.6 µmol/L, which was higher and significant than children of healthy individuals of less than 10 year in males. Similarly, the individuals of age 20–30-year, plasma oxalate content was found to be in the range of 8.2 µmol/L in male stone formers, higher than those in plasma of healthy individuals in males.

Storage stability and reusability:

The enzyme electrode was reused upto 80 days, when stored dry at 4-8 °C, which is higher than Sorghum leaf OxOx immobilized on PVC membrane.

A comparison of analytical parameters of various oxalate biosensors is summarized in Table 4.

Table: 2 Oxalate values in 24 h urine samples of healthy individuals compared with stone formers as determined by OxOxNPs/AuE

Age group (Years)(n =5)	Sex	Urinary oxalate in mg in healthy individuals (Mean)±SD	Urinary oxalate in mg in stone formers individuals (Mean)±SD
>10	M	7.2±0.46	34.1±2.54
	F	6.75±0.32	31.2±2.05
20-30	M	14.4±0.26	46.9±0.87
	F	13.5±2.96	46.1±0.56
31-40	M	16.2±4.25	52.5±3.34
	F	15.7±4.80	51.7±2.56
41-50	M	20.6±2.85	56.2±4.76
	F	18.7±3.02	55.6±4.31

Table 3: Oxalate values in plasma samples of healthy individuals compared with stone formers as determined by OxOxNPs/AuE

Age group (Years)	Sex	Oxalate value µmol/L in plasma of healthy individuals	Oxalate value µmol/Lin plasma of stone formers individuals
>10	M	1.6	7.5
	F	1.2	6.4
20-30	M	2.6	8.0
	F	2.1	8.1
31-40	M	3.1	9.0
	F	2.9	8.4
41-50	M	5.1	9.8
	F	4.8	8.9

Table 4 A comparison of analytical characteristics of various oxalate biosensors.

S. N.	Support for immobilization	Method of immobilization	Optimum pH	Optimum temp(C)	Detection limit	Linear range	Response time	Application	References
1	Gold electrode modified by OxOxNPs	Electro-deposition	5.5	35	1µM	1µM to 400µM	3s	Urine, serum	Present
2	Mucin/carbapol gel	Physio-sorption	2.8	ND	ND	ND	ND	Urine	Capra et al., 2007
3	Interdigitated gold electrode	Physio-sorption	3.6	ND	19.04 uM(in diluted samples) 4.67 uM in cells	2.5 to 100 uM(samples)	172 s (20 analysis per h)	Urine	Milardovic et al., 2008
4	Arylamine glass beads	Covalent immobilization	3.5	40	0.01 mM/l in urine 2.5 µM/l in serum	ND	ND	Urine, serum	Godara and Pundir, 2008
5	Mucin/chitosan gel	Physio-sorption	ND	ND	ND	ND	ND	Urine	Benavidez *et al.,* 2009
6	Carbon paste electrode	Adsorption	4.5	35	27.77µM	0-222µM	45 s		Mishra et al., 2009
7	PVC membrane	Physiosorption	5.0	30	1 µM	1-10 µM low conc. range 0.1-1mM high conc. range	10-12s	Urine and serum	Pundir and Phaugat, 2009

Conclusions

The use of nanoparticles of oxalate oxidase electrodeposited on the surface of Au electrode has resulted into improved performance of oxalate biosensor with a detection limit 1µM.The granular structural morphology of OxOxNPs provides very sensitive.

Ethics Approval

In the present work, we have collected left over human serum samples from Biochemistry Department, PtBDS PGIMS Rohtak hospital, affiliated to Pt. BDS University of Health Sciences, Rohtak under MoU between MDU Rohtak and Pt. BDS University of Health Sciences, Rohtak for research purpose. PtBDS PGIMS Rohtak hospital has its own ethical clearance from competent ethical committee for collecting biological samples for diagnosis of various diseases and their treatment.

Competing Interest

Authors declare no competing interest with the present research.

Authors' Contributions

Prof. CS Pundir planned the research work and supervised and guided the whole work. Practical work and initial writing of research was done by Ms. Rachita. Ms.Shikha Pundir checked and prepared the final draft.

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