Chapter 3
Materials and Methods
3.1. Sources of Chemicals and biomaterials

Glutamate oxidase (GluOx, EC 1.4.3.11, 5 units/mg, from Streptomyces sp.), 4-amino phenazone and gold chloride tetrahydrate were purchased from Sigma–Aldrich St. Louis, MO, USA. Carboxylated multiwalled carbon nanotubes (cMWCNT) (functionalized MWCNT, 12 walls, 15–30 μm length, 90% purity, nil metal content) were purchased from M/S Intelligent Materials (Panchkula, Haryana, India). Peroxidase from Horseradish, L-bovine serum albumin (BSA), glutamic acid, acetic acid, N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), chitosan (CHIT), phenol (solid), potassium ferrocyanide, potassium ferricyanide, potassium bromide (spectronic grade), nitric acid, sulphuric acid, hydrochloric acid, sodium carbonate, sodium bicarbonate, tris buffer, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate were purchased from Sisco Research Laboratories Mumbai, India. All other chemicals were of analytical reagent (AR) grade. Grass pea (Lathyrus sativus) seeds of two varieties (Fig. 7 a & b) were purchased from open market of Bhagalpur (Bihar) and the third variety (c) from Jhalawar, Rajasthan. Double distilled water (DW) was used in all experiments. All glasswares used were from M/S Borosil Glass Works Ltd. Mumbai.

![Fig.7](image-url) Seeds of different varieties of *Lathyrus sativus*

3.2. Instruments and Equipments used

The following instruments and laboratory items were used in the present study: Autolab Potentiostat/Galvanostat equipped with an Autolab PGSTAT-302N, general purpose electrochemical system (GPES) and frequency response analysis (FRA) software (Eco-Chemie, Utrecht, The Netherlands, Model: AUT83785), Fourier transform infrared (FTIR) spectrophotometer (Nicolet is10, Thermo Scientific USA) along with hydraulic
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press (cap 15T), Misonix ultrasonic liquid processor (XL-2000 series, Newtown City, USA) along with sonobox, Sigma refrigerated centrifuge (3-30K), biomedical freezer (-40°C, Sanyo), refrigerator (GL-325TMG4/2007, LG), all quartz double distillator apparatus (881B, Labco, India), electronic balance (Sartorius, BT224S), temperature controlled digital water bath shaker & oven (NSW, New Delhi), digital pH meter (335 D, systronics, Ahmadabad), Spectronic-20D+ (Thermo Scientific, USA), ice flaking machine (AICIL, Chandigarh) and magnetic stirrer with hot plate (Remi, N. Delhi).

3.3. Assay of free glutamate oxidase (GluOx)

The assay of glutamate oxidase was carried out as described by Satyapal and Pundir (1993) with modification and based on quantification of H2O2 generated from oxidation of L-glutamic acid by GluOx, using a color reaction consisting of 4-aminophenazone, phenol and peroxidase as chromogenic system. The reaction mixture containing 1.8 ml of 0.1 M potassium buffer pH 7.4 and 0.1 ml of GluOx (1 mg/ml) was pre-incubated at 37°C for 5 min. The reaction was started by adding 0.1 ml L-glutamate solution (0.1 M). After incubation at 37°C for 10 min in dark, 1.0 ml colour reagent was added to the reaction mixture and kept for 30 min at room temperature to develop the color. A520 was read and H2O2 concentration was extrapolated from standard curve between H2O2 concentrations and A520 (Fig. 8).

One unit (U) activity of GluOx is defined as the amount of enzyme required to generate 1 μmol of H2O2 per min under standard assay conditions.

3.3.1. Preparation of colour reagent

The colour reagent was prepared according to Bais et al. (1980), which consisted of 50 mg of 4-aminophenazone, 0.1 g solid phenol and 1 mg of horseradish peroxidase (RZ = 3.0) per 100 ml of 0.4 M sodium phosphate buffer, pH 7.0. It was stored in amber coloured bottle at 4°C until use and prepared fresh after one week.

3.3.2. Preparation of standard curve of H2O2

Different concentrations of H2O2 ranging from 50 μmoles to 250 μmoles at an interval of 50 μmoles in 2.0 ml 0.05 M sodium phosphate buffer, pH 7.0 were added to 1.0 ml of color reagent in test tubes. After incubation at 37°C for 30 min, A520 was read. A standard curve was drawn between H2O2 concentration and A520 (Fig. 8).
Fig. 8. Standard curve of H\textsubscript{2}O\textsubscript{2} using a colour reagent consisting of 4-aminophenazone, phenol and peroxidase as chromogenic system

3.3.3. Determination of protein: The concentration of protein in dissolved GluOx was determined by Lowry method. The following reagents were used in the method:

- Reagent A: 2\% Na\textsubscript{2}CO\textsubscript{3} in 0.1N NaOH
- Reagent B1: 1\% CuSO\textsubscript{4} in distilled water
- Reagent B2: 2\% sodium potassium tartarate

[Reagent B1 & B2 were stored at 4°C, when not in use]

- Reagent B: Regent B1 + Reagent B2 in 1:1 ratio (Prepared fresh).
- Reagent C: 50 ml of reagent A + 1 ml of Reagent B (Prepared fresh).
- Reagent D: 1 part of FC reagent + 1 part of distilled water. It was also prepared fresh

A stock solution of BSA (1 mg/ml) was prepared in DW. Different quantities of BSA in the range of 100 µg/ml to 500 µg/ml in a total volume of 0.5 ml were taken in a series of test tubes. For blank, 0.5 ml DW was taken in place of protein. Then 5 ml
reagent C was added to each tube, mixed well and allowed to stand for 10 min at room temperature. Reagent D (0.5 ml) was added and mixed immediately. The mixture was allowed to stand for 30 min at room temperature. The colour intensity of reaction mixture was measured in Spectronic-20D at 750 nm. A standard curve for protein was plotted between BSA concentration and $A_{750}$ (Fig. 9). The same procedure was followed with suitably diluted 0.5 ml of GluOx preparation and $A_{750}$ was measured. The amount of protein in enzyme preparation was interpolated from calibration curve.

![Standard curve of protein (BSA) by Lowry method](image.png)

**Fig.9.** Standard curve of protein (BSA) by Lowry method

**3.4. Construction of enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/AuE)**

**3.4.1. Preparation of AuNPs**

AuNPs were prepared as described by Zhang et al., (2009) with little modification. Briefly, 100 ml of aqueous HAuCl₄ (0.01%) was brought to boil and 2.5 ml of 1% trisodium citrate solution was added to it dropwise under vigorous stirring. A wine red coloured colloidal AuNPs suspension was obtained, which was stored in dark glass bottle at 4°C for further use. The AuNPs formed were kept at 40°C for drying.
3.4.2. Characterization of AuNPs

AuNPs were characterized using the following techniques:

3.4.2.1. UV spectroscopy: UV-visible light absorption pattern of AuNPs suspension was kinetically monitored in the range, 380–700 nm in a UV-Vis spectrophotometer.

3.4.2.2. Transmission electron microscopy (TEM): To study the size & morphology of AuNPs, the TEM images of AuNPs were taken in a transmission electron microscope at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi on commercial basis. Samples of AuNPs were milled together with anhydrous alcohol and then dispersed in anhydrous alcohol to form dilute suspension. The dilute suspension was dropped on the carbon-grid and the morphology of the AuNPs was observed using TEM. The average diameter of particles was calculated by measuring 100-300 individual particles with Soft Imaging GmbH image analysis software.

3.4.2.3. Dynamic light scaterring (DLS): Dynamic light scattering (DLS) is an analytical tool used routinely for measuring the hydrodynamic size of nanoparticles and colloids in a liquid environment. AuNPs are extraordinary light scatterers at or near their surface plasmon resonance wavelength. The hydrodynamic diameter/size of the AuNPs was measured at Institute of Biotechnology, Amity University, Noida (U.P.) using a Zetasizer Nano ZS90 DLS system equipped with a red (633 nm) laser and an Avalanche photodiode detector (APD) (quantum efficiency > 50% at 633 nm) (Malvern Instruments Ltd., England). A Hellma cuvette QS 3 mm was used as sample container. DTS applications 5.10 software was used to analyze the data. All sizes reported here were based on intensity average.

3.4.2.4. FTIR: AuNPs possess a wine red colouration due to the transverse oscillations of the surface electrons of the particle on interaction with light of suitable wavelength, called surface plasmon resonance. Surface plasmon band is greatly dependent on the nanoparticle size. The shape of the particles plays an important role in deciding the position of this band. When the shape of gold nanoparticles changes to the form of nanorods, the surface Plasmon resonance mode changes from 517 nm to 850 nm. Thus FTIR is an effective tool in detecting the shape of nanometer sized materials.

To record FTIR spectra, AuNPs were ground with dry potassium bromide (KBr) and this powder mixture was then pressed in a mechanical press to form a translucent pellet through which, the beam of the spectrometer could pass. The pellet was kept in the socket
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of FTIR spectrophotometer and its spectrum was recorded. The infrared spectrum of a sample was recorded by passing a beam of infrared light through the sample. Band intensities in IR spectrum were expressed as transmittance (T). Transmittance is the fraction of incident light (electromagnetic radiation) at a specified wavelength that passes through a sample. Transmittance is defined as the ratio of the radiant power transmitted by a sample to the radiant power incident on the sample. In most of the spectrum transmittance (T) versus wave number (cm⁻¹) were plotted.

3.4.3. Dispersion of cMWCNTs

cMWCNT (1 mg) was suspended in a 1.0 ml mixture of concentrated H₂SO₄ and HNO₃ in a 3:1 ratio and ultrasonicated for 2 hr to obtain a homogeneous black coloured solution (Fig. 10) and then washed with DW until the pH of the washing discard was 7.0. The dispersed cMWCNT solution (0.1ml) was mixed into a mixture of 0.5 ml EDC (0.2 M) and 0.5ml NHS (0.2 M). The pH was adjusted to 6.0 and kept at RT for 1 hr.

![Partially dispersed cMWCNT powder (a) and Completely dispersed cMWCNT (b)](image)

Fig.10. Partially dispersed cMWCNT powder (a) and Completely dispersed cMWCNT (b)

3.4.4. Electrodeposition of CHIT and cMWCNT/AuNPs composite film on a gold (Au) electrode

The surface of a Au electrode (0.36 cm²) was polished manually by alumina slurry (diameter 0.05µm) with a polishing cloth, followed by thorough washing with DW and then placed into ethanol, sonicated to remove adsorbed particles and finally washed with DW for 3-4 times. CHIT (0.5% in acetic acid, 200 µl) was added to 25 ml of 1M KCl and electrodeposited onto Au electrode through cyclic voltammetry using galvanostat by
applying 20 successive deposition cycles at -0.15 to 0.20 V at scan rate of 20 mV/s. AuNPs suspension (200 μl) and 400 μl of EDC and NHS treated cMWCNT suspension were added into 25 ml 1M KCl to get the cMWCNT/AuNPs mixture. Finally, electrodeposition of AuNPs and cMWCNT onto the Au electrode was carried out in an electrochemical cell applying 20 deposition cycles at -0.1 to 0.2 V at scan rate of 20 mV/s. During the electrochemical polymerization, the surface of Au wire became green gradually, indicating the deposition of cMWCNT/AuNPs/CHIT film on Au wire (Fig. 11). The resulting cMWCNT/AuNPs/CHIT modified gold electrode was washed thoroughly with DW to remove unbound matter and kept in a dry Petri-plate at 4°C.

![Fig.11. Au electrode without (a) and with (b) electrodeposited cMWCNT/AuNPs/CHIT composite film](image)

3.4.5. Immobilization of GluOx onto cMWCNT/AuNPs/CHIT modified Au electrode

cMWCNT/AuNPs/CHIT/Au electrode was placed into 2ml of sodium phosphate buffer (0.1M, pH 7.4) containing 100 μl of GluOx (2.3 U/ml) and kept overnight at 4°C for immobilization. The resulting bioelectrode (GluOx/cMWCNT/AuNPs/CHIT/Au electrode) was washed 3-4 times with 0.1 M sodium phosphate buffer, pH 7.4 to remove unbound enzyme and used as working electrode. The unbound enzyme was tested for activity and protein. The bioelectrode was stored at 4°C when not in use.
3.4.6. Characterization of enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/AuE)

The fabricated enzyme electrode was characterized using scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS). SEM enables the investigation of specimens with a resolution down to the nanometer scale. SEM studies were used for investigating the morphology of cMWCNT/AuNPs/CHIT electrode with and without immobilized enzymes. SEM studies were carried out on commercial/payment basis at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi. SEM used a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derived from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, crystalline structure and orientation of materials making up the sample. The electrodes were cut into small pieces (1 cm) and placed on a specimen chamber of 2 cm diameter using a spray gun, generally mounted rigidly on a specimen holder called a specimen stub and micrographs were taken with a scanning electron microscope. The samples were coated with an ultrathin coating of electrically conducting material gold. In SEM, data were collected over a selected area of the surface of the sample and 2-dimensional images were generated that displays spatial variations in these properties.

To record FTIR spectra of enzyme electrode at different stages of its construction, the deposited material was scrapped off the Au electrodes, their KBr pellets were prepared and their FTIR spectra were recorded as described in section 3.4.2.4.

EIS studies were carried out to investigate immobilization of enzyme onto cMWCNT/AuNPs/CHIT/Au electrode. The EIS studies were conducted on Autolab Potentiostat/Galvanostat after dipping the three electrodes system in 0.05 M PB (pH 7.5) containing 5 mM K₃Fe(CN)₆/K₄Fe(CN)₆ (1:1) as a redox probe. During an impedance measurement, a frequency response analyser (FRA) was used to impose a small amplitude AC signal to the electrode via the load. The AC voltage and current response of the electrode was analyzed by the FRA to determine the resistive, capacitive and inductive impedance behaviour of the electrode at that particular frequency. When conducted over a broad range of frequencies, impedance spectroscopy was used to identify and quantify the impedance associated with these various processes. EIS data for electrochemical cells such as enzyme electrode were most often represented in Nyquist plot. A complex plane
or Nyquist plot depicts the imaginary impedance, which was indicative of the capacitive and inductive character of the cell, versus the real impedance of the cell. Nyquist plots have the advantage that activation-controlled processes with distinct time-constants show up as unique impedance arcs and the shape of the curve provides insight into possible mechanism or governing phenomena.

3.5. Construction and testing of amperometric β-ODAP biosensor

An amperometric β-ODAP biosensor was constructed using a three-electrode electrochemical cell system, consisting of an enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/Au) as working electrode, a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxiliary electrode. These electrodes were connected through Autolab Potentiostat/Galvanostat (Fig. 12). The electrode system was dipped into a reaction mixture containing 10 ml 0.05 M PB, pH 7.5 and 0.5 ml L-glutamate solution (0.1 mM). The electrode response was measured in terms of milliampere (mA) applying a potential range of 0 to 0.3 V vs Ag/AgCl. The maximum current was obtained at 0.135V vs Ag/AgCl and hence subsequent studies were carried at this potential. The reaction involved oxidation of L-glutamate into H$_2$O$_2$ by immobilized GluOx. Then H$_2$O$_2$ was splitted into O$_2$, H$^+$ and 2e$^-$ at applied potential. The electrons released were transferred to the working electrode, to be relayed to potentiometer, in which it was read as current in mA. The following electrochemical reactions occurred during the response measurement:

$$\text{L-glutamate} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GluOx}} \text{2-oxoglutarate} + \text{NH}_3 + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 \xrightarrow{0.135 \text{ V}} \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$$

3.6. Optimization of working conditions of β-ODAP biosensor

Various kinetic properties of GluOx immobilized onto cMWCNT/AuNPs/CHIT composite film coated Au electrode were studied such as effect of pH, incubation temperature for maximum activity, time of incubation and effect of substrate (glutamate) concentration to optimum working conditions of enzyme electrode. All measurements were carried out with electrode through potentiostat/Galvanostat at a constant potential of 0.135 V vs Ag/AgCl.
3.6.1. Effect of pH

To determine the optimum pH of the enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/Au), the pH of reaction mixture was varied from 5.0 to 10.0 at an interval of 0.5 using the following buffer, each at a final concentration of 0.1 M: pH 5.0 to 6.0 sodium acetate buffer, pH 6.5 to 7.5 sodium phosphate buffer, pH 8.0 to 9.0 Tris-HCl buffer and pH 9.5 to 10.0 glycine buffer.

![Autolab Potentiostat/Galvanostat equipped with an Autolab PGSTAT-302N, general purpose electrochemical system (GPES) and frequency response analysis (FRA) software [A] and three-electrode electrochemical cell system, consisting of a working electrode (GluOx/cMWCNT/AuNPs/CHIT/Au), a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxiliary electrode [B]](image)

3.6.2. Effect of incubation temperature

To determine the optimum incubation temperature for maximum response of the enzyme electrode, the reaction mixture was incubated at different temperatures ranging from 20 to 50°C at 5°C interval. The current response in terms of mA was measured using Autolab potentiostat/galvanostat.

3.6.3. Effect of incubation time

To study the effect of incubation time for maximum response of the enzyme electrode, the reaction mixture was incubated at different time. The current in terms of mA was measured using Autolab potentiostat/galvanostat upto 40 s at an interval of 2 s.
3.6.4. Effect of substrate (L-glutamate) concentration

The effect of L-glutamate concentration on the initial velocity of GluOx reaction was studied by varying the final concentration of glutamate from 2 µM to 700 µM. The immobilized enzyme current responses were measured in mA by enzyme electrode using Autolab potentiostat/galvanostat.

3.6.5. Determination of $K_m$ and $I_{\text{max}}$

A Lineweaver Burk plot was made between reciprocal of glutamate concentration ($1/[S]$) vs enzyme electrode current response ($1/I$) of the enzyme reaction. Apparent $K_m$ and $I_{\text{max}}$ were calculated from the plot using the following Michaelis-Menten equation:

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

Where slope $= \frac{K_m}{I_{\text{max}}}$; intercept $= \frac{1}{I_{\text{max}}}$

$K_m = $ Michaelis-Menten constant

$I_{\text{max}} = $ Maximum current response of enzyme electrode

3.7. Amperometric determination of β-ODAP in seed extracts of *Lathyrus sativus*

The *Lathyrus sativus* seeds (400 g) were ground to powder and then homogenised in DW (2 l) in a pestle and mortar at room temperature (25°C). The homogenate/slurry was kept at 60-65°C for 15 hr with occasional stirring for evaporation of DW and then passed through filter paper. The residue from the filtered slurry was further extracted with DW (1 l). The combined water extracts were treated with alcohol to achieve 75% concentration, stirred, and filtered after half an hour. The filtrate was concentrated under reduced pressure to about 1.5 l. The concentrate was extracted first with ether (1 liter) and subsequently with chloroform (1 liter) (Rao et al., 1964). Then aqueous phase was separated and loaded onto a Dowex column ($r \times h = 0.5 \text{ cm} \times 25 \text{ cm}$) pre-equilibrated with sodium phosphate buffer (pH 2.5, 0.1 M). The column was washed in the same buffer until no protein was detected in the eluting buffer. The column was eluted using a linear gradient of 0.1 M-0.8 M KCl in sodium phosphate buffer (0.1 M, pH =2.5) and fractions (1 ml) each were collected at a flow rate of 0.5 ml/min. Each fraction was analysed.
amperometrically for β-ODAP using the present biosensor in the similar manner as described above for its testing/response measurement, under its optimal working conditions except that Glu was replaced by fraction. A plot between fraction no. and current response was drawn and total current response was calculated from the area of peak for β-ODAP (Fig. 13).

Fig.13. Current response in different fractions of *Lathyrus* seed extract eluted from ion exchanger
3.7.1. Preparation of standard curve for glutamate using enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/AuE) electrode

The standard curves using enzyme/working electrode was prepared with different concentrations of glutamate in the range 2 µM to 550 µM under optimal working conditions, as described above. A curve was plotted between glutamate concentration vs current in mA (Fig. 14). The β-ODAP concentration was interpolated from a standard curve of Glu concentrations v/s current prepared under optimal conditions.

3.8. Evaluation of β-ODAP biosensor employing enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/AuE)

The following parameters were studied in order to evaluate β-ODAP biosensor employing enzyme electrode for β-ODAP determination.

3.8.1. Detection limit (LOD)

In order to check limit of detection of the method, amperometric responses for enzyme electrode were studied at varying glutamate concentration ranging from 2µM to 700µM
and current response was measured through potentiostat/galvanostat. LOD was calculated as follows:

$$LOD = 3.3 \left( \frac{SD}{S} \right)$$

$SD = $ standard deviation of the response

$S = $ slope of the calibration curve

### 3.8.2. Precision

To study reproducibly of the method, the $\beta$-ODAP content was determined in *Lathyrus sativus* seed extract repeatedly six times on the same day (within batch) and in the same samples after their storage at -20°C for one week (between batch) by the present enzyme electrode. Both within and between batches coefficients of variation (CV) in *Lathyrus* seed extract were calculated as follows:

**Coefficient of variation (CV)**

$$CV = \frac{\sigma \times 100}{\alpha}$$

Where $\sigma = $ SD

$\alpha = $ means of series

**Standard deviation (SD)**

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}$$

Where $x = $ deviation from mean

$n = $ number of samples

**Standard error (SE)**

$$SE = \frac{\sigma}{\sqrt{n}}$$
3.8.3. Accuracy

In order to determine accuracy of present method, the glutamate values in *Lathyrus sativus* seed extract was determined by the present enzyme electrode method \((y)\) and compared with those obtained by chemical spectrophotometric method \((x)\), the values obtained by both the methods were co-related using regression equation. A regression plot was drawn between the two methods and the correlation coefficient was calculated using following formula:

\[
\text{Correlation coefficient } (r) = \sqrt{\frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{n\Sigma x^2 - (\Sigma x)^2} \sqrt{n\Sigma y^2 - (\Sigma y)^2}}}
\]

Where \(x\) = value obtained by reference chemical spectrophotometric method

\(y\) = values obtained by present enzyme electrode method

3.8.4. Determination of *Lathyrus* seed extracts \(\beta\)-ODAP by chemical spectrophotometric method (Rao et al., 1978)

The standard chemical spectrophotometric method for \(\beta\)-ODAP determination was performed as follows:

It involves alkaline hydrolysis of \(\beta\)-ODAP to L-\(\alpha\), \(\beta\)-diaminopropanoic acid (DAP) followed by reaction with orthopthalaldehyde (OPT), which, in the presence of ethane thiol gives a colored adduct which can be measured at 420 nm. The method, however, does not discriminate between the non-toxic \(\alpha\) form and the toxic \(\beta\) isomeric form as both of them hydrolyse to DAP.

Assay of \(\beta\)-ODAP: In 15 ml test tube, 1 ml of *Lathyrus* seed extract mixed with 0.2 ml of 3 N KOH was added and the tubes were kept in a boiling water bath for 30 min. After cooling the tubes to room temperature 2 ml of the OPT reagent was added. The absorbance of the yellow solution was measured after 30 min at 420 nm against a reagent blank.
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OPT reagent: 100 mg of OPT in 1 ml of 95% ethanol and 0.2 ml of mercaptoethanol were added to 99 ml of potassium borate buffer. This reagent although freshly made and used can also be used for as long as 3 days.

3.9. Interference study

The following metabolites such as cysteine, methionine, lysine, aspartic acid, glycine, histidine, leucine, isoleucine, asparagine, proline, phenylalanine, valine, threonine, tyrosine, tryptophan, arginine, serine, alanine were added individually in the reaction mixture each at a final concentration of 1 mM. The biosensor response i.e. current (μA) was measured and compared with that where none was added (control) and % relative response was calculated considering the control as 100 %.

3.10. Reusability and storage stability of enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/AuE)

To reuse the working electrode, it was washed by dipping it in 0.1 M, pH 7.5 sodium phosphate buffer. The long-term storage and stability of the biosensor was investigated over a 3 months period, when enzyme electrode was stored in a refrigerator at 4°C in 0.1 M PB, pH 7.5.