List of Research publications based on Ph.D work


Construction and application of $\beta$-(3-$N$-oxalyl-$L$-2,3-diaminopropanoic acid) biosensor based on carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan/Au electrode

Bhawna Batra and C. S. Pundir*

We describe the construction of a new enzyme electrode (glutamate oxidase/carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan/gold electrode: GluOx/cMWCNT/AuNPs/CHIT/Au) and its application for the amperometric determination of $p$-ODAP (3-$N$-oxalyl-$L$-2,3-diaminopropanoic acid) in Lathyrus seeds. The enzyme electrode was characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The biosensor showed an optimum response within 2 s at pH 7.5 and 35 °C when operated at 0.135 V. A good linear relationship was observed between the biosensor response, i.e. the current (mA), and $\gamma$-glutamate (Glu) concentration in the range of 2–550 gM. The biosensor had a detection limit of 2.32 gM with a high sensitivity of 486.31 gA cm$^{-2}$ gM$^{-1}$. The biosensor measured $p$-ODAP in a glutamate-free Lathyrus seed extract. The results for ODAP efficiently correlated ($r = 0.9969$) with those by the standard colorimetric method. The enzyme electrode lost only 35% of its initial activity after its 70 regular uses over a period of 90 days.

1. Introduction

$\beta$-ODAP (3-$N$-oxalyl-$L$-2,3-diaminopropanoic acid or $\beta$-$N$-oxalyl-$L$-$\alpha$, $\beta$-diaminopropanoic acid), a neurotoxin, occurs in the seeds of the grass pea (Lathyrus sativus L. family Fabaceae syn Leguminosae), which is a high-yielding, drought resistant legume.$^{1,2}$ Grass pea seeds are consumed as a food in Northern India and in the neighboring countries, as well as in Ethiopia. However, grass peas could not be developed as an important food legume due to the presence of this neurotoxin in the seeds. The consumption of Lathyrus seeds in large quantities for a prolonged period can cause neurolathyrism, which is characterized by spastic paraparesis, in particular involving the lower limbs.$^{3,4}$

In spite of its neurotoxicity, $\beta$-ODAP exhibits several physiological functions in grass peas, such as a carrier molecule for zinc ions,$^{5}$ as a scavenger for hydroxyl ions$^{6}$ and as a protector of photosynthesis at high light intensity.$^{7}$ ODAP exists in the plant in two isomeric forms, the $\alpha$-$N$-oxalyl derivatives, but only the $\beta$-isomer is toxic. The content of $\beta$-ODAP in the seeds of L. sativus has been reported in the range of 0.5–2.5%. The safe concentration of ODAP for human consumption is lower than 0.2%.$^{8}$

Therefore, the determination of $\beta$-ODAP in Lathyrus seeds is very important. Various analytical methods such as colorimetric,$^{9}$ flow-injection analysis,$^{10}$ high-performance liquid chromatography (HPLC),$^{11}$ reversed-phase high-performance liquid chromatography,$^{12}$ capillary zone electrophoresis$^{13,14}$ paper chromatography, liquid chromatography using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene$^{15}$ and 6-aminoquinolyl-$N$-hydroxysuccinimidyl carbamate (AQC)$^{16}$ and high-pressure thin-layer chromatography (HPTLC)$^{17}$ have been reported for the determination of $\beta$-ODAP in grass pea seeds. However, all of these methods suffered from glutamate interference. Either liquid chromatography (LC)$^{18,19}$ or an enzymatic method using glutamate decarboxylase$^{20}$ were employed to remove glutamate interference but it was time-consuming, expensive and complicated due to the enzyme/protein. Moreover, most of these methods did not satisfy the requirements for a simple, fast, accurate and specific method. However, biosensing methods have many advantages over these methods because of their simplicity, rapidity, high sensitivity and specificity. These $\beta$-ODAP biosensors were based on graphite rods modified with Os$_2$Cl$_4$,$^{21}$ graphite electrodes,$^{22}$ screen-printed carbon electrodes modified with MnO$_2$,$^{23}$ Os containing a mediating polymer, polyethylene glycol diglycidyl ether$^{24}$ and prussian blue-modified GC electrode.$^{25}$ However, these electrodes suffered from limited electron communication, complexity of immobilization and low stability of enzymes. The use of nanomaterials in the construction of a biosensor has offered great opportunities to improve their sensitivity, stability and anti-interference ability. Recently, carbon nanotubes (CNTs) have also been incorporated into the transducers of

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electrochemical sensors due to their high surface-to-volume ratios and chemical functionalization. These CNT-based sensors have shown comparatively higher sensitivities, lower limits of detection, and faster electron-transfer kinetics. Among the metallic nanomaterials employed in the biosensors, gold nanoparticles (AuNPs) have received the greatest attention due to their high surface-to-volume ratio and high surface energy, which allow a stable immobilization of biomolecules and ability to permit fast and direct electron transfer between a wide range of electroactive species and electrode materials.

Chitosan (CHIT), a cationic polysaccharide with abundant amines has excellent film-forming ability, good biocompatibility and adhesion, thus employed as an excellent matrix for the immobilization of enzyme to fabricate enzyme electrodes.

The present report describes a new strategy for the removal of l-glutamate from Lathyrus sativus (gramineae) and ability to permit fast and direct electron transfer between a novel enzyme electrode comprising glutamate oxidase immobilized onto carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan composite film, electrodeposited on a Au electrode for the amperometric determination of β-O-DAP.

2. Experimental

2.1. Materials

l-Glutamic acid, gold chloride (HAuCl₄), N-ethyl-Y-(3-dimethylamino propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), CHIT obtained from SISCO Research Lab., Mumbai, India; carboxylated multi-walled carbon nanotubes (cMWCNT) obtained from Intelligent Materials Pvt. Ltd., Panchkula, (Haryana) India; GluOx, anion exchanger “Dowex® 1 x 8 chloride form” (mesh size:50–100) from Sigma-Aldrich, St Louis, Mo., USA, were used. Grass pea (Lathyrus sativus) seeds were purchased from an open market of Bhagalpur (Bihar). Double-distilled water (DW) was used throughout the experimental studies.

2.2. Apparatus

UV Spectrophotometer (Shimadzu, Model 160 A), X-ray diffractometer (XRD), (122 Rigaku, D/Max5500, Tokyo, Japan), transmission electron microscope (TEM JEM 2100 F), dynamic light scattering (DLS) (Wyatt Technology, Amity University, Noida, India). TEM at Jawahar Lal Nehru University, New Delhi, on a commercial basis and DLS at the Amity Institute of Nanotechnology, Amity University, Noida, India.

2.3. Construction of GluOx/cMWCNT/AuNP/CHIT-modified Au electrode

2.3.1. Preparation of AuNP. AuNPs were prepared as described in the literature with slight modifications. Briefly, 100 ml aqueous HAuCl₄ (0.01%) was heated to boil on a temperature controlled magnetic stirrer, and 2.5 ml 1% trisodium citrate solution was added to it with vigorous stirring until wine red coloured colloidal gold nanoparticles (AuNPs) suspension was obtained. This AuNPs suspension was stored in the dark in a glass bottle at 4 °C until further use.

2.3.2. Characterization of AuNPs. AuNPs were characterized by UV-visible spectroscopy and XRD at the Department of Physics, Guru Jambheshwar University, Hisar, Haryana (India), TEM at Jawahar Lal Nehru University, New Delhi, on a commercial basis and DLS at the Amity Institute of Nanotechnology, Amity University, Noida, India.

2.3.3. Electrodeposition of cMWCNT/AuNP/CHIT onto Au electrode. 1 mg of c-MWCNT was suspended into a 4 ml mixture of concentrated H₂SO₄ and HNO₃ in 3 : 1 ratio and ultra-sonicated for 2 h to obtain a homogeneous mixture. The dispersed cMWCNT solution (0.1 ml) was then added into a mixture of 0.5 ml EDC (0.2 M) and 0.5 ml NHS (0.2 M); its pH was adjusted to 6.0 and maintained at room temperature for 1 h. The surface of a Au electrode (0.30 cm²) was manually polished by alumina slurry (diameter of 0.05 μm) with a polishing cloth, thoroughly washed with DW, placed into ethanol, sonicated to remove adsorbed particles and finally washed three to four times with DW. CHIT (0.5% in acetic acid, 200 μl) was added to 25 ml 1 M KCl and electrodeposited onto a Au electrode through cyclic voltammetry using the galvanostat, applying 20 successive deposition cycles between −0.15 to 0.20 V at a scan rate of 20 mV s⁻¹. During the electrochemical polymerization, the surface of Au wire gradually became green, indicating the deposition of cMWCNT–AuNPs onto CHIT film-modified Au wire. The resulting cMWCNT/AuNP/CHIT-modified gold electrode was thoroughly washed with DW to remove the unbound matter and stored in a dry Petri-plate at 4 °C.

2.3.4. Immobilization of GluOx onto cMWCNT/AuNP/CHIT-modified Au electrode. A cMWCNT/AuNP/CHIT/Au electrode was placed into 2 ml of sodium phosphate buffer (0.1 M, pH 7.4) containing 100 μl of GluOx (2.3 U ml⁻¹) and stored overnight at 4 °C for immobilization. The resulting bioelectrode (GluOx/cMWCNT/AuNP/CHIT/Au electrode) was washed three to four times with 0.1 M sodium phosphate buffer, pH 7.4 to remove unbound enzyme and used as working electrode. It was stored at 4 °C when not in use.

2.3.5. Characterization of GluOx/cMWCNT/AuNP/CHIT/Au electrode. The fabricated enzyme electrode was characterized by the scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS).

To investigate the morphology of a cMWCNT/AuNP/CHIT/Au electrode with and without immobilized enzymes, the electrodes were cut into small pieces (1 cm) and placed on a specimen chamber of diameter 2 cm using a spray gun, in general, rigidly mounted on a specimen holder called a specimen stub, and their images were taken by a scanning electron microscope.
To record the FTIR spectra of an enzyme electrode at the different stages of its construction, the deposited material was scrapped off the Au electrodes, ground with dry potassium bromide (KBr). This powder was then pressed in a mechanical press to form a translucent pellet transparent to the beam of the spectrometer. The pellet was kept in the socket of the FTIR spectrophotometer, and its spectrum was recorded.

The EIS studies of the enzyme electrodes before and after the immobilization were carried out in a Potentiostat/Galvanostat equipped with FRA in the frequency range, 0.01 to 105 Hz, amplitude 5 mV, after dipping the three-electrode 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, an FRA was used to impose a low-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.

2.3.6. Cyclic voltammetric measurements and testing of β-ODAP biosensor. Cyclic voltammogram (CV) of the GluOx/cMWCNT/AuNPs/CHIT/Au electrode was recorded in a galvanostat between −0.1 to 0.6 V vs. Ag/AgCl as the reference and Pt wire as the counter electrode in 30 ml 0.1 M sodium phosphate buffer (pH = 7.4) containing 1 mM Glu (100 μl). The maximum response was observed at 0.135 V, therefore the subsequent amperometric studies were carried out at this voltage.

2.4. Optimization of β-ODAP biosensor

To determine the optimum pH, the pH of the reaction buffer was varied from 5.0 to 10.0 with an interval of 0.5 each buffer at a final conc. of 0.1 M: using sodium acetate buffer for pH 5.0 to 6.0, sodium phosphate buffer for pH 6.5 to 7.5, Tris-HCl buffer for pH 8.0 to 9.0 and glycine buffer for pH 9.5 to 10.0. Similarly, optimum temperature was estimated by incubating the reaction mixture at various temperatures (20–50 °C) and durations (1–40 s). The effect of the Glu concentration on the biosensor response was determined by varying its concentration from 2 μM to 700 μM.

2.5. β-ODAP determination in seed extracts by GluOx/cMWCNT/AuNPs/CHIT/AuE

The grass pea (Lathyrus sativus) seeds (400 g) were ground into powder and then homogenised in DW (2 L) with a mortar and pestle at room temperature (25 °C). The homogenate/slurry was maintained at 60–65 °C for 15 h with occasional stirring for the evaporation of DW and then passed through a 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 30 min. The filtrate was concentrated under a reduced pressure to about 1.5 liters. The concentrate was first extracted with ether (1 liter) and subsequently with chloroform (1 liter). The aqueous phase was then separated and loaded onto a Dowex column (r x h = 0.5 cm x 25 cm) pre-equilibrated with a sodium phosphate buffer (pH = 2.5, 0.1 M). The column was washed with the same buffer and was eluted using a linear gradient of 0.1 M–0.8 M KCl and each fraction (1 ml) was collected at a flow rate of 0.5 ml min⁻¹. Each fraction was amperometrically analysed for β-ODAP using the present biosensor in the similar manner as previously described for evaluating its testing/response measurement under the optimal working conditions, except that Glu was replaced by certain fraction. A plot between the fraction number and current response was drawn, and the total

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Fig. 1 Current response in various fractions of Lathyrus seed extract eluted from ion exchanger.

Fig. 2 (A) Calibration curve in a concentration range of 2–10 μM l-glutamate. (B) Standard curve for l-glutamate by β-ODAP biosensor based on GluOx/cMWCNT/AuNPs/CHIT-based Au electrode.
current response was calculated from the area of the peak for β-ODAP (Fig. 1). The β-ODAP concentration was calculated from a standard curve of Glu concentrations vs. a current prepared under optimal conditions (Fig. 2).

2.6. Interference study
The interferents, such as cysteine, methionine, lysine, aspartic acid, glycine, histidine, leucine, isoleucine, asparagine, proline, phenylalanine, valine, threonine, tyrosine, tryptophan, arginine, serine, and alanine, were individually added 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 no interferent was added (control) and % relative response was calculated considering the control as 100%.

2.7. Reusability and storage stability of GluOx/cMWCNT/AuNP/CHIT/Au electrode
To reuse the working electrode, it was washed by dipping in 0.1 M sodium phosphate buffer of pH 7.5. The long-term storage and stability of the biosensor was investigated over a three-month period, during which the enzyme electrode was stored in a refrigerator at 4 °C in 0.1 M PB, pH = 7.5.

3. Results and discussion
3.1. Characterization of AuNPs
AuNPs were characterized by UV-visible spectroscopy, XRD, TEM and DLS (Fig. 3A–D). UV-visible spectra showed strong absorbance peaks at 560 nm, confirming the synthesis of AuNPs (Fig. 3A). The XRD patterns of AuNPs depicted a well-defined diffraction pattern, which was in agreement with JCPDS card no. 089-3697. The spherical shapes of AuNPs with an average size of 20 nm were confirmed by TEM images (Fig. 3C). DLS utilizes the temporal variations of scattered light fluctuations to measure an average hydrodynamic diameter of particles suspended in a liquid medium. AuNPs are extraordinary light scatterers at or near their surface plasmon resonance wavelength. The dynamic light scattering provided the diameter of the gold nanoparticles to be 78.5 nm, which might be due to their aggregation (Fig. 3D).

3.2. Morphological characterization of Au electrode by SEM studies
Fig. 4 shows the stepwise modification of the electrode. The SEM image of the bare Au electrode showed uniform morphology (Fig. 4a). SEM images of cMWCNT/AuNP/CHIT/Au exhibited homogenous and cable-like morphology of the nanostructure of cMWCNT/AuNP/CHIT/Au composite film, which showed that c-MWCNTs were well-dispersed in the composite film (Fig. 4b). After the immobilization of GluOx on cMWCNT/AuNP/CHIT/Au composite film, the hybrid bio-electrode showed the sporadic appearance of a beaded structure, indicating that GluOx was successfully immobilized on the surface of cMWCNT/PANI composite film (Fig. 4c).

![Fig. 3](image-url)
3.3. Fourier transform infrared (FTIR) spectroscopy

Fig. 5 (A) shows FTIR spectra for the CHIT/Au electrode (curve i), cMWCNT/AuNP/CHIT/Au electrode (curve ii) and GluOx/cMWCNT/AuNP/CHIT/Au electrode (curve iii). The FTIR spectra of electrodeposited CHIT/Au composites show a peak at 1745 cm\(^{-1}\) due to the C=O stretching. The peak at 1650 cm\(^{-1}\) can be attributed to the C-O stretching along with the N-H deformation mode and 1096 cm\(^{-1}\) to the stretching vibration mode of the hydroxyl group. Curve (ii) shows the FTIR spectra of the cMWCNT/AuNP/CHIT/Au electrode, revealing several significant peaks. The peak at 1558 cm\(^{-1}\) corresponds to the stretching mode of the C=C double bond that formed the framework of the carbon nanotube sidewall. The peaks at 1730 and 1028 cm\(^{-1}\) correspond to the stretching modes of the C=O groups. The FTIR spectrum of GluOx/cMWCNT/AuNP/CHIT/Au bioelectrode (curve iii) shows the appearance of additional band at 1695 cm\(^{-1}\) assigned to the
carbonyl stretch, indicating the covalent binding of GluOx with cMWCNT.

### 3.4. Electrochemical impedance measurements

EIS provides an effective method for probing the electronic features of surface-modified electrodes. Fig. 6 shows Nyquist plots obtained for (a) bare Au electrode (b) cMWCNT/AuNP/CHIT/Au electrode and (c) GluOx/cMWCNT/AuNP/CHIT/Au electrode, respectively. Nyquist diameters (real axis value at lower frequency intercept) indicated the value of charge-transfer resistance ($R_{\text{ct}}$), i.e. hindrance, provided by the electrode material to transfer the charge from the solution to the electrode, which is correlated with the modification of the surface. The $R_{\text{ct}}$ value obtained for the GluOx/cMWCNT/AuNP/CHIT/Au electrode (curve c), 570 Ω, was higher than that of the cMWCNT/AuNP/CHIT/Au electrode (curve b), 400 Ω, which can be attributed to the insulating nature of GluOx that inhibits the permeability of $\text{[Fe(CN)₆]}^{3−}$ to the electrode surface.

### 3.5. Construction and response measurement of β-ODAP biosensor

An enzyme (GluOx) electrode was fabricated by covalently immobilizing GluOx from *Streptomyces* onto a cMWCNT/AuNP/CHIT/Au electrode using EDC and NHS chemistry. EDC was used to conjugate the free carboxyl (−COOH) groups of cMWCNT to the amine (−NH₂) groups of the GluOx, using NHS as a catalyst. Fig. 7 showed a larger redox peak of the GluOx/cMWCNT/AuNP/CHIT/Au electrode than that of the GluOx/cMWCNT/CHIT/Au electrode and GluOx/AuNP/CHIT/Au electrodes, which corresponded to the oxidation of Glu on the surface of the modified electrode, as well as the enhancement of the current. It appeared that the cMWCNT/AuNP/CHIT/Au nanocomposite provided a biocompatible environment to the GluOx, cMWCNT and AuNP to act as an electron mediator, resulting in an accelerated electron transfer between the enzyme and electrode.

During the response measurement of the present biosensor, GluOx catalysed the oxidation of β-ODAP into an α-keto acid and H₂O₂, which get dissociated into O₂ + 2H⁺ + 2e⁻ at 0.135 V. These electrons flow through the cMWCNT and AuNPs to the Au electrode and generate a signal in the form of current. It appeared that the cMWCNT/AuNP/CHIT/Au nanocomposite provided a biocompatible environment to the enzyme, whereas the cMWCNT and AuNPs acted as an electron mediator, resulting in an accelerated electron transfer between the enzyme and electrode surface. All of these electrochemical reactions are summarised in Fig. 8.

### 3.6. Optimization of biosensor

The experimental conditions affecting the biosensor response were studied in terms of the effect of pH, incubation...
temperature and substrate (Glu) concentration. The biosensor showed optimum response, i.e. current, at pH 7.5 (Fig. 9a), which is almost similar to that of earlier biosensors (Table 1). The optimum incubation temperature for the biosensor was 35 °C (Fig. 9b). There was a hyperbolic relationship between the electrode response and Glu concentration in the range of 2-700 μM with linearity in the range of 2-550 μM (Fig. 2), which is comparatively better than those of the earlier biosensors (Table 1). Similarly, the detection limit (LOD) of the present biosensor was 2.32 pM (S/N = 3), which is also better/ lower than that for the earlier biosensors (Table 1). The lower LOD of the present biosensor might be due to the film of carboxylated multiwalled carbon nanotubes and gold nanoparticles attached on the Au electrode through chitosan film, which provided a high biocompatibility and rapid electron transfer rate between the enzyme and electrode.

3.7. Determination of β-ODAP in seed extract

3.7.1. Preparation of glutamate-free sample of Lathyrus seeds. A new method has been developed to remove the glutamate interference from β-ODAP containing grass pea extract using Dowex 1 x 8, an anion exchanger. The removal was based on the different isoelectric points of glutamate (pI = 3.22) and β-ODAP (pI = 2.02). When a sodium phosphate buffer (0.1 M, pH = 2.5, pK_a = 2.12) was passed through this anion exchanger, glutamate and β-ODAP acquire positive and negative charges, respectively. At this stage, glutamate (positively charged at pH = 2.5) passes through the column, while β-ODAP (negatively charged at pH = 2.5) remains attached to the ion exchanger (positively charged at pH = 2.5) leading to the removal of glutamate from β-ODAP. The glutamate-free sample containing β-ODAP was eluted from the Dowex ion exchanger column in the same buffer using a continuous ionic gradient (0.1 M-0.8 M KC1) and analysed for the amperometric determination of β-ODAP by the present biosensor (Fig. 10). The present method for the removal of glutamate interference is better than the earlier LC method based on the Carbopac anion exchange column, as the former has no chance of mixing

Table 1. Comparison of analytical properties of β-ODAP biosensors

<table>
<thead>
<tr>
<th>Properties</th>
<th>Belay et al., 1997</th>
<th>Yigzaw et al., 2001</th>
<th>Yigzaw et al., 2002</th>
<th>Beyene et al., 2003</th>
<th>Beyene et al., 2004</th>
<th>Marichamy et al., 2005</th>
<th>Varma et al., 2006</th>
<th>Present work</th>
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</thead>
<tbody>
<tr>
<td>Support of immobilization rod</td>
<td>Graphite rod modified with Os</td>
<td>Graphite rod containing mediating polymer with polyethylene glycol diglycidyl ether</td>
<td>Screen-printed carbon electrode modified with MnO_2</td>
<td>Screen-printed carbon electrode modified with MnO_2</td>
<td>Graphite electrode</td>
<td>Prussian blue-modified GC</td>
<td>cmWNT/ AuNP/ CHIT/Au</td>
<td></td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.0</td>
<td>7.5</td>
<td>7.5</td>
<td>7.75</td>
<td>7.75</td>
<td>7.5</td>
<td>7</td>
<td>7.5</td>
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<tr>
<td>Optimum voltage</td>
<td>-50 mV</td>
<td>-</td>
<td>-50 mV</td>
<td>-</td>
<td>440 mV</td>
<td>-50 mV</td>
<td>-</td>
<td>135 mV</td>
</tr>
<tr>
<td>Linearity</td>
<td>1-250 μM</td>
<td>1-250</td>
<td>0.284-28.4 mM</td>
<td>195-1950 μM</td>
<td>1-600 μM</td>
<td>0.05-1 mM</td>
<td>2-550 μM</td>
<td></td>
</tr>
<tr>
<td>Detection limit</td>
<td>4 μM</td>
<td>2 μM</td>
<td>2 μM</td>
<td>0.164 μM</td>
<td>111 μM</td>
<td></td>
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<tr>
<td>Response time</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td></td>
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<tr>
<td>Sensitivity</td>
<td>4.6 mA M(^{-1}) cm(^{-2})</td>
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<td>Storage stability</td>
<td>2 days</td>
<td>2 days</td>
<td>65 days</td>
<td>40 days</td>
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Fig. 10 (i-ODAP concentration in fractions 1 to 6 (each 1.5 ml) as measured by β-ODAP biosensor.

glutamate with β-ODAP, whereas the latter method provides such chances (due to closeness of elution peaks for glutamate and β-ODAP). The method is also significantly better than the previous method, which included the treatment of sample with a glutamate decarboxylase/protein, which might undergo hydrolysis to generate the interfering amino acids.  

3.7.2. Measurement of β-ODAP. The β-ODAP content in the seed extract measured by the present biosensor was 19.2 μM, which amounts to 1.62 ± 0.06 g kg⁻¹ of seed powder (mean ± SD) (on a fresh-weight basis). There was a good correlation (r = 0.9969) between these values and the values obtained by colorimetric method (Fig. 11).

3.8. Long-term stability of enzyme electrode
The enzyme electrode lost 35% of its initial activity after its 70 regular uses over a period of 90 days. This stability of the enzyme electrode is better than the earlier enzyme electrodes (Table 1).

3.9. Interference study
Among the various substances tested, including cysteine, methionine, lysine, aspartic acid, glycine, histidine, leucine, isoleucine, asparagine, proline, phenylalanine, valine, threonine, tyrosine, tryptophan, arginine, serine and alanine (each at 1 mM), practically none had any interference on the present biosensor response.

4. Conclusion
A new method was developed for the removal of glutamate interference for the measurement of β-ODAP in Lathyrus seed extract, which is better than the previously reported methods. An improved β-ODAP biosensor was constructed based on a nanocomposite thin film of a carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan. The biosensor showed a rapid response (2 s), lower detection limit (2.32 μM), higher sensitivity (486.31 μA cm⁻² μM⁻¹), broader working range (2-550 μM) and longer stability (three months). This nanocomposite film could also be used for the improvement of other biosensors.

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References
An amperometric glutamate biosensor based on immobilization of glutamate oxidase onto carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan composite film modified Au electrode

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ABSTRACT
A method is described for the construction of a novel amperometric glutamate biosensor based on covalent immobilization of glutamate oxidase (GluOx) onto, carboxylated multi walled carbon nanotubes (cMWCNT), gold nanoparticles (AuNPs) and chitosan (CHIT) composite film electrodeposited on the surface of a Au electrode. The GluOx/cMWCNT/AuNP/CHIT modified Au electrode was characterized by scanning electron microscopy (SEM), fourier transform infra-red (FTIR) spectroscopy, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The biosensor measured current due to electrons generated at 0.135 V against Ag/AgCl from H2O2, which is produced from glutamate by immobilized GluOx. The biosensor showed optimum response within 2 s at pH 7.5 and 35 °C. A linear relationship was obtained between a wide glutamate concentration range (5-500 pM) and current (μA) under optimum conditions. The biosensor showed high sensitivity (155 nA/pM/cm2), low detection limit (1.6uM) and good storage stability. The biosensor was unaffected by a number of serum substances at their physiological concentrations. The biosensor was evaluated and employed for determination of glutamate in sera from apparently healthy subjects and persons suffering from epilepsy.

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1. Introduction
Glutamate (Glu) is one of the main excitatory neurotransmitter in the central nervous system (CNS) (Salt and Eaton, 1996), playing a major role in various neural functions, thus being an important analyte in medicine (Maeda et al., 1997; Haxhiu et al., 2000) and food analysis (Mankasingh et al., 2000; Perez-Ruiz et al., 2000). It is also involved in cognitive functions such as memory and learning (Jain et al., 2002). As Glu concentrations in blood are correlated with those in cerebrospinal fluid (CSF), (McGale et al., 1977; Alfredsson et al., 1988) serum levels may influence Glu concentration and function in brain. Prolonged elevated extracellular levels of Glu have been shown to be excitotoxic with the result of neuronal cell death ultimately. It is involved in epileptogenesis, initiation and spreading of seizures and seizure-related neuronal damage (Meldrum, 1994). Furthermore, alterations in Glu levels have been shown to be linked to several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, as well as ischemic stroke and amyotrophic lateral sclerosis. Hence the accurate measurement of Glu levels in vitro and in vivo for a better understanding of the physiological and pathological role of Glu in neurotransmission has remained challenging (Rieben et al., 2009). Different techniques have been developed to determine Glu, e.g. chromatographic (Zhang and Sun, 2005; Clarke et al., 2007; Shah et al., 2008; Acuna and Trias, 2009; Buck et al., 2009), spectrophotometric (Kampha et al., 2004; Tang et al., 2007; Acebal et al., 2008) and fluorimetric (Sanchez et al., 1992; Tcherkas and Denisenko, 2001; Tsukatani and Matsumoto, 2005; Chakraborty and Raj, 2007). Besides being highly sensitive, these methods have some limitations, including labor-intensive, requiring expert handling, involving pre-treatment of the sample, and being time consuming. Electrochemical methods are considered as one of the most potential approach, because of their simplicity, rapidity, high sensitivity and specificity. With advances in biosensor technology, it has become a popular and reliable technique for the estimation of different metabolites. With numerous advances reported in the field of biosensors, the immobilization of enzymes on electrodes in the designing and optimization of biosensors has attracted many researchers (Bhambi et al., 2010). But still there are a few issues in which improvement is needed, such as lack of stability, high response time, low reproducibility and the requirement of high working electrode potential. The biocompatible nanomaterials have opened a bright field towards the development of third generation biosensors based on the direct electron transfer between the enzyme and the electrode. Among these, Carbon nanotubes (CNTs) are promising materials for sensing
applications due to several intriguing properties. In particular, their large length-to-diameter aspect ratios provide high surface-to-volume ratios. Moreover, CNTs have an outstanding ability to mediate fast electron-transfer kinetics for a wide range of electroactive species, such as hydrogen peroxide or NADH (Balasubramanian and Burghard, 2006). They have established themselves as good materials for immobilization of enzymes due to the desirable microenvironment and enhance the direct electron transfer between the enzyme’s active sites and the electrode. Gold nanoparticles (AuNPs) are used increasingly in many electrochemical applications, since they have the ability to enhance the electrode conductivity and facilitate the electron transfer, thus improving the analytical selectivity and sensitivity (Guo and Wang, 2007; Cao et al., 2010). Chitosan (CHIT) has been gradually used for constructing sensors due to its attractive properties that include excellent film-forming ability, high permeability, good adhesion, nontoxicity, cheapness and a susceptibility to chemical modification (Ling et al., 2009; Yuan et al., 2008). It also facilitates the electron transfer after its swelling in reaction mixture due to its hydrophilic nature. We describe herein the construction of an improved amperometric Glu biosensor by immobilizing covalently glutamate oxidase (GluOx) onto carboxylated multiwalled carbon nanotubes (cMWCNT), AuNPs and CHIT hybrid film electrodeposited onto the Au electrode.

2. Materials and method

2.1. Materials

GluOx from Sigma-Aldrich St. Louis, USA, carboxylated multiwalled carbon nanotubes (cMWCNT) (Functionalized MWCNT) from Intelligent Materials Pvt. Ltd., Ranchkula (Haryana) India, Glutamic acid, acetic acid, N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), and CHIT from SISCO Research Lab., Mumbai, India, were used. Double distilled water (DW) was used throughout the experimental studies.

2.2. Apparatus

Potentiostat/Galvanostat (Autolab, model: AUTB378S, manufactured by Eco Chemie) with a three electrode system composed of a Pt wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and a GluOx/cMWCNT/AuNP/CHIT modified Au electrode as the working electrode. A transmission electron microscope (TEM) (JEOL 2100F) and scanning electron microscope (SEM) (Zeiss EV040), UV Spectrophotometer (Shimadzu, Model 160A), X-ray diffractometer (XRD), (122 Rigaku, D/Max2550, Tokyo, Japan) and Fourier transform infra-red spectrophotometer (FTIR) (Thermo Scientific, USA) were also used.

2.3. Construction of GluOx/cMWCNT/AuNP/CHIT modified Au electrode

2.3.1. Preparation of AuNP

AuNPs were prepared as described (Zhang et al., 2009), with some modification. Briefly, a 100 mL sample of aqueous HAuCl₄ (0.018) was brought to boil and 2.5 mL of 1% trisodium citrate solution was added to it with vigorous stirring. A wine-red colored colloidal gold nanoparticles suspension was obtained, which was stored in a dark glass bottle at 4 °C for further use.

2.3.2. Electrocdeposition of cMWCNT/AuNP/CHIT onto gold electrode

One milligram of cMWCNT was suspended into a 4 mL mixture of concentrated H₂SO₄ and HNO₃ in a 3:1 ratio, and ultrasonicated for 2 h to obtain a homogeneous mixture. The dispersed cMWCNT solution (0.1 mL) was mixed into a mixture of 0.5 mL EDC (0.2 M) and 0.5 mL NHS (0.2 M). The pH was adjusted to 6.0 and it was kept at RT for 1 h. The surface of a Au electrode (0.36 cm²) was polished manually by alumina slurry (diameter 0.05 μm) 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 three-four times. Chitosan (0.5% in acetic acid, 200 μL) was added to 25 mL of 1 M KCl and electrodeposited onto the Au electrode through cyclic voltammetry using a galvanostat by applying 20 successive deposition cycles at −0.15 to 0.20 V at a scan rate of 20 mV/s (Supplementary Fig. 1). The AuNPs suspension (200 μL) and 400 μL of EDC and NHS treated cMWCNT suspension were added into 25 mL 1 M KCl to get the cMWCNT/AuNP mixture. Finally, electrodeposition of AuNPs and cMWCNT onto the gold electrode was carried out in an electrochemical cell system applying 20 deposition cycles at −0.31 to 0.2 V at a 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. The resulting cMWCNT/AuNP/CHIT modified gold electrode was washed thoroughly with DW to remove unbound matter and kept in a dry Petri-plate at 4 °C.

2.3.3. Immobilization of GluOx onto cMWCNT/AuNP/CHIT modified Au electrode

GluOx/cMWCNT/AuNP/CHIT/Au electrode was placed into 2 mL of sodium phosphate buffer (0.1 M, 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/AuNP/CHIT/Au electrode) was washed three-four times with 0.1 M sodium phosphate buffer, pH 7.4, to remove unbound enzyme and used as the working electrode. It was stored at 4 °C when not in use.

2.3.4. Characterization of GluOx/cMWCNT/AuNP/CHIT/Au electrode by FTIR

To record FTIR spectra of the enzyme electrode at different stages of its construction, the deposited material was scrapped off the Au electrodes, grinded 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 can pass. Then this pellet was kept in the socket 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).

2.3.5. Construction, cyclic voltammetry measurement and testing of Glu biosensor

Cyclic voltammogram (CV) of GluOx/cMWCNT/AuNP/CHIT/Au electrode was recorded in a galvanostat from −0.15 to 0.3 V vs. Ag/AgCl as the reference and Pt wire as the counter electrode in 30 mL 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM Glu (100 μL). The maximum response was observed at 0.135 V (Fig. 1), and hence subsequent amperometric studies were carried out at this voltage.

2.4. Optimization of Glu biosensor

To determine optimum pH, the pH was varied from 5.0 to 10.0 at an interval of 0.5 each at a final concentration of 0.1 M: pH 5.0–5.5 sodium acetate buffer, pH 6.0–8.0 sodium phosphate buffer, pH 8.5–9.0 Tris–HCl buffer and pH 9.5–10.0 glycine buffer. Similarly, optimum temperature was studied by incubating the reaction mixture at different temperatures (20–50 °C) and times (1–40 s).
The effect of Glu concentration on biosensor response was determined by varying its concentration from 5 μM to 700 μM.

2.5. Serum Glu determination by Glu biosensor

Fresh serum samples from apparently healthy individuals (both male and female) and persons suffering from epilepsy were collected from the hospital at the local Pt. BDS Post Graduate Institute of Medical Science (Rohtak) and analyzed for Glu using the current electrode. The procedure was the same as described for response measurement of electrode under optimal working conditions and replaced by serum. The Glu concentration was determined by varying its concentration from 5 pM to 700 pM.

2.6. Storage stability of GluOx/cMWCNT/AuNP/CHIT/Au electrode

The stability of the working electrode was studied for 4 months by performing the assay on a weekly basis. The present electrode system was stored in dried condition at 4°C when not in use.

3. Results and discussion

3.1. Characterization of AuNP

The characterization of nanoparticles was carried out by UV and visible spectroscopy, XRD and TEM (Fig. 3A–C). UV and visible spectra exhibited a strong absorbance peak at 560 nm, confirming the synthesis of AuNP (Fig. 3A). The XRD patterns of the prepared AuNP clearly showed a well defined diffraction pattern, which was in agreement with JCPDS card no. 089-3697. (Fig. 3B). The typical TEM images of AuNP nanoparticles (Fig. 3C) showed the spherical shape of AuNP with an average size of 20 nm.

3.2. SEM studies of Au electrode during modification

The SEM images of the surface of bare Au electrode, cMWCNT/AuNP/CHIT/Au electrode and GluOx/cMWCNT/AuNP/CHIT/Au electrode are shown in Fig. 4(a)–(c). The stepwise modification of electrode could be seen clearly from these SEM images. The SEM image of the bare Au electrode showed a smooth and featureless morphology. The cMWCNT/AuNP/CHIT/Au composite film exhibited a net structure. Film is more uniform and porous, hence effective surface area is larger. On immobilization of GluOx, the globular structural morphology of GluOx/cMWCNT/AuNP/CHIT/Au changes into the regular form due to the interaction between cMWCNT/AuNP/CHIT/Au electrode and GluOx.

3.3. FTIR spectra

Fig. 5(A) shows FTIR spectra for the CHIT/Au electrode (curve i), cMWCNT/AuNP/CHIT/Au electrode (curve ii) and GluOx/cMWCNT/AuNP/CHIT/Au electrode (curve iii). FTIR spectra of the electrode-deposited CHIT/Au composite shows that the peak at 1745 cm⁻¹ was assigned to C=O stretching, 1650 cm⁻¹ is attributed to C=O stretching along with N-H deformation mode and 1096 cm⁻¹ to the stretching vibration mode of the hydroxyl group. Curve (ii) shows the FTIR spectra of the cMWCNT/AuNP/CHIT/Au electrode, revealing several significant peaks. The peak at the 1558 cm⁻¹ corresponds to the stretching mode of the C=C double bond that forms the framework of the carbon nanotube sidewall. The peak at 1730 and 1028 cm⁻¹ apparently corresponds to the stretching modes of the carboxylic acid groups. The FTIR spectrum of the GluOx/cMWCNT/AuNP/CHIT/Au electrode (curve iii) shows the appearance of additional bands at 1695 cm⁻¹ assigned to the carbonyl stretch, indicating the GluOx binding.

3.4. Electrochemical impedance measurements

Fig. 5(B) shows electrochemical impedance spectra (EIS) of (a) bare Au electrode (b) cMWCNT/AuNP/CHIT/Au electrode and (c) GluOx/cMWCNT/AuNP/CHIT/Au electrode, respectively. The charge transfer process in GluOx/cMWCNT/AuNP/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, cMWCNT/AuNP/CHIT/Au electrode and GluOx/cMWCNT/AuNP/CHIT/Au electrodes were 650 Ω, 400 Ω and 570 Ω respectively (the average of three replications). The decrease in Rct value of the cMWCNT/AuNP/CHIT/Au electrode after electrodeposition of cMWCNT/AuNP/CHIT/Au was due to the attachment of nanomaterials onto the electrode. The increase in RCT value of the modified electrode (cMWCNT/AuNP/CHIT/Au) after immobilization of GluOx can be attributed to the fact that most biological molecules, including enzyme, are poor electrical conductors at low frequencies and cause hindrance to electron transfer. These results also confirmed the attachment of enzyme onto the cMWCNT/AuNP/CHIT/Au composite.

3.5. Construction of Glu biosensor

Fig. 6 summarizes the construction of the Glu biosensor based on immobilization of GluOx on the AuNP decorated cMWCNT and CHIT composite film electrodeposited onto the Au electrode. Firstly, CHIT
Fig. 3. (A) UV spectrum of AuNP (B) transmission electron microscopic (TEM) images, of AuNP and (C) X-ray diffraction (XRD) pattern of taken from AuNP and (D) FTIR spectrum of AuNP.

Fig. 4. SEM images of (a) bare Au electrode (b) cMWCNT/AuNP/CHIT/Au electrode Au electrode and (c) GluOx/cMWCNT/AuNP/CHIT/Au electrode.

was electrodeposited on the surface of the Au electrode, as this method is easy and the layer thickness could be controlled, and then cMWCNT and AuNP were co-electrodeposited on the CHIT coated Au electrode. To construct the working electrode, GluOx was immobilized covalently onto cMWCNT/AuNP/CHIT/Au electrode. Incubation of GluOx solution with activated cMWCNT/AuNP/CHIT/Au electrode leads to effective collision between −COOH group of cMWCNT and −NH₂ groups on the surface of the GluOx to form an amide (−CO-NH) bond through EDC and NHS chemistry. EDC and NHS activate the free −COOH groups of cMWCNT/AuNP/CHIT composite layer. The CVs of cMWCNT/AuNP/CHIT/Au exhibited higher currents than CHIT/Au, which indicate that cMWCNT/AuNP/CHIT/Au composite film, have large effective surface area than CHIT/Au composite film and cMWCNT/AuNP/CHIT/Au composite film could provide a conducting path through the composite matrix for faster kinetics. Hence, the cMWCNT/AuNP, acting as electron transfer mediator help in enhancing the biosensor response and thus increase its sensitivity. These observations also suggest that the cMWCNT/AuNP/CHIT composite film provided a large surface area for immobilization of the GluOx.

3.6. Response measurements of Glu biosensor

To evaluate the catalytic activity of GluOx at the GluOx/cMWCNT/AuNP/CHIT/Au electrode, the modified electrode was characterized by a cyclic voltammogram in 30 mL 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM Glu (100 μL). No redox peaks appeared in the case of bare gold electrode (Supplementary Fig. 2, curve a), while a well-defined redox peak was observed in the case of cMWCNT/AuNP/CHIT modified Au electrode (Supplementary Fig. 2, curve b) due to nanocomposite of cMWCNT and AuNP and CHIT, which facilitate the electron transfer. However, curve c showed a bigger redox peak of the GluOx/cMWCNT/AuNP/CHIT/Au electrode (Supplementary Fig. 2, curve c) than the cMWCNT/AuNP/CHIT/Au electrode, which corresponded to the
oxidation of Glu on the surface of the modified electrode as well as the enhancement of the current. It appeared that the cMWCNT/AuNP/CHIT/Au nanocomposite provided a biocompatible environment to the enzyme, cMWCNT and AuNPs act as an electron mediator, resulting in an accelerated electron transfer between enzyme and electrode. When the glutamic acid was added into the buffer solution, the oxidation current rose steeply to reach a stable value (Supplementary Fig. 3). The biosensor responded very rapidly, producing 95% of the steady-state current within 2 s.

3.7. Optimization of biosensor

The experimental conditions affecting the biosensor response were studied in terms of effect of pH, incubation temperature and substrate (Glu) concentration. The optimum current was obtained at pH 7.5, which is almost similar to that of earlier biosensors (Supplementary Table 1). The optimum temperature of sensor was 35 °C, which is higher than DO metric biosensors (Supplementary Table 1). There was a hyperbolic relationship between sensor response and Glu concentration in the range 5–700 μM, the response was constant after 500 μM.

3.8. Evaluation of biosensor

3.8.1. Linearity

There was a linear relationship between the current (in μA) and the Glu concentration in the range 5–500 μM (Fig. 1), which is a better linear range than those of earlier biosensors (Supplementary Table 1).

3.8.2. Detection limit

The detection limit of the present biosensor was 1.6 μM (S/N=3), which is lower than that for earlier biosensors (Supplementary Table 1).

3.8.3. Analytical recovery

The average recoveries of Glu added to serum (at levels of 20 and 40 μM) varied from 95.40% to 97.56%, demonstrating the satisfactory accuracy of the present biosensor.
3.8.4. Precision
Within-sample and between-sample coefficients of variation (CVs) for the determination of Glu in serum on the same day and after 1 week storage at -20 C were 2.2% and 4.3%, respectively. These high precisions highlighted the good reproducibility and consistency of the present method, which can be attributed to the excellent immobilization of GluOx onto the cMWCNT/AuNP/CHIT/Au electrode.

3.8.5. Correlation
The accuracy of the present method was tested by comparing Glu values in serum samples by the present method (y) with those obtained by standard colorimetric method (x). The values obtained by both methods showed a very good correlation with r = 0.9919 with the regression equation y = 1.0281x - 0.4658. These results indicate a very good analytical performance of the present biosensor in biological samples.

3.8.6. Study of interferences
The effect of several possible interfering substances, such as ascorbic acid, bilirubin, urea, uric acid, triglycerides and glucose on the present biosensor was investigated in phosphate buffer (pH 7.5). The value of Imax does not vary significantly in the presence of interferants indicating non-influence of the individual interferants.

3.9. Detection of Glu in sera
The Glu content in serum samples, was in the range 10.3 μM to 26.8 μM in apparently healthy persons with a mean of 20.68 μM in males and 17.04 μM in females, but ranged from 34.8 μM to 48.5 μM in epileptic patients with a mean of 39.56 μM in males and 42.5 μM in females.

4. Conclusion
An improved amperometric Glu biosensor was constructed by immobilization of GluOx onto hybrid of carboxylated multiwalled carbon nanotubes and gold nanoparticles attached on to Au electrode through chitosan film, which showed remarkably enhanced sensitivity (155 nA/μM/cm²) and selectivity for Glu. The biosensor showed relatively rapid response (2 s), broad linear range (5–500 μM), low detection limit (1.6 μM), good reproducibility and long stability (4 months).

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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.03.063.

References
List of Research publications based on other than Ph.D work


