Chapter 5

Summary
Summaxif, P-N-oxalyl-L-α,β-diaminopropionic acid (P-ODAP), a neurotoxin, occurs as a free non-protein amino acid in the seeds of *Lathyrus sativus*, a hardy crop of countries like India, Bangladesh and Ethiopia and also some countries of Europe. Prolonged excessive consumption of grass pea seeds may cause a drastic paralytic disease known as ‘lathyrisms’ or ‘neurolathyrism’ manifesting as paralysis of the leg muscles, muscular rigidity and weakness. Among the various analytical methods available for measurement of β-ODAP content, biosensing method is most simple, rapid, sensitive and specific. The aim of present work was to construct an improved ODAP biosensor based on covalent immobilization of glutamate oxidase (GluOx) onto carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan (cMWCNT/AuNPs/CHIT) composite film electrodeposited on the surface of an Au electrode. To achieve this aim, AuNPs were prepared from HAUCl₄ solution by citrate reduction method and characterized by transmission electron microscopy (TEM). A nanocomposite film of cMWCNT/AuNPs/CHIT was electrodeposited on the surface of an Au wire. Commercial GluOx from *Streptomyces* sp. was immobilized covalently onto this Au electrode using N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) chemistry. This enzyme electrode (GluOx/cMWCNT/AuNPs/CHIT/Au) as working electrode, Ag/AgCl as standard electrode and Pt wire as auxiliary electrode were connected through potentiostat/galvanostat to construct an amperometric β-ODAP biosensor.

The enzyme electrode was characterized by scanning electron microscopy (SEM), fourier transform infra red spectroscopy (FTIR) and electrochemical impedance spectroscopy (EIS) at different stages of its construction. The SEM image of the bare Au electrode exhibited a smooth and featureless morphology, while the SEM image of cMWCNT/AuNPs/CHIT/Au composite film was a net structure, providing larger surface area. After immobilization of GluOx onto cMWCNT/AuNPs/CHIT/Au composite film, the SEM image of hybrid bioelectrode showed the sporadic appearance of globular/beaded structure on uniform structure of cMWCNT/AuNPs/CHIT/Au composite film to confirm the immobilization of GluOx.

The electrode response was measured in terms of milliampere (mA) applying a potential range of -0.1 to 0.9 V vs Ag/AgCl. The optimal current response was obtained at 0.135 V and hence for subsequent electrochemical studies were carried out at this potential. The
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low working potential in the present biosensor might be due to the presence of cMWCNT in the matrix of CHIT, which provides an environment for the enhanced electrocatalytic effect and a fast electron-transfer rate. The bare Au electrode, showed negligible redox peak. But, when a layer of CHIT and cMWCNT/AuNPs/CHIT was electrodeposited over bare Au electrodes, it showed excellent redox properties in CV. The following electrochemical reactions occurred during response measurement:

L-glutamate + O$_2$ + H$_2$O $\rightarrow$ GluOx $\rightarrow$ 2-oxoglutarate + NH$_3$ + H$_2$O$_2$

H$_2$O$_2$ $\rightarrow$ 0.135 V $\rightarrow$ O$_2$ + 2H$^+$ + 2 e$^-$

β-ODAP + O$_2$ + H$_2$O $\rightarrow$ GluOx $\rightarrow$ α-keto acid + NH$_3$ + H$_2$O$_2$

FTIR spectra of electrodeposited CHIT/Au composite curve (i) showed peak at 1745 cm$^{-1}$ assigned to C=O stretching, 1650 cm$^{-1}$ attributed to C-O stretching along with N-H deformation mode and 1096 cm$^{-1}$ for stretching vibration mode of the hydroxyl group. Curve (ii) showed the FTIR spectra of cMWCNT/AuNPs/CHIT/Au electrode, revealing several significant peaks. The peak at the 1558 cm$^{-1}$ corresponded 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. FTIR spectrum of GluOx/cMWCNT/AuNPs/CHIT/Au bioelectrode (curve iii) showed the appearance of additional bands at 1695 cm$^{-1}$ assigned to the carbonyl stretch indicating the covalent binding of GluOx.

The EIS provided useful information on impedance changes of the electrode surface during the fabrication process. The $R_{ct}$ values for the CHIT/Au, cMWCNT/AuNPs/CHIT/Au and GluOx/cMWCNT/AuNPs/CHIT/Au electrodes were 650Ω, 400Ω and 570Ω respectively. The $R_{ct}$ of cMWCNT/AuNPs/CHIT/Au electrode (curve ii) was lower than CHIT/Au electrode (curve i), revealing its decreased resistance and high electron transfer efficiency, which might be due to cMWCNTs inside the CHIT matrix. This may also be ascribed to the excellent conductivity of the AuNPs attached to cMWCNT. All these resulted in enhanced conductivity, lowering the resistance and facilitating the charge-transfer of the composite. However, the $R_{ct}$ of GluOx/cMWCNT/AuNPs/CHIT/Au (curve iii) bioelectrode increased compared with that of cMWCNT/AuNPs/CHIT/Au electrode, which could be attributed to the fact that most biological
molecules, including enzymes, are poor electrical conductors at low frequencies and cause hindrance to electron transfer.

The β-ODAP biosensor showed optimum response i.e. current (mA) within 2s at pH 7.5 and 35°C. There was a linear relationship between current (mA) and L-glutamate concentration in reaction mixture ranging from 2-550 μM. Km app for L-glutamate and I_max were 94.8 μM and 23.8 μA respectively. The biosensor was evaluated. The detection limit (LOD) of the present biosensor was 2.32 μM (S/N=3). The within and between batch coefficient of variation for glutamate determination in Lathyrus sp. seeds extract were 0.051% and 0.052% respectively, revealing the high reproducibility of the method. There was a good correlation i.e. r = 0.9969 between β-ODAP content as measured by standard colorimetric method and the present biosensor. Among the various metabolites substances tested such as cysteine, methionine, lysine, aspartic acid, glycine, histidine, leucine, isoleucine, asparagine, proline, phenylalanine, valine, threonine, tyrosine, tryptophan, arginine, serine, alanine (each at 1 mM), none had practically any interfering effect on the present biosensor response. The biosensor was employed for determination of β-ODAP level in seeds of three varieties of Lathyrus sp. prior to β-ODAP analysis, the seed extract was prepared in distilled water and then L-glutamate contamination was removed by Dowex anion exchanger chromatography. This glutamate removal was based on different isoelectric points of glutamate (pI = 3.22) and β-ODAP (pI = 2.02). The β-ODAP level in seeds of three varieties of Lathyrus sativus as measured by present biosensor, was 1.62 ± 0.06 to 1.89 ± 0.04 g/Kg seed powder (mean ± SD) (on fresh weight basis). The enzyme electrode lost 35% of its initial activity after its 70 regular uses over a period of 90 days, when stored at 4°C.

Conclusion

The use of GluOx/cMWCNT/AuNPs/CHIT/Au has resulted into an improved analytical performance of β-ODAP biosensor, respectively in terms of low working potential (0.135 V), detection limit (2.32 μM), short response time (2 s), higher sensitivity (486.31μA/cm²/μM), broader working range (2-550 μM) and longer stability (3 months) compared to earlier biosensors. Based on these results, these composite could be exploited for the improvement of other biosensors, also.