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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 H_2O_2, 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 μM) and current (μA) under optimum conditions. The biosensor showed high sensitivity (155 nA/μM/cm^2), low detection limit (1.6 μM) 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.

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 (Jamain 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

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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., Panchkula (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: AUT83785, manufactured by Eco Chemie) with a three electrode system composed of a Pt wire as an auxillary 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 $HAuCl_4$ (0.01%) 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. Electrodeposition of cMWCNT/AuNP/CHIT onto gold electrode

One milligram of cMWCNT was suspended into a 4 mL mixture of concentrated H_2SO_4 and HNO_3 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 cm^2) 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/AuNPs mixture. Finally, electrodeposition of AuNPs and cMWCNT onto the gold electrode was carried out in an electrochemical cell system applying 20 deposition cycles at −0.1 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/AuNPs/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

cMWCNT/AuNPs/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/AuNPs/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/AuNPs/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 voltametry measurement and testing of Glu biosensor

Cyclic voltammogram (CV) of GluOx/cMWCNT/AuNPs/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).

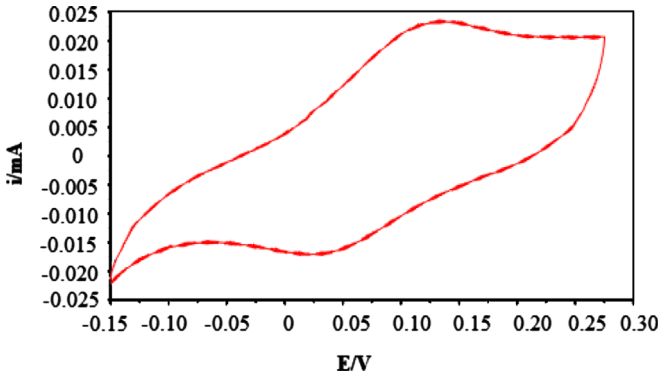

Fig. 1. Cyclic voltamogram for GluOx/cMWCNT/AuNP/CHIT/Au electrode 1 mM glutamate in 0.1 M sodium phosphate buffer (pH 7.5) with scan rate 20 mV/s.

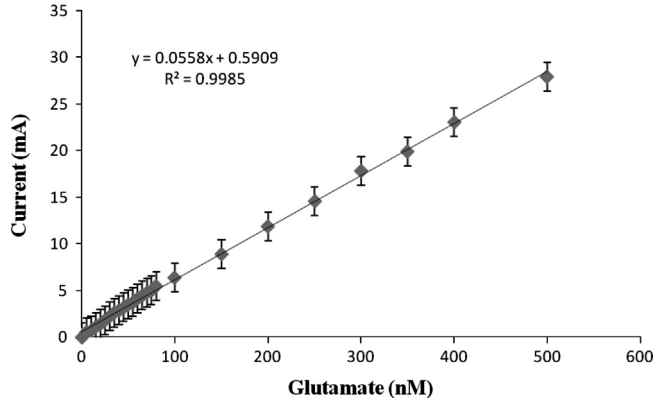

Fig. 2. Standard curve of glutamate by glutamate biosensor based on glutamate oxidase bound to cMWCNT/AuNPs/CHIT composite film electrodeposited on Au wire.

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 interpolated from a standard curve of Glu concentrations prepared under optimal conditions (Fig. 2).

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 NP 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 electrodeposited 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 bioelectrode (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 (R_{CT}) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (R_{CT}) depends on the dielectric and insulating features at the electrode/electrolyte interface. The R_{CT} 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 R_{CT} 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

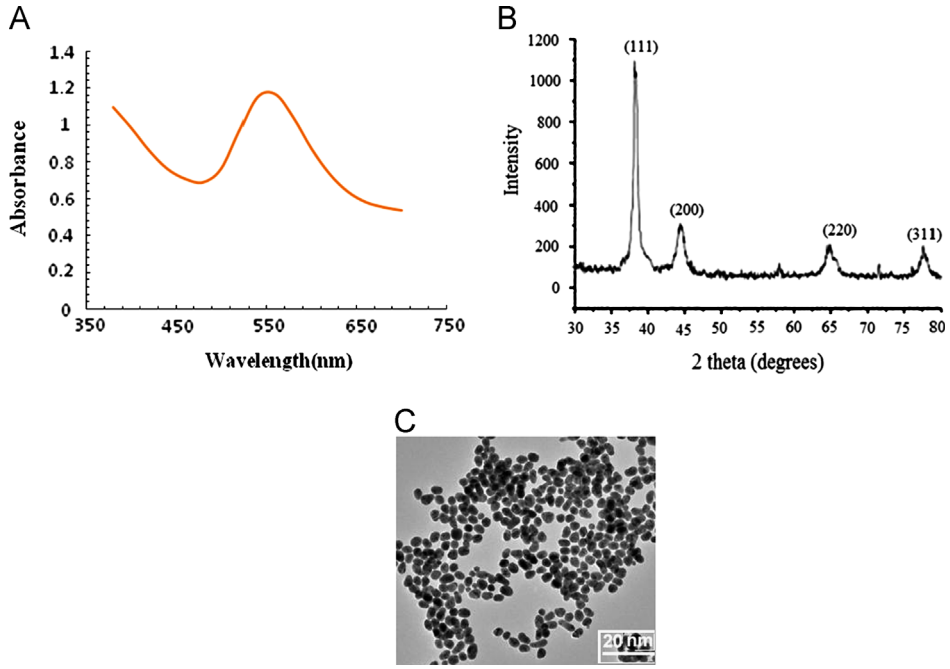

Fig. 3. (A) UV spectrum of AuNP (B) transmission electron microscopic (TEM) images, of AuNP and (C) X-ray diffraction (XRD) pattern of taken from AuNP and (D) FTIR spectrum of AuNP.

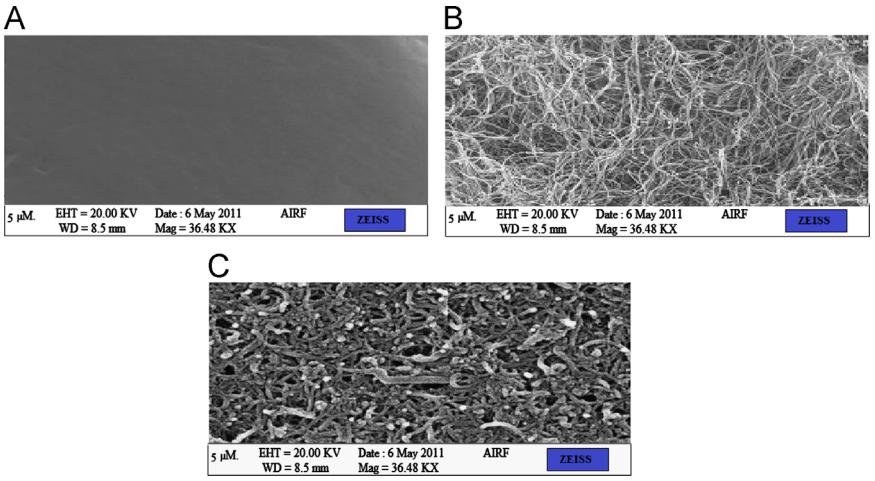

Fig. 4. SEM images of (a) bare Au electrode (b) cMWCNT/AuNP/CHIT/Au electrode Au electrode and (c) GluOx/cMWCNT/AuNP/CHIT/Au electrode .

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 –NH_2 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

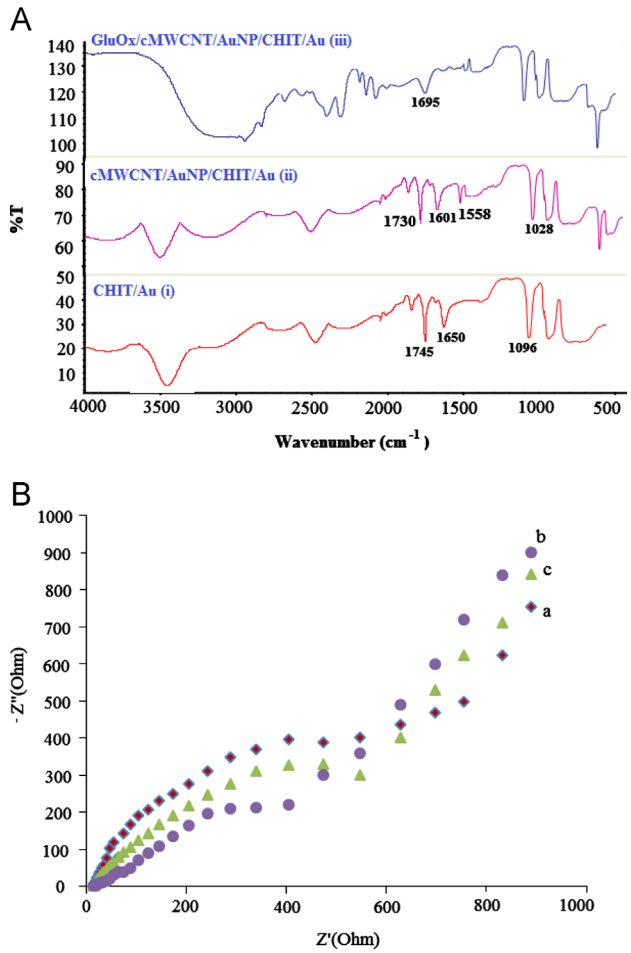

Fig. 5. (A) FTIR spectra of CHIT/Au (i) cMWCNT/AuNP/CHIT/Au (ii) and GluOx/cMWCNT–AuNP/CHIT/Au electrode (iii). (B) Impedance spectra of (a) bare Au electrode (b) GluOx/cMWCNT/AuNP/CHIT/Au electrode and (c) cMWCNT/AuNP/CHIT/Au electrode.

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 of 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).

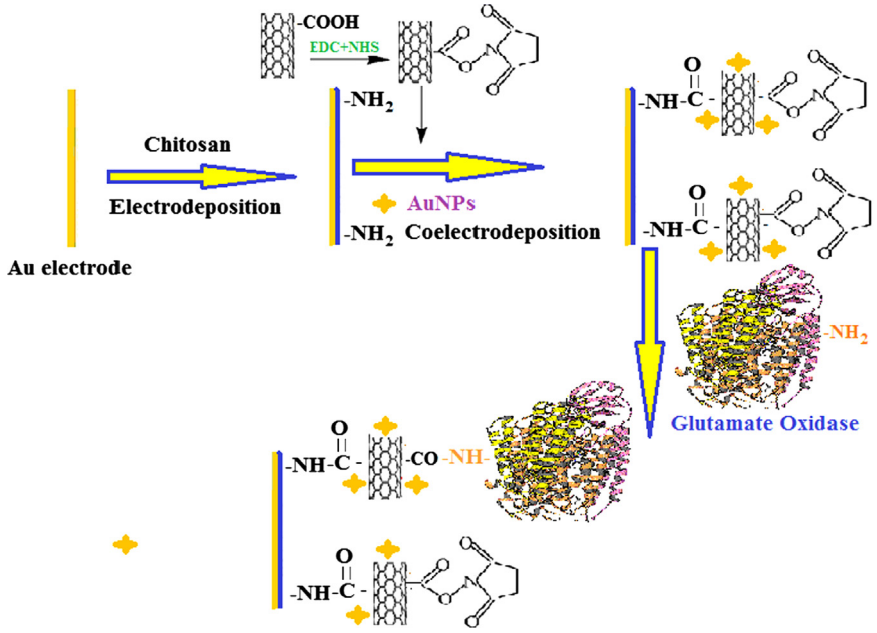

Fig. 6. Schematic representation of chemical reaction involved in the fabrication of GluOx/cMWCNT/AuNP/CHIT/Au electrode.

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 $r=99.19$ 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/cm^2) 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.

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