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RESEARCH ARTICLE

Graphene and tricobalt tetraoxide nanoparticles based biosensor for electrochemical glutamate sensing

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ABSTRACT

An amperometric biosensor based on tricobalt tetraoxide nanoparticles (Co_3O_4), graphene (GR), and chitosan (CS) nanocomposite modified glassy carbon electrode (GCE) for sensitive determination of glutamate was fabricated. Scanning electron microscopy was implemented to characterize morphology of the nanocomposite. The biosensor showed optimum response within 25 s at pH 7.5 and 37°C , at +0.70 V. The linear working range of biosensor for glutamate was from 4.0×10^{-6} to 6.0×10^{-4} M with a detection limit of 2.0×10^{-6} M and sensitivity of $0.73 \mu\text{A}/\text{mM}$ or $7.37 \mu\text{A}/\text{mMcm}^2$. The relatively low Michaelis–Menten constant (1.09 mM) suggested enhanced enzyme affinity to glutamate. The glutamate biosensor lost 45% of its initial activity after three weeks.

ARTICLE HISTORY

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KEYWORDS

Amperometry; biosensor;
 Co_3O_4 ; glutamate; graphene

Introduction

L-Glutamate is an amino acid, which plays an important role in food processing and clinical applications. It is an important neurotransmitter in the central nervous system and neuronal pathways in the brain which is related to several neurological disorders, such as epilepsy, stroke, Alzheimer's and Parkinson's diseases (Batra and Pundir 2013, Gholizadeh et al. 2012, Jamal et al. 2010). Moreover, its sodium salt, monosodium glutamate (MSG) is widely used as a flavor enhancing food additive and it has been associated with the Chinese restaurant syndrome. The excessive intake of MSG can cause allergic effects such as headache and stomach pain (Basu et al. 2006, Beyene et al. 2003). Therefore, developing sensitive and rapid methods is highly demanded and plays an important role in food industry and clinical analysis. Various methods are available for determination of glutamate like chromatography (Acuna and Trias 2009, Buck et al. 2009, Demirhan et al. 2015), spectrophotometry (Acebal et al. 2008) and fluorimetry (Tsukatani and Matsumoto 2005), electrophoresis (Tucci et al. 1998) and potentiometry (Gündüz et al. 1988). However, most of these methods are cumbersome, time consuming, and require expensive apparatus and skilled person to operate. Compared with other analytical methods, enzyme-based electrochemical biosensors possess simplicity of operation, high sensitivity, and selectivity. Different types of biosensors have been reported for detection of glutamate (Batra and Pundir 2013, Şimşek et al. 2016, Tian et al. 2009, Varma et al. 2006, Yu et al. 2011, Yılmaz and Karakuş 2011).

Graphene (GR) is a two dimensional layer of graphite with sp^2 carbon atoms arranged in a hexagonal lattice (Abbasi et al. 2016). Because of the unique properties including fast electron transfer kinetics, excellent thermal conductivity, large surface area and good biocompatibility, GR and its derivatives

have demonstrated attractive application in electrochemical sensing and biosensing (Lim et al. 2010, Liu et al. 2010, Pumera et al. 2010, Zhou et al. 2009). Metal and metal oxide nanoparticles have recently been investigated intensively in so many fields including the construction of biosensors being mainly due to their large surface-to-volume ratio, good electrical properties, high surface reaction activity, high catalytic efficiency, chemical stability and strong adsorption ability (Casero et al. 2014, Devi et al. 2013). Among different metal oxides, tricobalt tetraoxide nanoparticles (Co_3O_4) have been widely used in biosensors as they have advantages such as low cost, high reactivity, wide availability, and excellent electrocatalytic properties (Ali et al. 2015, Kaçar et al. 2014, Wang et al. 2011). Recent research interest has focused on the preparation of nanocomposites involving the combination of GR and metal or metal oxide nanoparticles. Various metal or metal oxide nanoparticles and GR composites such as Pd–graphene (Zeng et al. 2011), TiO_2 –reduced GR (Casero et al. 2014), NiO–carboxylic graphene (Yang et al. 2013), Au–graphene (Zhou et al. 2010) Pt–graphene (Sun et al. 2011) have been reported for electrochemical biosensing applications.

The aim of this work was to develop an amperometric biosensor for glutamate measurement that possesses properties such as low cost, good stability, and rapid response. The biosensor was constructed by immobilizing glutamate oxidase (GLOx) on a Co_3O_4 /chitosan (CS)/GR composite film modified glassy carbon electrode (GCE). The experimental conditions related to the construction and the characterization of the biosensor were studied in detail. Optimum parameter studies such as enzyme loading, pH, and working potential were conducted. The analytical performance parameters of the biosensor including the linear range, detection limit, sensitivity, response time, repeatability, and stability were also described.

Experimental

Materials

GR solution (DRP-GPHSOL) (2 mg/mL) was obtained from Dropsense (Llanera, Spain). GLOx (E.C. 1.4.3.11 from *Streptomyces sp.*), Co_3O_4 nanoparticles (<50 nm particle size), potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, L-glutamic acid monosodium salt, lysine, methionine, and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Sodium monohydrogenphosphate and sodium dihydrogenphosphate were supplied from Riedel-de Haën (Seelze, Germany). CS (90% deacetylation, medium molecular weight) and aspartic acid were from Aldrich (Steinheim, Germany). Glucose was from Fluka (Buchs, Switzerland). All other chemicals were obtained from Merck (Darmstadt, Germany). Standard solutions of glutamate were prepared by dissolving glutamate in phosphate buffer solution.

Apparatus and measurements

Electrochemical measurements were performed with IVIUM electrochemical analyzer (Ivium Technologies, the Netherlands). A conventional three electrode system was used for all electrochemical measurements. Ag/AgCl electrode (BAS MF 2052) and platinum wire (BAS MW 1034) electrode were used as reference electrode and counter electrode, respectively. The working electrode was a modified GCE (diameter 3 mm). All measurements were carried out in a thermostated cell at 37 °C, containing 0.05 M phosphate buffer solution (PBS pH 7.5) under stirring at an applied potential of +0.70 V. The morphology of the modified electrodes was observed using scanning electron microscope (SEM) from Carl Zeiss AG, EVO® 50 Series. pH measurements were performed using a ORION Model 720A pH/ion meter and ORION combined pH electrode (Thermo Scientific, Waltham, MA). Double-distilled water from a Human Power I+, Ultra Pure Water System (ELGA PURELAB Option-S, London, UK) was used for the preparation of all aqueous solutions.

Biosensor preparation and modification

Prior to coating, the bare GCE was carefully polished with 0.05 μm alumina slurry, then rinsed thoroughly with double

distilled water, followed by ultrasonication in ethanol and double distilled water for 5 min, respectively and dried in air before use. About 0.025 g of CS was dissolved in 5.0 mL of acetate buffer solution (pH 5.0) via magnetic stirring at room temperature for 4 h. Co_3O_4 nanoparticles/CS suspension (1 mg mL^{-1}) was prepared by dispersing Co_3O_4 nanoparticles in CS solution with ultrasonication for 4 h. About 5 μL of GR solution (2 mg/mL) was dropped on the cleaned surface of GCE and dried in air at room temperature. About 5 μL of Co_3O_4 /CS solution was dropped on the surface of GR modified GCE to prepare the Co_3O_4 /CS/GR/GCE electrode. 10 μL of GLOx solution (0.025 Units/ μL) was dropped on the surface of Co_3O_4 /CS/GR/GCE and the resulting electrode was placed in 2.5% glutaraldehyde vapor for 15 min for the crosslinking of the enzymes. 7.5 μL of nafion solution (0.5%) was casted and allowed to dry on the enzyme electrode. Finally, the biosensor was immersed in 0.05 M PBS (pH 7.5) to wash out the unbound components from the electrode surface. The glutamate biosensor was stored at +4 °C in a refrigerator when not in use. The fabrication steps of the biosensor are presented in Scheme 1.

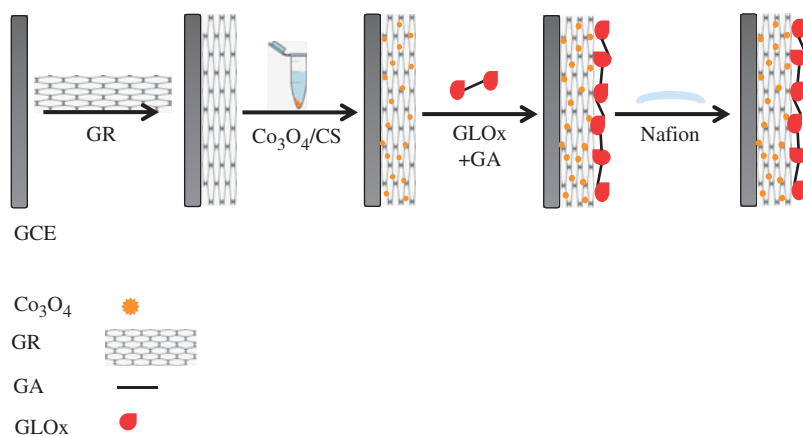
Results and discussion

Morphology of composite electrode

The surface morphologies of Co_3O_4 /CS/GR/GCE and GLOx/ Co_3O_4 /CS/GR/GCE were investigated by SEM (Figure 1). Image a shows that the Co_3O_4 nanoparticles are uniformly embedded in the porous CS network. The porous nature of the resulting film is suitable for the immobilization of enzymes. The surface of GLOx/ Co_3O_4 /CS/GR/GCE (image b) shows globular structures on Co_3O_4 /CS/GR composite indicating that enzymes were successfully immobilized on the surface of Co_3O_4 /CS/GR/GCE.

Electrochemical characteristics of the electrodes

CV of the ferricyanide system is a convenient and valuable tool to monitor the characteristics of the surface modified electrodes. The electron transfer properties of bare GCE, and Co_3O_4 /CS/GR/GCE were investigated in 0.1 M KCl solution containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ at 50 mVs^{-1} . As shown in



Scheme 1. The fabrication steps of the biosensor.

Figure 2(A) well-defined redox peaks of $\text{Fe}(\text{CN})_6^{3-/4-}$ were observed for bare GCE. The modification of the bare GCE with $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}$ resulted in an increase in peak currents and a decrease in peak separation which can be attributed to the high electric conductivity and large surface area of GR and Co_3O_4 nanoparticles (Dalkiran et al. 2014, Kuila et al. 2011, Pérez-López and Merkoçi 2012). Figure 2(C) shows cyclic voltammograms (CVs) of the $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ electrode in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution containing 0.1 M KCl over the potential range from -0.1 to 0.6 V at various scan rates. As shown in Figure 2(C) inset, the relationship between the peak current (I_p) and the square root of the scan rate ($v^{1/2}$) is linear for the $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$. This suggests that the mass transfer phenomenon in the double layer region of the electrodes is mainly controlled by diffusion (Qiu et al. 2011). The average

electroactive surface area could be calculated according to the Randles-Sevcik equation (Bard and Faulkner 2001):

$$I_p = 2.69 \times 10^{-5} n^{3/2} A D^{1/2} v^{1/2} C$$

where n is the number of electrons participating in the redox reaction, A is the area of the electroactive surface area (cm^2), D is the diffusion coefficient of the molecule in solution ($\text{cm}^2 \text{s}^{-1}$), C corresponds to the bulk concentration of the redox probe (mol cm^{-3}), and v is the scan rate (Vs^{-1}). The $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple used in this study exhibits a heterogeneous one-electron transfer ($n=1$). C is equal to 5 mM, and D is ($6.70 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$) (Zeng et al. 2006). According to the equation, the average value of the electroactive surface area for $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ is 0.238cm^2 . The effective electrochemical surface area of $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ (0.238cm^2) is 3.35 times higher than the surface area

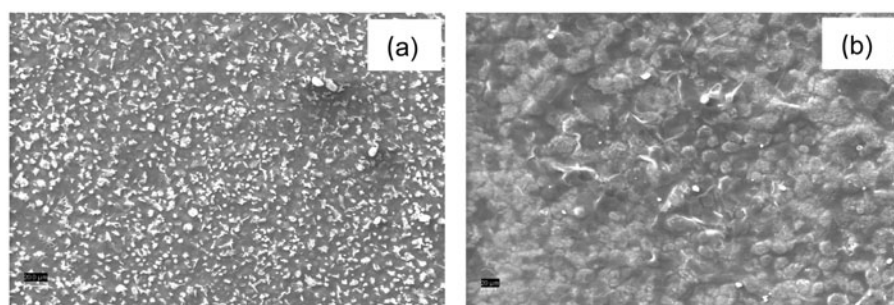


Figure 1. SEM images of (a) $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ and (b) $\text{GLOx}/\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ surfaces.

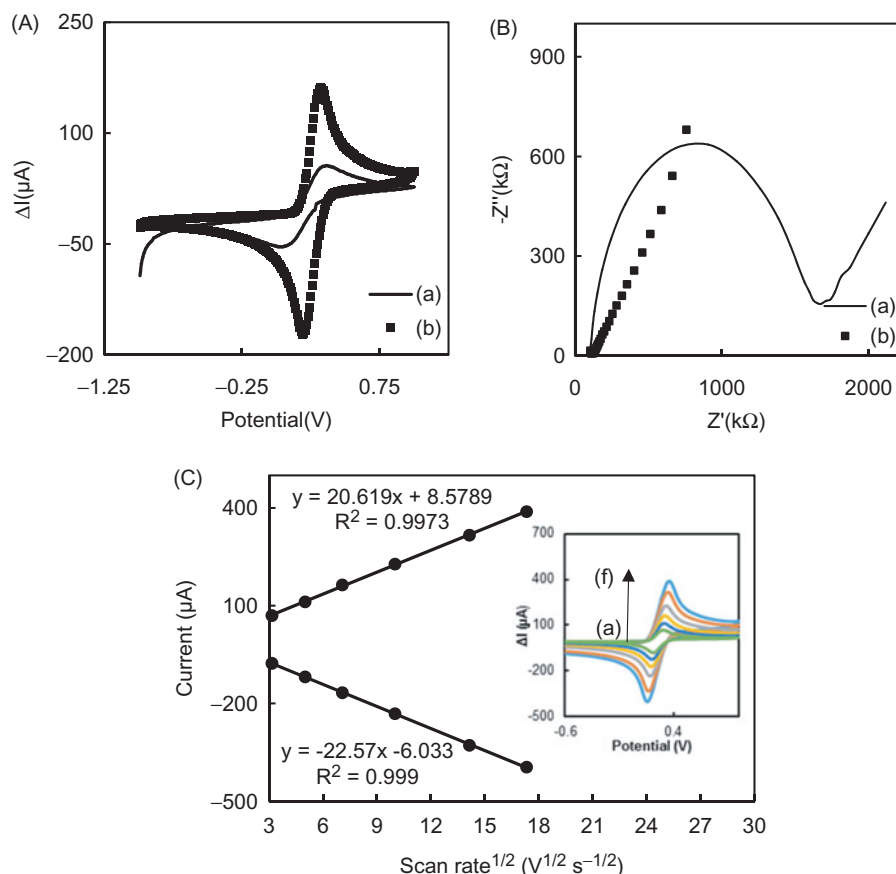


Figure 2. (A) Cyclic voltammograms of (a) GCE and (b) $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ at 50 mV s^{-1} (B) The Nyquist curves of (a) GCE and (b) $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ in 0.1 M KCl solution containing 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ (C) Peak currents of $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ as a function of scan rate for the determination of the effective working surface area. Inset: Cyclic voltammograms of the $\text{Co}_3\text{O}_4/\text{CS}/\text{GR}/\text{GCE}$ at scan rates of 10, 20, 50, 75, 100, 200, and 300 mV s^{-1} in a 0.1 M KCl solution containing 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$.

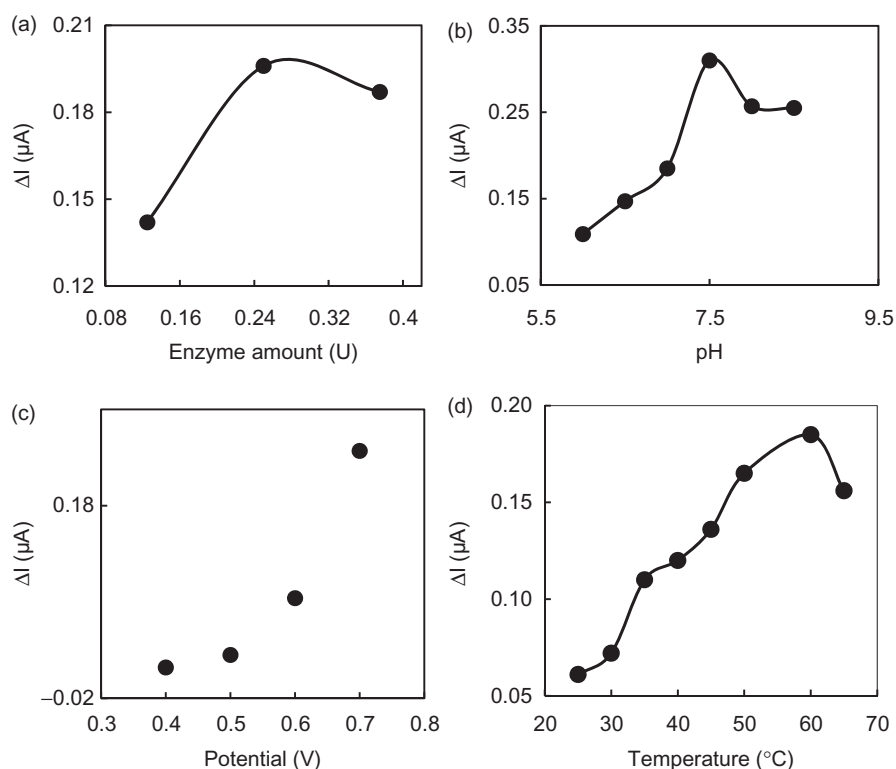


Figure 3. The effects of (a) enzyme amount (+0.70 V; pH 7.5), (b) buffer pH (+0.70 V), (c) applied potential (pH 7.5), and (d) temperature (+0.70 V; pH 7.5) on the response of GLOx/Co₃O₄/CS/GR/GCE (0.05 M PBS containing 0.2 mM glutamate).

of bare GCE (0.071 cm²). This result revealed that the Co₃O₄/CS/GR composite is more suitable to facilitate electron transfer between redox probe and working electrode. This may result from the high electron transfer kinetics and the large surface area of Co₃O₄/CS/GR/GCE and synergistic effects of GR and Co₃O₄.

EIS, a powerful tool for studying the interface characteristics of the modified electrodes, was employed to probe the electron transfer kinetics at the bare GCE and Co₃O₄/CS/GR/GCE. Figure 2(B) shows the Nyquist plots of the EIS of (a) bare GCE and (b) Co₃O₄/CS/GR/GCE in 0.10 M KCl solution containing 5.0 mM [Fe(CN)₆]^{3-/4-}. In Nyquist plots the imaginary impedance (Z'') is plotted against the real impedance (Z'). The typical impedance spectrum includes a semicircle portion and a linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer limited process, and the linear portion at lower frequencies represents the diffusion-limited process. The diameter of the semicircle in the impedance spectrum is equal to the electron-transfer resistance, R_{ct} , which controls the electron-transfer kinetics of the redox probe at the electrode interface (Li et al. 2014). It can be seen from the Figure 2(B) that a well-defined semicircle was obtained with the bare GCE (curve a). When the electrode was modified with Co₃O₄/CS/GR composite (curve b), smaller semicircle was obtained which indicated that the electron transfer resistance of [Fe(CN)₆]^{3-/4-} on Co₃O₄/CS/GR/GCE was greatly decreased as compared with that on the bare GCE. The impedance results were consistent with the conclusion from the CV experiments, indicating that the nanocomposite was successfully coated on electrode surface.

Optimization of experimental parameters

Zhang et al. (2006) have reported the high affinity of CS to GLOx postulated by strong electrostatic interactions between the polycationic CS ($pK_a = 6.5$) and polyanionic GLOx ($pI = 6.2$). To investigate the effect of the enzyme amount on the response current, the enzyme electrodes were prepared with three different GLOx amounts as 0.125, 0.25, and 0.375 Units (U). With the increase of the GLOx concentration from 0.125 U to 0.25 U, the response current of the glutamate biosensor in the presence of 0.2 mM glutamate increased accordingly, which implies that higher enzyme amount resulted in higher sensitivity (Figure 3a). The decrease in the response current of the biosensor at high enzyme amount may be attributed to the increase of the diffusional resistance for the substrate to arrive at the electrode surface. The optimal amount of 0.25 U of GLOx was found for the immobilization on the working electrode. This amount was consequently used for all biosensors in further experiments. About 0.23 U (Batra and Pundir 2013), 0.27 U (Chakraborty and Raj 2007) 0.1–0.2 U (Backer et al. 2013), 0.40 U (Cui et al. 2007), 0.50 U (Tseng et al. 2013), 0.58 U (Jamal et al. 2010) were reported as optimum enzyme amount for glutamate biosensors in the literature. It can be concluded that the result of optimum enzyme amounts study is consistent with the results reported in the literature.

The influence of solution pH was investigated to obtain the optimum conditions for the determination of glutamate. This study was conducted in 0.05 M PBS containing 0.2 mM glutamate between pH 6.0 and 8.5. As can be seen from Figure 3(b) the response increases significantly from pH 6.0

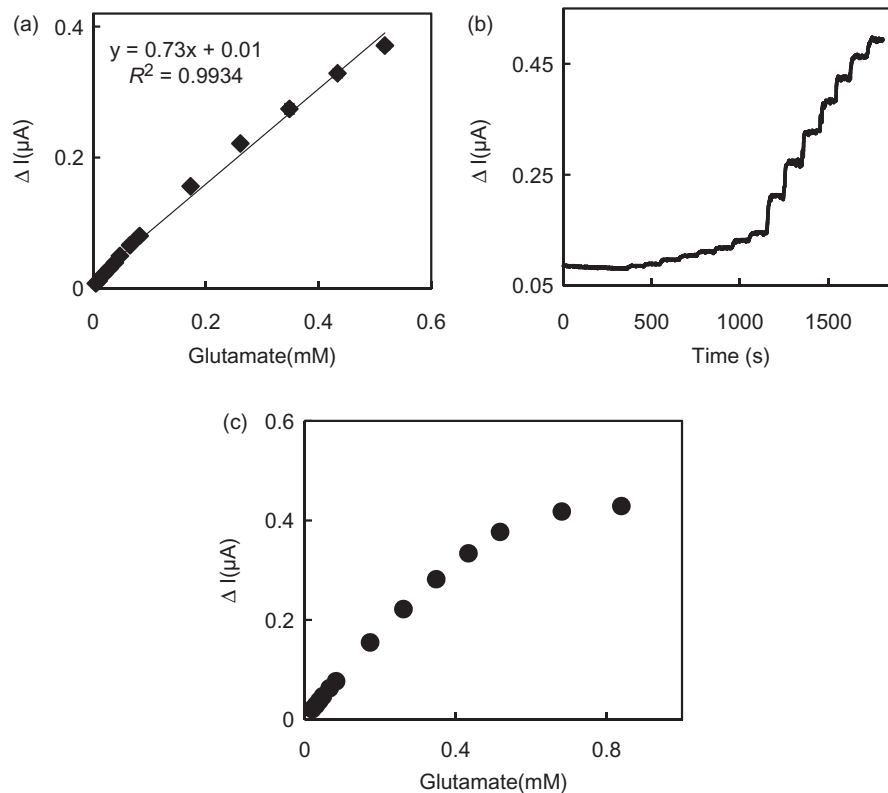


Figure 4. (a) Calibration curve of the GLOx/Co₃O₄/CS/GR/GCE, (b) Typical current–time response of the on successive injection of glutamate into a stirred solution of 0.05 M PBS (pH 7.5) at applied potential +0.70 V, and (c) The effect of glutamate concentration upon the amperometric response of the biosensor (0.05 M pH 7.5 PBS, 37 °C).

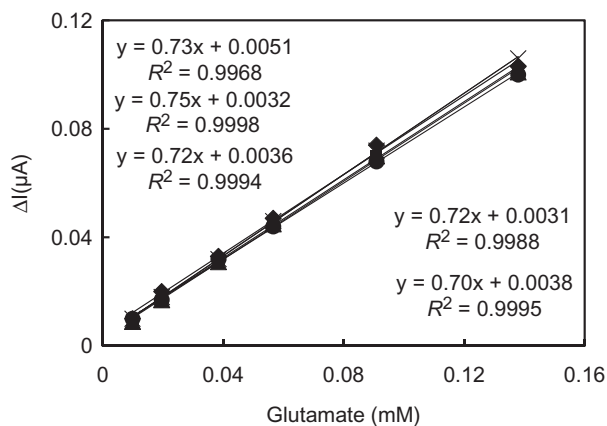


Figure 5. The repeatability of the GLOx/Co₃O₄/CS/GR/GCE (0.05 M pH 7.5 PBS, 37 °C).

to 7.5. The best amperometric response of the biosensor was obtained at pH 7.5. At pH values higher than 7.5, a decrease of the response was observed. Therefore, PBS with pH 7.5 was considered as the optimal electrolyte. This pH is in good agreement with the data reported in the literature (Batra and Pundir 2013). This optimum value is also compatible with the pH range (7.0–7.5) reported for the free GLOx (Chang et al. 2003, Kwong et al. 2000; Villarta et al. 1991). This study indicates that the immobilization procedure has no significant effect on the optimum pH of GLOx. Different pH values such as 6.5 (Cui et al. 2007), 7.0 (Basu et al. 2006, Chang and Chen 2007, Pauliukaite et al. 2006) 7.4 (Claussen et al. 2011, Jamal et al., 2010, Ryan et al. 1997, Tian, et al. 2009, Zhang et al. 2006), 8.0 (Backer

Table 1. The performance parameters and the optimum working conditions of the biosensor.

Optimum working conditions	
Enzyme amount (U)	0.24
Temperature (°C)	37
pH	7.5
Working potential (V)	+0.70 versus Ag/AgCl
Performance factors	
Linear range	4.0×10^{-6} M – 6.0×10^{-4} M
Limit of detection (LOD)	2.0×10^{-6} M
Sensitivity (μ A/mM and μ A/mM cm ²)	0.73; 7.37
K_m (mM)	1.09
Response time (s)	25
Repeatability (RSD%)	1.0

et al. 2013) were also reported for glutamate enzyme electrodes.

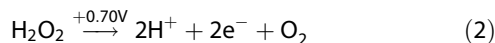
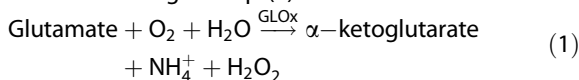
The effect of applied potential on the response currents of GLOx/Co₃O₄/CS/GR/GCE in the presence of 0.2 mM glutamate was examined at different potentials between +0.40 and +0.70 V. With the increase of the applied potential from +0.40 V to +0.70 V, the response current increased regularly and a maximum response current was observed at +0.70 V (Figure 3c). Thus, a potential of 0.70 V was chosen for glutamate detection in the following experiments. GLOx catalyzes the oxidation reaction of L-glutamate to α -ketoglutarate according to Eq. (1) (Pauliukaite et al. 2006). The electron transfer mechanism of the presented biosensor is based on the formation of H₂O₂ from

Table 2. Characteristics of various amperometric glutamate biosensors.

Electrode	Working potential V	Detection limit	Linear range	Sensitivity	Response time	K_m	pH	References
c-MWNT/AuNPs/CS modified Au electrode	+0.135 versus Ag/AgCl	1.6 μ M	5–500 μ M	155 nA/ μ M/ cm^2	2 s	–	7.5	Batra and Pundir (2013)
Pt nanoparticle modified Au nanowire array electrode	+0.65 versus Ag/AgCl	14 μ M	Up to 0.8 μ M	10.76 μ A/ mM cm^2	<5 s	–	7.4	Jamal et al. (2010)
Chip-based amperometric enzyme sensor	+0.6 versus Ag/AgCl	0.05 mM	Up to 10 mM	3.68 μ As/ mM	–	–	8.0	Backer et al. (2013)
p-hydroxybenzoate hydroxylase on a Clark-type electrode	–0.6 versus Ag/AgCl	5 μ M	10 μ M–1.5 mM	–	2 s	–	8.0	Cui et al. (2007)
A photo-crosslinkable polymer membrane modified biosensor	+0.4 versus Ag/AgCl	0.05 μ M	0.05–100 μ M	12.18 nA/ μ M	–	–	7.0	Chang and Chen (2007)
A L-GLOx–L-GLDH coupled enzymatic sensor based on oxygen electrode	–	–	0.02–1.2 mg/L	–	2 min	1.92 mM	7.0 $\pm$ 2.0	Basu et al. (2006)
Nafion/methyl viologen modified glassy carbon electrode	–0.65 versus Ag/AgCl	20 μ M	Up to 75 mM	–	–	2.84 mM	7.4	Maalouf et al. (2007)
Glutamate dehydrogenase/vertically aligned CNTs electrode	–	57 nM	0.1–20 μ M	0.976 mA/ mM cm^2 0.182 mA/ mM cm^2	–	–	7.0	Gholizadeh et al. (2012)
A tetrafulvalene-tetracyanoquinodimethane carbon paste electrode	–	0.15 mmol L^{-1}	–	–	20–50 s	–	7.0	Pauliukaite et al. (2006)
GLDH and poly(amidoamine) dendrimer-encapsulated platinum nanoparticle onto CNTs	+0.2 versus Ag/AgCl	10 nM	0.2–250 μ M	433 μ A/ mM cm^2	3 s	–	7.4	Tang et al. (2007)
Microbiosensor based on our sol-gel electrodeposition technique	+0.6 versus Ag/AgCl	5 nM	0.5–100 μ mol/L	279.4 $\pm$ 2.0 A (mmol L^{-1}) $^{-1}$ cm^{-2}	10 s	0.776 mM	7.4	Tian et al. (2009)
CS enzyme film modified Pt electrode	+0.4 versus Ag/AgCl	1 $\times 10^{-7}$ M	5 $\times 10^{-7}$ –2 $\times 10^{-4}$ M	85 mA/M cm^2	2 s	–	7.4	Zhang et al. (2006)
GLOx/nano conducting polymer/Pt microbiosensor	+0.45 versus Ag/AgCl	0.1 $\pm$ 0.03 μ M	0.2–100 μ M	–	10 s	–	7.4	Rahman et al. (2005)
Co ₃ O ₄ nanoparticles and GR modified GCE	+0.7 versus Ag/AgCl	2 $\times 10^{-6}$ M	4 $\times 10^{-6}$ –6 $\times 10^{-4}$ M	0.73 μ A/ mM 7.37 μ A/ mM cm^2	25 s	1.09 mM	7.5	This work

c-MWCNTs: Carboxylated carbon nanotubes; GR: Graphene; CNTs: Multiwall carbon nanotubes; AuNPs: Gold nanoparticles; CS: Chitosan; Au: gold electrode; L-GLDH: L-glutamate dehydrogenase.

glutamate by GLOx and its oxidation at an applied potential of +0.70 V according to Eq. (2).



The influence of temperature on the electrochemical response of biosensor was investigated over the range of 25–65 °C in the presence of 0.2 mM glutamate. Figure 3(d) shows that the response current of the biosensor increased up to 60 °C. Further increase in temperature caused a decrease in the response current of the electrode, which is caused by the denaturation of the enzyme. The best current response obtained at 60 °C glutamate measurements were performed at body temperature (37 °C) because the response current was stable between 35 and 40 °C. Moreover, the repeatability of the calibration curves obtained over 40 °C was not satisfactory. Different temperatures such as 37 °C (Chang and Chen 2007), 35 °C (Basu et al. 2006, Batra and Pundir 2013, Rahman et al. 2005), 42 °C (Gholizadeh et al. 2012), 25 °C (Maalouf et al. 2007, Tang et al. 2007, Tian et al. 2009) were selected as optimum for glutamate biosensors.

Performance parameters of glutamate biosensor

The amperometric response of the presented biosensor was given in Figure 4 on successive injection of glutamate into the stirring 0.05 M PBS (pH 7.5) at an applied potential of +0.70 V. Immediately after the addition of glutamate, the anodic current increased and reached a steady state within 25 s. This response time is quite fast and highly suitable for biosensor response. There are longer; 2 min (Almeida and Mulchandani 1993), 120 s (Basu et al. 2006), 20–50 s (Pauliukaite et al. 2006), 20–40 s (Kulagina et al. 1999), and shorter response times; 10 s (Frey et al. 2010; Rahman et al. 2005, Ryan et al. 1997), 5 s (Jamal et al. 2010), 2 s (Batra and Pundir 2013) reported for glutamate biosensors.

The response current of GLOx/Co₃O₄/CS/GR/GCE was linear in the range from 4.0×10^{-6} M to 6.0×10^{-4} M with a correlation coefficient of 0.9934. The sensitivity obtained from calibration curve was 0.73 $\mu\text{A}/\text{mM}$ for glutamate. Also, the sensitivity calculated by considering the effective electrochemical surface area of GLOx/Co₃O₄/CS/GR/GCE (0.099 cm²) was found as 7.37 $\mu\text{A}/\text{mMcm}^2$ for glutamate. The limit of detection (LOD) of the biosensor, which is the minimum concentration at which the ratio of signal to noise is not less than 3, is 2.0×10^{-6} M ($S/N=3$). Compared with some previously reported glutamate biosensors this detection limit is lower than that of poly(carbamoyl) sulfonate hydrogel modified bienzymatic Clark electrode (5.0×10^{-6} M) (Cui et al. 2007) and electrodeposited CS modified microelectrodes ($2.5 \pm 1.2 \times 10^{-6}$ M) (Tseng et al. 2013) but comparable to carboxylated multiwalled carbon nanotubes (c-MWCNT), gold nanoparticles (AuNPs) and CS modified Au electrode (1.6×10^{-6} M) (Batra and Pundir 2013). The apparent Michaelis–Menten constant (K_m^{app}) was calculated to evaluate

the catalytic activity of immobilized enzyme by using the Lineweaver–Burk equation:

$$\frac{1}{I_{ss}} = \frac{1}{I_{\text{max}}} + \frac{K_m^{\text{app}}}{I_{\text{max}}} \frac{1}{C}$$

where I_{ss} is the steady current after the addition of substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under the saturated substrate condition (Xu et al. 2003). The apparent Michaelis–Menten constant, in this study was calculated to be 1.09 mM for the GLOx/Co₃O₄/CS/GR/GCE. This value was smaller than those of a GLOx immobilized polycarbonate membrane based biosensor (1.92 mM) (Basu et al. 2006), and nafion and methyl viologen modified GCE based biosensor (2.84 mM) (Maalouf et al. 2007). These results show that the current biosensor possesses higher affinity to glutamate compared with some of the earlier biosensors.

The repeatability and long-term stability of our proposed biosensor have also been studied. During the repeatability study, five calibration curves were plotted by the use of the same enzyme electrode sequentially (Figure 5). The relative standard deviation (RSD) of the sensitivities was calculated to be 1.0% which confirms the reliable repeatability of GLOx/Co₃O₄/CS/GR/GCE.

The long-term stability of the GLOx/Co₃O₄/CS/GR/GCE was determined by measuring response current of 0.2 mM glutamate during three weeks. The electrode was stored in dry atmosphere at +4 °C when not in use. There were not obvious changes of amperometric response during the first five days and then an activity loss of 45% was observed after three weeks. The satisfactory stability of this biosensor may be attributed to the chemical and mechanical stability of Co₃O₄/CS/GR nanocomposite and thus it can be used as a useful platform for immobilizing enzymes to enhance biosensor performances. The effect of possible interfering substances on GLOx/Co₃O₄/CS/GR/GCE was investigated at +0.70 V in 0.05 M PBS (pH 7.5) by injection of 0.1 mM glutamate and 0.01 mM interfering species. The interference caused by lysine, glucose, methionine, aspartic acid, valine, glutamine, serine, leucine, isoleucine, phenylalanine, arginine to 0.1 mM glutamate was <5%. This result indicated that the presented biosensor has acceptable anti-interferent ability. The performance parameters and the optimum working conditions of the presented biosensor are summarized in Table 1.

A comparison of the performance of different glutamate biosensors was summarized in Table 2. The results presented in Table 2 show that the different characteristics of the proposed glutamate biosensor are better in some cases or comparable with the other glutamate biosensors reported so far.

Conclusion

In this paper, a novel amperometric biosensor based on GR/Co₃O₄/CS nanocomposite for monitoring glutamate was described. The use of a GR and Co₃O₄ nanoparticles resulted in the improved analytical performance of the glutamate biosensor in terms of low response time (25 s), wide linear range (4.0×10^{-6} M – 6.0×10^{-4} M), good repeatability (1.0%) and no interference by common interfering substances. Moreover,

the cost of the presented biosensor is lower than that of the conventional techniques and the preparation procedure of the biosensor is very simple. The purposed strategy can be extended for the development of other enzyme-based biosensors. It can be concluded that the presented biosensor can be used for glutamate determination in food samples.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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