



Iron oxide nanoparticles–chitosan composite based glucose biosensor

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ABSTRACT

Iron oxide (Fe₃O₄) nanoparticles prepared using co-precipitation method have been dispersed in chitosan (CH) solution to fabricate nanocomposite film on indium–tin oxide (ITO) glass plate. Glucose oxidase (GOx) has been immobilized onto this CH–Fe₃O₄ nanocomposite film via physical adsorption. The size of the Fe₃O₄ nanoparticles estimated using X-ray diffraction (XRD) pattern and transmission electron microscopy (TEM) has been found to be ~22 nm. The CH–Fe₃O₄ nanocomposite film and GOx/CH–Fe₃O₄/ITO bioelectrode have been characterized using UV–visible and Fourier transform infrared (FTIR) spectroscopic and scanning electron microscopy (SEM) techniques, respectively. This GOx/CH–Fe₃O₄/ITO nanocomposite bioelectrode has response time of 5 s, linearity as 10–400 mg dL^{−1} of glucose, sensitivity as 9.3 μA/(mg dL cm²) and shelf life of about 8 weeks under refrigerated conditions. The value of Michaelis–Menten (*K_m*) constant obtained as 0.141 mM indicates high affinity of immobilized GOx towards the substrate (glucose).

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1. Introduction

Applications of nanomaterials to biosensors have recently aroused much interest. This is because these interesting materials exhibit large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency, and strong adsorption ability that are helpful for the immobilization of desired biosensing molecules including glucose oxidase. Moreover, nanoparticles have a unique ability to promote fast electron transfer between electrode and the active site of the enzyme (Pandey et al., 2008). In this context, different types of nanoparticles based on gold (AuNPs) (Pandey et al., 2007), ZnO (Wei et al., 2006; Wang et al., 2006), Fe₃O₄ (Rossi et al., 2004; Lu and Chen, 2006), etc., have been suggested as promising matrices for enzyme immobilization to improve stability and sensitivity of a biosensor. Among the various biosensors, development of glucose biosensor based on nanoparticles has been considered very important due to its application in clinical diagnostics (Pandey et al., 2007; Wei et al., 2006; Wang et al., 2006; Rossi et al., 2004; Lu and Chen, 2006).

Among the various nanomaterials, magnetic nanoparticles have recently gained increased interest due to promising applications as drug delivery, hyperthermia treatment, cell separation, biosensors, enzymatic assays, etc. (Rossi et al., 2004; Lu and Chen, 2006;

Cao et al., 2003; Gupta and Gupta, 2005; Cheng et al., 2005; Kouassi et al., 2005; Pisanic II et al., 2007). In this context, Fe₃O₄ nanoparticles have been considered as interesting for immobilization of desired biomolecules (GOx) because of biocompatibility, strong superparamagnetic property, low toxicity, etc. (Rossi et al., 2004; Lu and Chen, 2006; Cao et al., 2003). Immobilization of bioactive molecules on the surface of magnetic nanoparticles is of great interest, because magnetic behavior of these bioconjugates is likely to improve delivery and recovery of biomolecules for biomedical applications (Rossi et al., 2004). Recent advances in clinical diagnostics have stimulated demand for highly sensitive and precise analytical methods for estimation of desired analytes including glucose. It may be noted that the existing problem of aggregation and rapid biodegradation of Fe₃O₄ nanoparticles onto a desired matrix containing GOx can perhaps be overcome by modifying these interesting magnetic nanoparticles using inorganic semiconductors, conducting polymers and biopolymers (polysaccharides), etc.

Among the various biopolymers, chitosan (CH) along with nanoparticles has been utilized as a stabilizing agent due to its excellent film-forming ability, mechanical strength, biocompatibility, non-toxicity, high permeability towards water, susceptibility to chemical modifications, cost-effectiveness, etc. for enzyme (GOx) immobilization. Moreover, amino groups of CH provide a hydrophilic environment compatible with the biomolecules. Many attempts have been made to improve the biocompatibility and activity of the CH by structural modification by fabrication of

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nanocomposites with metal oxide nanoparticles, etc. (Miao and Tan, 2000; Xu et al., 2001).

CH-Fe₃O₄ nanocomposites have recently attracted much attention since surface functionalization of the nanoparticles allows their covalent attachment, self-assembly and organization on surfaces making them promising for the loading of biomolecules in a favorable microenvironment for the development of a biosensor (glucose). Lin and Leu (2005) have fabricated CH-Fe₃O₄ nanocomposite-based chemical sensor for cathodic determination of hydrogen peroxide (H₂O₂). Hrbac et al. (2007) have reported carbon electrode modified by nanoscopic Fe₃O₄ particles to assemble chemical sensor for estimation of H₂O₂ using amperometric technique. Zhang et al. (2007) have studied direct electrochemistry of hemoglobin immobilized on carbon-coated Fe₃O₄ nanoparticles for amperometric estimation of H₂O₂. Zhao et al. (2006) have reported that multilayer thin film of CH-Fe₃O₄ promotes direct electron transfer of hemoglobin. Liang et al. (2007) have synthesized polysaccharide-modified iron oxide nanoparticles as an effective magnetic affinity adsorbent for the bovine albumin serum. Wang and Tan (2007) have reported amperometric immunosensor based on CH-Fe₃O₄ composite film for estimation of ferritin. We report results of the studies relating to preparation, characterization of GOx immobilized onto CH-Fe₃O₄ nanocomposite film deposited onto ITO-coated glass surface.

2. Materials and methods

2.1. Reagents

Ferrous chloride, ferric chloride and triethyl amine were purchased from Sigma–Aldrich and have been used for the preparation of Fe₃O₄ nanoparticles. Chitosan, glucose oxidase (200 U mg⁻¹), and horseradish peroxidase (HRP, EC 1.11.1.7, and 200 U mg⁻¹) were purchased from Sigma–Aldrich (USA). All these chemicals have been used to prepare solutions in deionized water without further purification.

2.2. Preparation of Fe₃O₄ nanoparticles

Fe₃O₄ nanoparticles have been prepared using co-precipitation method. Ten milliliters of ferrous chloride (3×10^{-2} M) and 20 mL of ferric chloride (3×10^{-2} M) were thoroughly mixed using magnetic stirring to obtain a clear solution of pH 4 at room temperature. Solution (5 mL) of triethyl amine (5N) was added dropwise into continuously stirred solution for about 5 h until the black precipitate was obtained. Fe₃O₄ nanoparticles were collected with the help of a powerful magnet on the round-bottomed flask. These Fe₃O₄ nanoparticles were washed several times with distilled water and were followed by methanol. The prepared nanoparticles were kept in a vacuum oven at 40 °C for drying.

2.3. Preparation of CH-Fe₃O₄ nanocomposite film

A CH (0.50%) solution was prepared by dissolving CH (50 mg) in 100 mL of acetate buffer (0.05 M, pH 4.2) solution. The calculated amount of Fe₃O₄ nanoparticles was dispersed in the CH solution by stirring at room temperature after which it was sonicated. Finally, a highly viscous solution of CH with uniformly dispersed Fe₃O₄ nanoparticles was obtained. The film of nanocomposite was fabricated by dispersing 15 μ L solution of CH-Fe₃O₄ composite onto an ITO (0.25 cm²) surface and allowing it to dry at room temperature for about 12 h in controlled environment. The surface area of the ITO-coated glass plate is maintained as 0.25 cm². This CH-Fe₃O₄ nanocomposite film was washed with deionized water to remove any unbound particles.

It has been found that optimized ratio of CH and Fe₃O₄ nanoparticles taken as 10:1 to prepare CH-Fe₃O₄ nanocomposite film exhibits maximum amperometric current and that 15 μ L of CH-Fe₃O₄ nanocomposite solution standardized for dispersion onto ITO surface.

2.4. Characterization

The structure and particle size of Fe₃O₄ nanoparticles have been investigated using X-ray diffraction (XRD) and transmission electron microscopy (TEM) (Jeol, model JEM 200 CX), respectively. Fourier transform infrared (FTIR) spectrophotometer (PerkinElmer, Spectrum BX II) has been used to characterize CH-Fe₃O₄ nanocomposite and its interaction with enzyme (GOx). The cyclic voltammetry, electrochemical impedance spectroscopy (EIS) and amperometric measurements have been recorded on an Autolab Potentiostat/Galvanostat (Eco Chemie, The Netherlands). The electrochemical measurements have been conducted on a three-electrode system in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) as the electrolyte. The activity of the immobilized enzyme on CH-Fe₃O₄/ITO electrode has been estimated using UV–vis spectrophotometer (Phoenix 2200 DPCV). The surface morphological studies have been investigated using scanning electron microscope (LEO-440).

2.5. Immobilization of GOx on CH-Fe₃O₄ nanocomposite film

The fresh solution of glucose oxidase (1 mg mL⁻¹) was prepared in phosphate buffer (50 mM, pH 7.0) and 10 μ L solution of freshly prepared GOx was mechanically spread onto the desired CH-Fe₃O₄/ITO electrodes. The GOx/CH-Fe₃O₄/ITO bioelectrode was kept undisturbed at room temperature for about 4 h. Finally, these bioelectrodes were washed by phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) to remove any unbound enzyme from the electrode surface. It has been found that the GOx/CH-Fe₃O₄/ITO bioelectrode shows highest catalytic behavior in phosphate buffer at pH 7 (50 mM, 0.9% NaCl).

3. Results and discussion

3.1. Characterization of Fe₃O₄ nanoparticles, CH-Fe₃O₄ nanocomposite and GOx/CH-Fe₃O₄/ITO bioelectrode

X-ray diffraction pattern of synthesized Fe₃O₄ nanoparticles (Fig. 1A) shows reflection planes that are consistent with the standard pattern for Fe₃O₄ (ASTM 19-629) (Wing et al., 2004). However, the broad reflection planes are perhaps due to the nano-size of the Fe₃O₄ particles. The average particle size of Fe₃O₄ nanoparticles estimated using Debye–Scherrer formula is obtained as about 22 nm. The TEM image (Fig. 1B) indicates that magnetite nanoparticles are nanocrystalline, though their shape is predominantly spherical with some hexagonal shaped nanoparticles. The size of the spherical nanoparticles varies from ~10 to 30 nm with average particle size of ~22 nm and corresponds to the size estimated using X-ray diffraction measurements. Moreover, Fe₃O₄ nanoparticles are agglomerates due to high surface area and magnetic dipole–dipole interactions between particles (Reddy et al., 2007).

The UV–visible absorption spectroscopy has been employed to characterize the CH-Fe₃O₄ nanocomposite and to ascertain the presence of GOx on CH-Fe₃O₄ nanocomposite. The absorption band seen at 220 nm arising due to the π – π^* transition associated with –C=O group is attributed to the oligomer (Lu et al., 2006) originating from the degradation of product CH (Fig. 2A, curve a). A broad and featureless adsorption band seen at ~330 nm (Fig. 2A, curve b) originates primarily from the absorption and scattering of light by the Fe₃O₄ nanoparticles and is characteris-

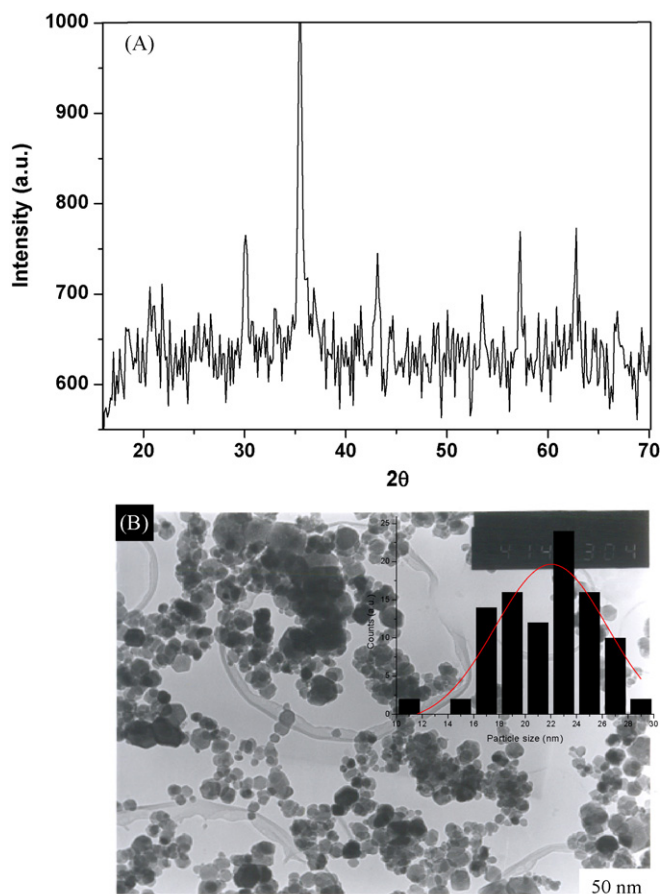


Fig. 1. X-ray diffraction pattern (A) and transmission electron micrograph (B) of Fe_3O_4 nanoparticle.

tic of the indirect band gap of semiconductors (Zhao et al., 2006). The absorbance of 220 nm band increases and the peak position shifts to higher wavelength region in the $\text{CH-Fe}_3\text{O}_4$ nanocomposite (Fig. 2A, curve c). This increase in the absorbance can be associated with successive loading of Fe_3O_4 nanoparticles in CH. The resulting enhanced Fe atom binding with CH molecules via complex formation (degradation of CH by Fe_3O_4 oligosaccharide containing C=O groups) indicates the electroactivity of bioelectrode (Khan and Dhayal, 2008). The additional absorption band appearing at 275 nm (curve d) in the spectra of $\text{GOx/CH-Fe}_3\text{O}_4$ bioelectrode indicates the immobilization of GOx indicating that the natural structure of enzyme (GOx) is preserved (Luo et al., 2004).

The FTIR spectra of pure CH (curve a, Fig. 2B) displays bands at $3200\text{--}3400\text{ cm}^{-1}$ due to the stretching vibration mode of OH and NH_2 group, 1745 cm^{-1} peak is assigned to the C=O stretching (C=O in carboxylic acid), 1650 cm^{-1} is due to amide I group (C=O stretching along with N-H deformation mode), 1560 cm^{-1} peak is attributed to the NH_2 group due to N-H deformation, 1460 cm^{-1} is assigned to the symmetrical deformation of CH_3 and CH_2 group, 1425 cm^{-1} peak is due to C-N axial deformation (amine group band), 1380 cm^{-1} peak is due to COO^- group in carboxylic acid salt, 1151 cm^{-1} is assigned to the special broad peak of $\beta(1\text{--}4)$ glucosidic band in polysaccharide unit, 1096 cm^{-1} is attributed to the stretching vibration mode of the hydroxyl group, 1028 cm^{-1} stretching vibration of C-O-C in glucose circle and $1060\text{--}1015\text{ cm}^{-1}$ bands corresponds to CH-OH in cyclic compounds (Tian et al., 2003).

The FTIR spectra (curve b, Fig. 2B) of the $\text{CH-Fe}_3\text{O}_4$ nanocomposite film exhibits characteristic IR bands of the functional group corresponding to pure CH and the Fe_3O_4 nanoparticles. The IR

bands corresponding to the -NH and OH stretching modes are shifted to the lower wave number in the IR spectra of the $\text{CH-Fe}_3\text{O}_4$ nanocomposite film (curve b), corresponding to pure CH. This indicates that amine group of CH is involved in the assembling of Fe_3O_4 nanoparticles. The absorption band of Fe_3O_4 nanoparticle appears at 582 cm^{-1} belonging to the stretching vibration mode and the torsional vibration mode of Fe-O bonds in the tetrahedral sites and in the octahedral sites. The FTIR spectra of the $\text{GOx/CH-Fe}_3\text{O}_4$ /bioelectrode (curve c, Fig. 2B) exhibits characteristic infrared bands of GOx (curve d) and $\text{CH-Fe}_3\text{O}_4$ nanocomposite (curve b, Fig. 2B). In $\text{CH-Fe}_3\text{O}_4$ nanocomposite, the presence of CH facilitates immobilization of biomolecules via amine and hydroxyl group. In addition, the electrostatic interaction between CH and Fe_3O_4 nanoparticles through hydrogen bonding and weak interactions reduces leaching of the enzyme leading to the improved stability of the bioelectrode. However, the shape of the absorption peaks becomes broader due to the overlapping of the functional group of the GOx and $\text{CH-Fe}_3\text{O}_4$ nanocomposite film indicating the immobilization of GOx on nanocomposite matrix.

Surface morphological studies of CH/ITO film, $\text{CH-Fe}_3\text{O}_4$ /ITO nanocomposite and $\text{GOx/CH-Fe}_3\text{O}_4$ /ITO bioelectrode have been investigated using scanning electron microscopy (Fig. 2C). The porous film of chitosan contains pin holes as shown in SEM micrograph (image a). Image b shows that Fe_3O_4 nanoparticles are uniformly embedded in the porous CH network and are stable with minimum aggregation. The granular porous surface of the $\text{CH-Fe}_3\text{O}_4$ /ITO nanocomposite film is suitable for the immobilization of biomolecules. As GOx is immobilized onto the $\text{CH-Fe}_3\text{O}_4$ nanocomposite matrix, the morphology of the surface changes to the well-regulated form. The surface of GOx immobilized $\text{CH-Fe}_3\text{O}_4$ nanocomposite shows a homogeneous globular morphology (image c) revealing the immobilization of GOx.

3.2. Mechanism of immobilization of GOx onto $\text{CH-Fe}_3\text{O}_4$ /ITO electrode

Fig. 3 shows the proposed mechanism for the immobilization of GOx onto $\text{CH-Fe}_3\text{O}_4$ nanocomposite film. It is known that CH is a cationic amine-rich polysaccharide (pH 4.2). It appears that the surface charged Fe_3O_4 nanoparticles (Reddy et al., 2007) interact with $\text{-NH}_2/\text{OH}$ groups of CH (Zhao et al., 2006) via electrostatic interaction and hydrogen bonding. These electrostatic interactions between Fe_3O_4 nanoparticles and CH have been confirmed by FTIR spectra. However, GOx is negatively charged biomolecule at pH 7.0 (Pandey et al., 2007) that can be easily immobilized onto the positively charged $\text{CH-Fe}_3\text{O}_4$ nanocomposite matrix via electrostatic interaction. The immobilization of GOx has been confirmed by UV-vis and IR studies (Fig. 2A and B).

3.3. Electrochemical impedance spectroscopic studies

Electrochemical impedance spectroscopy (EIS) studies of CH/ITO film, $\text{CH-Fe}_3\text{O}_4$ /ITO electrode and $\text{GOx/CH-Fe}_3\text{O}_4$ /ITO bioelectrodes have been investigated in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) in the frequency range, $0.01\text{--}10^5$ Hz. In the EIS, the semicircle part corresponds to the electron transfer limited process; its diameter is equal to the electron transfer resistance, R_{CT} , which controls the electron transfer kinetics of the redox probe at the electrode interface. Fig. 4A presents the Nyquist plots of the impedance spectroscopy of the CH/ITO film, $\text{CH-Fe}_3\text{O}_4$ /ITO electrode and $\text{GOx/CH-Fe}_3\text{O}_4$ /ITO bioelectrode, respectively. The diameter of semicircle for $\text{CH-Fe}_3\text{O}_4$ nanocomposite (curve b) is smaller than that of CH film (curve a). These results suggest that the electron transfer in the $\text{CH-Fe}_3\text{O}_4$ nanocomposite film is easier between the solution and electrode, i.e. Fe_3O_4 nanoparticles not

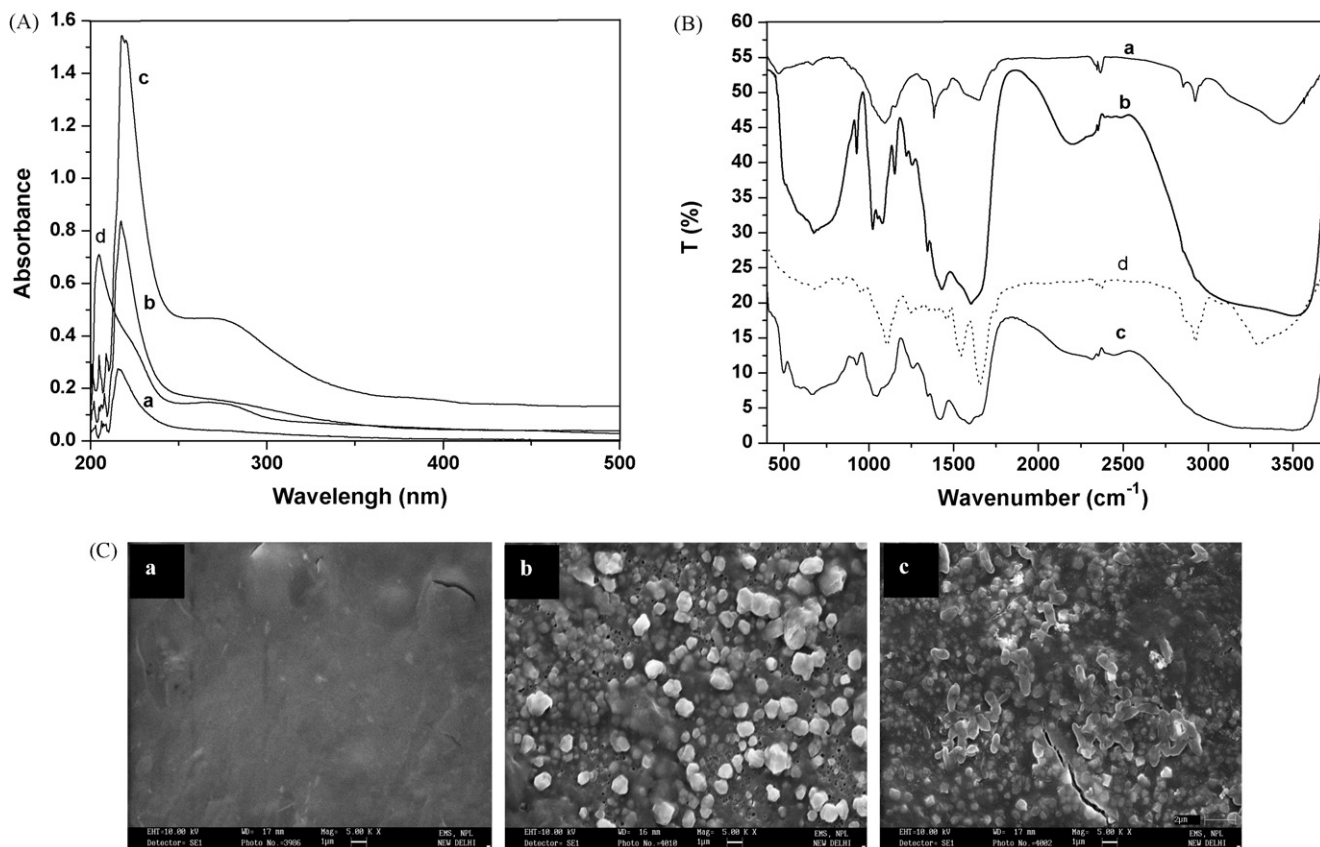


Fig. 2. UV-visible absorption (A) and FTIR transmission spectra (B) of (a) CH, (b) nanocomposite-CH-Fe₃O₄/ITO, (c) GOx/CH-Fe₃O₄/ITO bioelectrode and (d) GOx; (C) scanning electron microscopic images of (a) CH, (b) CH-Fe₃O₄ and (c) CH-Fe₃O₄-GOx on ITO-coated glass.

only provide the hydrophilic surface, but also act as a nanoscale electrode and promote electron transfer due to permeable structure of CH/ITO. However, the diameter of the semicircle obtained for the GOx/CH-Fe₃O₄/ITO bioelectrode further increases (curve c).

It appears that GOx layer acts as a barrier for the electron transfer between the electrode surface and the redox probes in the solution confirming immobilization of GOx onto the CH-Fe₃O₄ nanocomposite matrix.

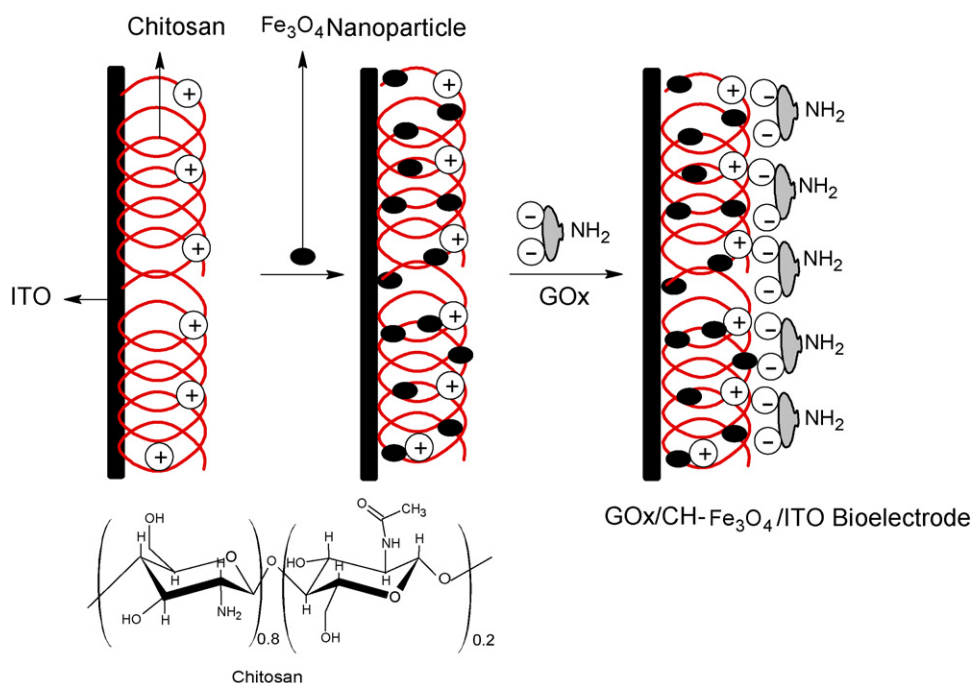


Fig. 3. Proposed mechanism of formation of CH-Fe₃O₄ nanocomposite and immobilization of glucose oxidase on nanocomposite matrix.

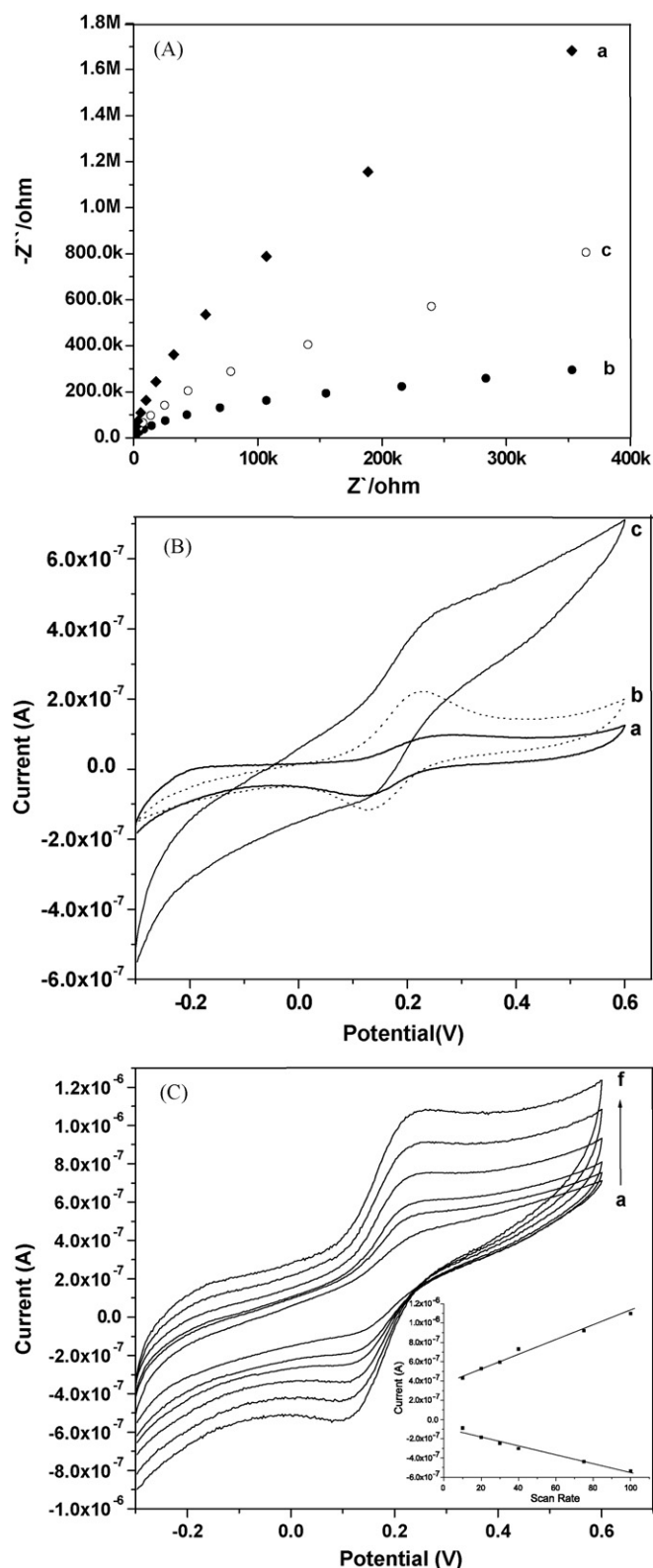


Fig. 4. (A) Electrochemical impedance spectra of CH (a), CH-Fe₃O₄ (b) and GOx/CH-Fe₃O₄/ITO bioelectrode. (B) Cyclic voltammogram of CH (a), CH-Fe₃O₄ (b) and GOx/CH-Fe₃O₄ (c). (C) Cyclic voltammogram of GOx/CH-Fe₃O₄ bioelectrode on increasing scan rate from 10 to 100 (a–g), in phosphate buffer solution (50 mM, pH 7) containing 0.9% NaCl.

3.4. Cyclic voltammetric studies

The cyclic voltammograms of CH/ITO, on CH-Fe₃O₄/ITO nanocomposite film and GOx/CH-Fe₃O₄/ITO bioelectrodes in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) are shown in Fig. 4B. In case of the CH/ITO electrode surface, oxidation and reduction peaks are obtained at 0.22 and 0.13 V, respectively (curve a). When Fe₃O₄ nanoparticles are incorporated into the CH matrix, the increased oxidation peak current is obtained (curve b). This may be assigned to the presence of Fe₃O₄ nanoparticles with increased electron mobility at the electrode surface resulting in enhanced electron transfer. The CV spectra of GOx/CH-Fe₃O₄/ITO (curve c) bioelectrode shows increased peak current with stable and well-defined redox peaks obtained at about 0.22 and 0.13 mV as compared to that of CH-Fe₃O₄/ITO electrode. This result is attributed to the redox characteristics of the electroactive centers of the immobilized GOx indicating enhanced electron transfer between GOx and CH-Fe₃O₄/ITO electrode. It appears that CH-Fe₃O₄ nanocomposite provides a biocompatible environment for GOx, and that Fe₃O₄ nanoparticles act as electron mediator accelerate electron transfer between GOx and electrode (Zhang et al., 2007). The results of experiments repeated a number of times have been found to be reproducible.

The CV studies have been carried out on GOx/CH-Fe₃O₄/ITO bioelectrode as a function of scan rate varying from 10 to 100 mV s⁻¹ (Fig. 4C). The variation of the redox peak height with sweep rate, is shown in the inset of Fig. 4C. It is observed that the peak height varies directly with the sweep rate (with linear regression coefficient 0.9937), indicating improved electrocatalytic behavior. It has been shown that this novel composite of CH-Fe₃O₄ can be used for the immobilization of GOx providing sufficient accessibility to electrons to shuttle between the enzyme and the electrode.

The surface concentrations of the CH/ITO, CH-Fe₃O₄/ITO electrode and GOx/CH-Fe₃O₄/ITO bioelectrode have been calculated using Brown–Anson model by following equation (Solanki et al., 2007):

$$I_p = \frac{n^2 F^2 I^* A V}{4RT} \quad (1)$$

where n is the number of electrons transferred (1), F is the Faraday constant (96,485 C/mol), I^* is the surface concentration (I^*) of the CH-Fe₃O₄/ITO film (mol cm⁻²), A is the surface area of the electrode (0.14 cm²), V is the scan rate (10 × 10⁻³ V s⁻¹), R is the gas constant (8.314 J mol⁻¹ K⁻¹) and T is the absolute temperature (298 K). The surface concentration of GOx/CH-Fe₃O₄/ITO bioelectrode (2.46 × 10⁻¹⁰ mol cm⁻²) is higher than that of CH-Fe₃O₄/ITO (1.48 × 10⁻¹⁰ mol cm⁻²) and pure CH (1.644 × 10⁻¹¹ mol cm⁻²). The higher surface concentration of CH-Fe₃O₄ electrode suggests that loading of Fe₃O₄ nanoparticles increases the electroactive surface area. However, the increase in the surface concentration of GOx/CH-Fe₃O₄ bioelectrode suggests high loading of glucose oxidase onto CH-Fe₃O₄ nanocomposite matrix.

4. Response characteristics of GOx/CH-Fe₃O₄/ITO bioelectrode

4.1. Electrochemical response studies

Fig. 5A shows results of the cyclic voltammetric (CV) studies carried out to investigate the activity of the GOx/CH-Fe₃O₄/ITO bioelectrode as a function of glucose concentration (10–400 mg dL⁻¹) in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) at scan rate of 10 mV s⁻¹. It can be seen that no redox peak relating to the generation of H₂O₂ is observed in the CV measurements (Fig. 5A). This could perhaps be attributed to the nanocomposite matrix of

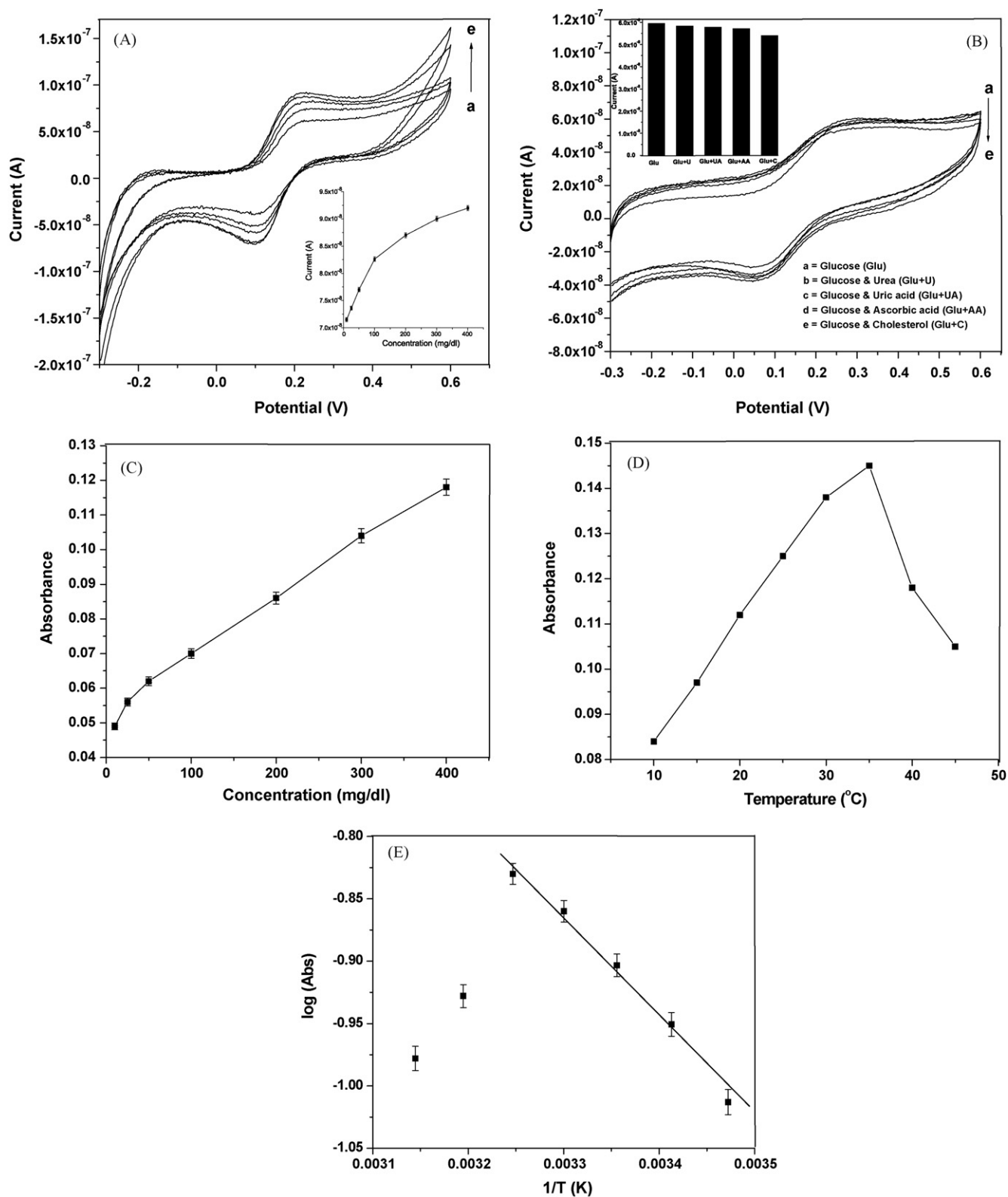


Fig. 5. (A) Electrochemical response studies of GOx/CH-Fe₃O₄/ITO bioelectrode (inset: variation in current obtained as a function of glucose concentration). (B) Effect of interference on GOx/CH-Fe₃O₄/ITO bioelectrode (inset: variation in current obtained as a function of interference). (C) Photometric response of GOx/CH-Fe₃O₄ bioelectrode as a function of glucose concentration (10–400 mg dL⁻¹) in phosphate buffer (50 mM, pH 7.0) containing 0.9% NaCl. (D) Effect of temperature on GOx/CH-Fe₃O₄/ITO bioelectrode from 10 to 45 °C. (E) Arrhenius plot between $\log(1/\text{Abs})$ and $1/T$ (K) for GOx/CH-Fe₃O₄/ITO bioelectrode calculated the activation energy.

Table 1
The glucose sensing characteristics of GOx/CH–Fe₃O₄/ITO bioelectrode are summarized along with those reported in literature (based on nanoparticle, CH and nanocomposite of CH and nanoparticles)

Material	Detection limit	Linearity	Sensitivity	K _m value	Response time (s)	Shelf life	Ref.
Thiolated gold		50–400 mg dL ⁻¹		3.75 mM	30	180 days	Pandey et al. (2007)
ZnO nanocomb	0.02 mM	2.02–4.5 mM	15.33 μ A mM ⁻¹	2.19 mM			Wei et al. (2006)
ZnO nanorod	0.01 mM	0.001–3.45 mM	23.1 μ A mM ⁻¹ cm ⁻²	2.6 mM	5		Wang et al. (2006)
Fe ₃ O ₄		1–100 mM					Rossi et al. (2004)
Fe ₃ O ₄ /SPCE		33.3 mM	1.74 μ A mM ⁻¹		15		Lu and Chen (2006)
Fe ₃ O ₄ @ SiO ₂	3.25 $\times 10^{-6}$ M	(1–4) $\times 10^{-5}$ mM			10		Qiu et al. (2007)
Sol–gel–CH		2–14 mM		21 mM	30	30 days	Chen et al. (2003)
Thiolated gold–CH	1 $\times 10^{-2}$ mM		4.9 nA mM ⁻¹		20		Miscoria et al. (2006)
Au–CH/Pt	7.0 μ M	0.5–16 mM	0.555 μ A mM ⁻¹	10.5 mM	8	30 days	Wu et al. (2007)
Au–CH	13 μ M	5 $\times 10^{-5}$ to 1.3 $\times 10^{-3}$ mM		3.5 mM	10		Du et al. (2007)
Au–Pt alloy–CH	0.2 μ M	0.001–7 mM	8.52 μ A mM ⁻¹		5		Kang et al. (2007)
Au–CH	2.7 μ M	5 μ M to 2.4 mM			7	5 weeks	Luo et al. (2004)
CH–Au/Pt	6.9 $\times 10^{-7}$ M	1 $\times 10^{-6}$ to 1.6 $\times 10^{-3}$ M	69.29 μ A mM ⁻¹	6.8 mM L ⁻¹	3		Xue et al. (2006)
CNT–CH		0–7.8 mM	0.52 μ A mM ⁻¹	8.2 mM		15 days	Liu et al. (2005)
CNT–PtNP–CH–MTOS	3 $\times 10^{-7}$ M	1.3 $\times 10^{-6}$ to 6 $\times 10^{-3}$ mM	2.08 μ A mM ⁻¹	7.9 mM	5	30 days	Kang et al. (2008)
Pt–MWCNT–CH–SiO ₂	1 μ M	1–23 μ M	58.9 μ A mM ⁻¹	14.4 mM	5		Zou et al. (2008)
Ferri–CH–SPCE	1.38 mM		0.677 μ A mM ⁻¹		20		Lee et al. (2006)
CH–ZrO ₂	1 $\times 10^{-5}$ M	1 $\times 10^{-5}$ to 9.5 $\times 10^{-3}$ M	0.028 μ A mM ⁻¹		10	30 days	Yang et al. (2004)
CH–Fe ₃ O ₄	0.5 mM	0.5–22 mM	9.3 μ A mM ⁻¹ cm ⁻²	0.141 mM	~5	8 weeks	Present work

CH and Fe₃O₄ nanoparticles that act as better electron acceptor over molecular oxygen from the reduced enzyme (Matharu et al., 2007). During the reoxidation of GOx after enzymatic reaction, the nanocomposite CH–Fe₃O₄ nanocomposite film accepts electrons from the reduced enzyme, thereby causing increase in the oxidation current of CH–Fe₃O₄ (Fig. 5A). The magnitude of the oxidation peak increases linearly with increase in the glucose concentration (Fig. 5A). The response time of the GOx/CH–Fe₃O₄/ITO bioelectrode has been found to be about 5 s and is attributed to faster electron communication feature of CH–Fe₃O₄ nanocomposite. It is revealed that GOx/CH–Fe₃O₄ film/ITO bioelectrode (inset of Fig. 5A) can be used to estimate glucose from 10 to 400 mg dL⁻¹. The sensitivity of the GOx/CH–Fe₃O₄ film/ITO bioelectrode calculated from the slope of curve has been found to be 9.3 μ A/(mg dL cm²). The standard deviation and correlation coefficient from the linear regression analysis for the bioelectrode have been found to be 0.00316 μ A and 0.9935, respectively.

The selectivity of GOx/CH–Fe₃O₄/ITO bioelectrodes has been determined by comparing the electrochemical response by adding the normal concentration of interferents (Fig. 5B) such as cholesterol (5 mM), ascorbic acid (0.05 mM), uric acid (0.1 mM) and urea (1 mM) with glucose (100 mg dL⁻¹) in phosphate buffer (50 mM, pH 7, 0.9% NaCl). It can be seen that the value of the amperometric current remains nearly same except for cholesterol wherein there is a decrease of about 6%.

4.2. Photometric response studies

The UV–visible experiments have been carried out to estimate the enzyme (glucose oxidase) activity, stability, effect of interferents and shelf life of GOx/CH–Fe₃O₄/ITO bioelectrodes. The bioelectrode is dipped in reaction mixture containing 3 mL PBS solution, 25 μ L of HRP (1 mg mL⁻¹ in PBS), 25 μ L of *o*-dianisidine dye (1% in deionized water), and 100 μ L of glucose solution (100 mg dL⁻¹) and is kept for about 3 min.

Fig. 5C shows the variation of the difference between the initial and final absorbance value obtained at 500 nm after 3 min of incubation of the substrate (glucose). It can be seen (Fig. 5C) that the value of absorbance increases linearly with glucose concentration in the range of 10–400 mg dL⁻¹. The experiments carried out in triplicate sets have been found to be reproducible within 1% error.

The value of apparent Michaelis–Menten constant (K_m) estimated using Lineweaver–Burke plot, i.e. graph between inverse of

absorption and inverse of glucose concentration has been found to be 2.54 mg dL⁻¹ (0.141 mM). The lower value of K_m compared to the value reported for metal oxide- and CH-based matrices (Table 1), suggests that the CH–Fe₃O₄ nanocomposite matrix helps the immobilized glucose oxidase to achieve better conformation for faster enzymatic reaction resulting in the enhanced enzymatic activity.

The apparent enzyme activity (U cm⁻²), defined as one unit of enzyme activity that results in the conversion of 1 μ mol of glucose into glucolactone per minute, has been calculated using the following equation (Solanki et al., 2007):

$$\alpha_{app}^{enz} (\text{U cm}^{-2}) = \frac{AV}{\varepsilon_{st}} \quad (2)$$

where A is the difference in absorbance before and after incubation, V is the total volume (3.015 cm³), ε is the millimolar extinction coefficient (7.5 for *o*-dianisidine at 500 nm), t is the reaction time (3 min), and s is the surface area (cm²) of the electrode. The apparent enzyme activity has been found to be ~0.0305 (U cm⁻²) indicating that 0.0305 units of enzyme per cm² actively participate in the enzymatic reaction.

The activity of GOx/CH–Fe₃O₄/ITO bioelectrode has been measured at temperature varying from 10 to 45 °C in phosphate buffer saline solution at pH 7.0 (Fig. 5D). The reaction rate is found to increase with temperature up to 35 °C (Fig. 5D), where after it shows a sharp decrease, indicating that GOx gets denatured after ~35 °C. The activation energy, estimated using the Arrhenius plot of log(absorbance) with reciprocal of temperature (K) (Fig. 5E) has been found to be 18 kJ mol⁻¹ in the lower temperature range.

The shelf life of GOx/CH–Fe₃O₄/ITO bioelectrode has been monitored as a function of absorbance with respect to time, with regular interval of 1 week. It is observed that these bioelectrodes retain about 80% of glucose oxidase activity even after about 8 weeks (data not shown) when stored in refrigerated conditions (4 °C). It has been found that this glucose biosensing electrode has higher affinity to glucose.

The glucose sensing characteristics of the GOx/CH–Fe₃O₄/ITO bioelectrode along with those reported in literature (based on nanoparticle, CH and nanocomposite of CH and nanoparticles) are summarized in Table 1. It can be seen that GOx/CH–Fe₃O₄/ITO bioelectrode reveals improved performance with fast response time, low K_m value, high sensitivity and better shelf life.

5. Conclusions

Fe₃O₄ nanoparticles have been successfully embedded in the chitosan matrix and GOx has been immobilized on the CH–Fe₃O₄/ITO electrode. CH prevents the aggregation of Fe₃O₄ nanoparticles without changing its optical and electrical properties. The immobilized GOx displays excellent catalytic property to glucose, and Fe₃O₄ nanoparticles in the biosensing interface offer not only friendly environment to immobilize GOx but also improve the electron transfer between analyte (glucose) and CH–Fe₃O₄/ITO electrode surface. GOx/CH–Fe₃O₄/ITO bioelectrode shows high sensitivity, low detection limit and fast response for glucose. In addition, the GOx/CH–Fe₃O₄/ITO glucose biosensor exhibits both good reproducibility and long-term stability. It should be interesting to investigate the effect of porosity of the CH–Fe₃O₄ nanocomposite on the performance of the glucose bioelectrode. Efforts should also be made to fabricate other biosensors based on this interesting nanocomposite.

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