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Amperometric choline biosensor based on multiwalled carbon nanotubes/zirconium oxide nanoparticles electrodeposited on glassy carbon electrode

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ABSTRACT

A bienzymatic choline biosensor was constructed by coimmobilizing acetylcholinesterase (AChE) and choline oxidase (ChO) onto nanocomposite of carboxylated multiwalled carbon nanotubes (c-MWCNTs) and zirconium oxide nanoparticles (ZrO_2NPs) electrodeposited on the surface of a glassy carbon electrode (GCE) and using it (AChE–ChO/c-MWCNT/ZrO_2NPs/GCE) as working electrode, Ag/AgCl as reference electrode, and Pt wire as auxiliary electrode connected through a potentiostat. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and cyclic voltammetry (CV) studies, optimized, and evaluated. The biosensor exhibited optimum response within 4 s at +0.2 V, pH 7.4, and 25 °C. The detection limit and working range of the biosensor were 0.01 μM and 0.05 to 200 μM, respectively. The half-life of the enzyme electrode was 60 days at 4 °C. The serum choline level, as measured by the biosensor, was 9.0 to 12.8 μmol/L (with a mean of 10.81 μmol/L) in apparently healthy persons and 5.0 to 8.4 μmol/L (with a mean of 6.53 μmol/L) in persons suffering from Alzheimer's disease. The enzyme electrode was unaffected by a number of serum substances.

Choline is a precursor of acetylcholine, a neurotransmitter that is involved in the signal transmission among nerves, muscles, and organs. Monitoring the levels of acetylcholine and choline in serum is very important to detect neurodegenerative diseases such as Alzheimer's and neuromuscular diseases, myasthenia gravis, and impaired cholinergic neurotransmission [1,2]. Among the various methods available for measurement of choline, biosensing methods are comparatively simpler and more sensitive, rapid, and specific. These acetylcholine/choline biosensors employed acetylcholinesterase (AChE)[1] and choline oxidase (ChO), which catalyzed the following electrochemical reactions [3–5]:

(1) Hydrolysis of acetylcholine:

$$\text{Acetylcholine} + H_2O \xrightleftharpoons{\text{Acetylcholinesterase}} \text{Acetate} + \text{Choline} + H^+$$

(2) Oxidation of choline:

$$\text{Choline} + 2O_2 + H_2O \xrightleftharpoons{\text{cholineoxidase}} \text{Betaine} + 2H_2O_2$$

(3) Electrolysis of H_2O_2:

$$2H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

The flow of electrons (i.e., current) is directly proportional to the acetylcholine/choline concentration. A number of amperometric choline biosensors have been reported based on immobilization of AChE/ChO onto photopolymerized polyvinyl alcohol (PVA)–SbQ [6], methacrylate–vinylene carbonate copolymer [7], polyvinylferrocenium [8], poly-5,2′:5′,2″-terthiophene-3-carboxylic acid (poly-TTCA) [9], poly(2-hydroxyethyl methacrylate) films [10], PVA–SbQ and covered with Nafion (perfluorosulfonated membrane) [11], PVA cryogel membrane [12], poly(pyrrole)/poly(2-naphthol) bilayer membrane [13], and Prussian blue on the surface of a Pt electrode by crosslinking with bovine serum albumin and glutaraldehyde [14]. All of these biosensors had low storage stability due to leakage of enzymes and sensitivity.

Nanomaterials have attracted much attention for designing novel biosensing systems to improve their bioanalytical performance. Multiwalled carbon nanotubes (MWCNTs) are one promising

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[1] *Abbreviations used:* AChE, acetylcholinesterase; ChO, choline oxidase; PVA, polyvinyl alcohol; MWCNT, multiwalled carbon nanotube; ZrO_2NP, zirconium oxide nanoparticle; c-MWCNT, carboxylated MWCNT; GCE, glassy carbon electrode; DW, double distilled water; CV, cyclic voltammetry; EIS, electrochemical impedance spectroscopy; TEM, transmission electron microscopy; FTIR, Fourier transform infrared; SEM, scanning electron microscopy; ChCl, choline chloride; HPLC, high-performance liquid chromatography.

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nanomaterial that has been explored for chemical and biological sensing applications. These nanotubes have been employed in biosensors as effective catalyst supports due to their large surface area, unique structural and electromechanical properties, good biocompatibility, ease of preparation, and surface renewability [15–17]. Recently, nanocomposites of conducting polymers [18] and nanoparticles [19,20] have attracted potential interest for such purposes. Zirconium oxide nanoparticles (ZrO_2NPs) are nontoxic due to their excellent chemical inertness and biocompatibility and, thus, are an ideal support for immobilization of biomolecules. We describe here the construction and application of a bienzymatic choline sensor by covalently immobilizing AChE and ChO onto nanocomposite of carboxylated MWCNTs (c-MWCNTs) and ZrO_2NPs electrodeposited on a glassy carbon electrode (GCE).

Materials and methods

Chemicals and reagents

AChE (EC 3.1.1.7, type VI-S, from electric eel, activity 200–600 U/mg solid), ChO (EC 1.1.3.17, from *Alcaligenes* species, activity 10 U/mg solid), acetylcholine chloride, and choline chloride were obtained from Sigma Chemical Co, St. Louis (USA). c-MWCNTs (functionalized MWCNTs, 12 walls, length 15–30 mm, purity 90%, no metal content) obtained from Intelligent Materials (Panchkula (Haryana), India), ZrO_2 nanopowder obtained from Sisco Research Laboratory (Mumbai, India), and GCE (disk diameter 3 mm) obtained from Metrohm–India (Delhi, India) were used. All other chemicals were of analytical reagent grade. Double distilled water (DW) was used throughout the experiments.

Apparatus

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements in a potentiostat/galvanostat (Autolab, model AUT83785, Eco Chemie, The Netherlands) with a three-electrode system consisting of enzyme electrode (AChE–ChO/c-MWCNT/ZrO_2NPs/GCE) as working electrode, Ag/AgCl as reference electrode, and Pt wire as auxiliary electrode, ultrasonication in Misonix Ultrasonic Liquid Processors (model XL-2000 series), transmission electron microscopy (TEM) images of ZrO_2NPs (1 mg/ml) in a transmission electron microscope at Punjab University (Chandigarh, India), Fourier transform infrared (FTIR) spectra in an FTIR spectrometer (model iS10, Thermo Electron, USA), and scanning electron microscopy (SEM) images of modified electrode in a scanning electron microscope (model Joel JSM-6510, Japan) were recorded.

Construction of c-MWCNT/ZrO_2NPs modified GCE

Prior to the electrodeposition, the bare GCE was polished with alumina slurry (diameter 0.05 μm) and then cleaned ultrasonically in ethanol and water, followed by thorough rinsing with DW. The powder of c-MWCNTs was then sonicated in 5.0 mM ZrO_2 solution containing 100 mM KCl (0.5 mg/ml) for approximately 15 min to form uniform c-MWCNT black-colored solution. The cleaned GCE was dipped into this ZrO_2 NPs/c-MWCNT suspension. A nanocomposite film of c-MWCNT/ZrO_2NPs was electrochemically deposited on the surface of polished GCE at a constant potential of −1.1 V for 5 min. The prepared electrode was rinsed gently with DW and dried in air [21].

Preparation of enzyme electrode

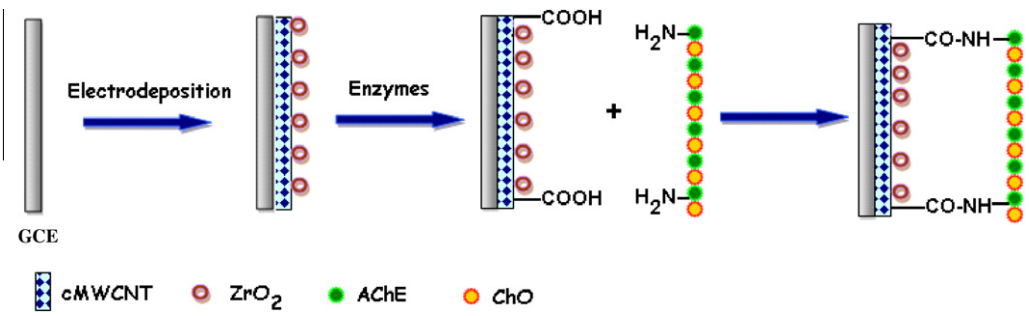
To prepare the enzyme electrode (AChE–ChO/c-MWCNT/ZrO_2NPs/GCE), a mixture of 10 μl of AChE solution (2 mg/ml) and 10 μl of ChO (10 mg/ml) was mounted on the surface of c-MWCNT/ZrO_2NPs modified GCE and kept at 4 °C for 24 h. The prepared enzyme electrode was rinsed with DW clearly, dried, and stored at 4 °C until use (Scheme 1).

Characterization of enzyme electrode

The enzyme electrode was characterized by SEM, FTIR, and EIS at different stages of its construction. EIS studies were carried out in a potentiostat/galvanostat in the frequency range of 0.01 Hz to 10 kHz with amplitude +0.2 V.

CV study, response measurements, and optimization of enzyme electrode

A cyclic voltammogram of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE was recorded in the potential range of +0.0 to +0.6 V at a scan rate of 50 mV s^{-1} versus Ag/AgCl as reference electrode and Pt as auxiliary electrode in 15 ml of 0.1 M phosphate buffer (pH 7.0) containing 1 ml of choline chloride (ChCl). The maximum response was observed at +0.2 V; hence, subsequent studies were carried out at this voltage. To test the functioning of the biosensor, the three-electrode system was immersed into 15 ml of 0.1 M phosphate buffer (pH 7.0) containing ChCl (1 ml of 0.5 mM solution) in a 50-ml beaker, and the current (mA) generated at +0.2 V was recorded. The effect of pH of the buffer was studied over the pH range 5.0 to 9.0 at an interval of pH 0.2 using 0.1 M sodium succinate buffer for pHs 5.0 to 5.6, sodium phosphate for pHs 5.8 to 8.0, and borate buffer for pHs 8.2 to 9.0. The effect of incubation temperature on AChE–ChO/c-MWCNT/ZrO_2NPs modified GCE was studied by incubating the reaction mixture at different tempera-



Scheme 1. Scheme for preparation of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE.

tures (20–50 °C at an interval of 5 °C). CV studies were recorded in 0.1 M phosphate buffer (pH 7.0) containing ChCl concentrations varying from 0.05 to 200 μM at 0.0 to +0.6 V with a scan rate of 30 mV s^{-1}. The amperometric response was also measured in the presence of the potential interferants/metabolites such as ascorbic acid, uric acid, dopamine, lactic acid, heparin sodium, $CuSO_4$, KCl, NaCl, and $MgCl_2$, each at 1.0 mM.

Amperometric determination of serum choline with enzyme electrode

Fresh serum samples from apparently healthy persons and persons suffering from Alzheimer's disease were collected in three age groups—(i) children up to 20 years, (ii) adults from 21 to 45 years, and (iii) older adults 46 years and above—from the hospital of the local Pt. Bhagwat Dayal Sharma Post Graduate Institute of Medical Science (Rohtak, India) and analyzed for choline using the current electrode. The procedure for measurement of choline in these samples was the same as described for response measurement of electrodes under optimal conditions except that ChCl was replaced by serum. Choline concentration was interpolated from the standard curve between ChCl concentration and current (mA).

Reusability and storage stability of enzyme electrode

To reuse the working electrode, it was washed by dipping it in a test tube containing 2 ml of reaction buffer. The storage stability of the enzyme electrode was investigated over 60 days by keeping it dry at 4 °C.

Results and discussion

SEM study of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE

The surface of bare GCE was smooth (Fig. 1A). Fig. 1B shows the presence of c-MWCNTs and ZrO_2NPs on the surface of modified GCE (c-MWCNT/ZrO_2NPs/GCE) as tubular structures and granular structures. Fig. 1C shows the globular shapes of enzymes (AChE–ChO) on the surface of the enzyme electrode (AChE–ChO/c-MWCNT/ZrO_2NPs/GCE), confirming the presence of enzyme layer after its immobilization.

FTIR analysis

FTIR spectra of c-MWCNTs show the characteristic absorption peaks near 1518, 1156, and 2320 cm^{-1} (Fig. 2A), which were originated from the graphitic component of c-MWCNTs. A sharp absorption peak was observed at 1640 cm^{-1} due to the attachment of –COOH groups of MWCNTs with $–NH_2$ groups on the surface of enzyme because –COOH groups attached to MWCNTs were merely partly replaced by ZrO_2NPs (Fig. 2, curve i). In the FTIR spectrum of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE (curve ii), enzyme (AChE) binding is indicated by the appearance of additional absorption bands at 1641 and 1539 cm^{-1} assigned to the carbonyl stretch. In addition, a broad band was seen around 3200 cm^{-1}, which is attributed to amide bond present in AChE.

EIS study

Fig. 2B shows electrochemical impedance spectra of bare GCE (curve i), c-MWCNT/ZrO_2NPs/GCE (curve ii), and AChE–ChO/c-MWCNT/ZrO_2NPs/GCE (curve iii). The charge transfer process in AChE–ChO/c-MWCNT/ZrO_2NPs/GCE was studied by monitoring charge transfer resistance (R_{ct}) at the electrode/electrolyte interface. The value of the electron transfer resistance (semicircle diameter, R_{ct}) depends on the dielectric and insulating features at the

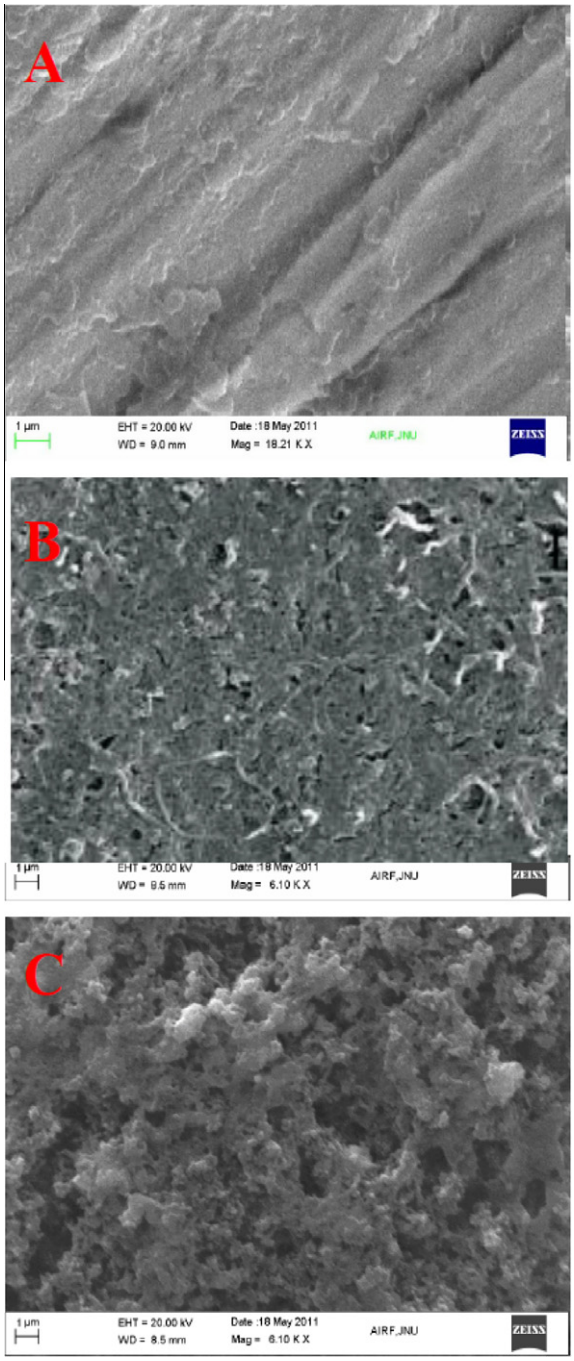


Fig.1. Scanning electron micrographs of bare GCE (A), c-MWCNT/ZrO_2NPs/GCE (B), and AChE-ChO/c-MWCNT/ZrO_2NPs/GCE (C).

electrode/electrolyte interface. The obtained R_{ct} values for the bare GCE, c-MWCNT/ZrO_2NPs/GCE, and AChE–ChO/c-MWCNT/ZrO_2NPs/GCE were 675, 452, and 929 Ω, respectively. The increased R_{ct} value of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE was due to the immobilization of enzymes onto ZrO_2NPs/c-MWCNT modified GCE surface. This increase in R_{ct} is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 KHz) and cause hindrance to the electron transfer.

CV study of enzyme electrode

The AChE/ChO electrode was characterized using ChCl as a standard substrate. In the developed enzyme electrode, AChE–ChO was

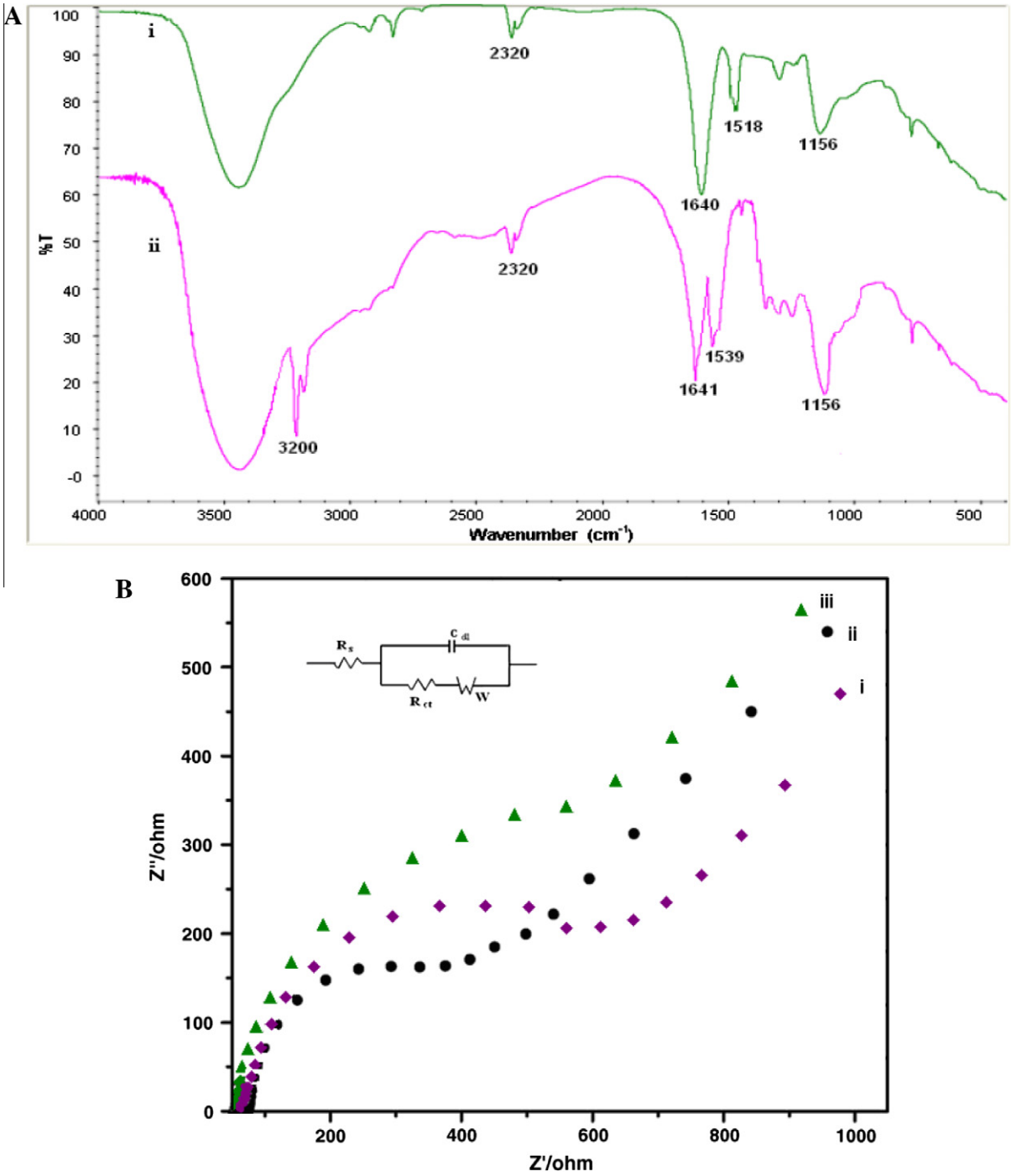


Fig.2. (A) Infrared spectra of AChE–ChO/c-MWCNT/ZrO_2NPs/GCE without enzyme (i) and with enzyme (ii). (B) Impedance spectroscopy study of bare GCE (i), c-MWCNT/ZrO_2NPs/GCE (ii), and AChE–ChO/c-MWCNT/ZrO_2NPs/GCE (iii) at 0.1 M phosphate buffer (pH 7.4) containing 5 mM $Fe(CN)_6^{3-/4-}$ and 0.5 mM ChCl. Frequency range: 0.01 Hz to 10 kHz. The inset shows the equivalent circuit for mixed kinetic and diffusion control.

covalently immobilized on ZrO_2NPs/c-MWCNT/GCE. The performance of the enzyme electrode was also evaluated by CV in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 M KCl at different stages of its construction. Fig. 3A shows the cyclic voltammograms of a GCE, c-MWCNT/ZrO_2NPs/GCE and AChE–ChO/c-MWCNT/ZrO_2NPs/GCE, in the presence of 100 µl of ChCl (0.6 mM) in 0.1 M sodium phosphate buffer (pH 7.4) at 0 to +0.6 V with a scan rate of 50 mV s^{-1}. No peak was observed for GCE (curve a) after 100 µl of ChCl was injected into the reaction cell. The cyclic voltammogram of c-MWCNT/ZrO_2NPs/GCE identified an oxidation peak current (curve b). The AChE–ChO/c-MWCNT/ZrO_2NPs/GCE show a higher oxidation peak current (curve c) than that of c-MWCNT/ZrO_2NPs/GCE (curve b).

Optimization of choline biosensor

The biosensor showed a maximum response at pH 7.4. The effect of the incubation temperature on the biosensor response showed a slight peak at 25 °C. Hence, the subsequent experiments

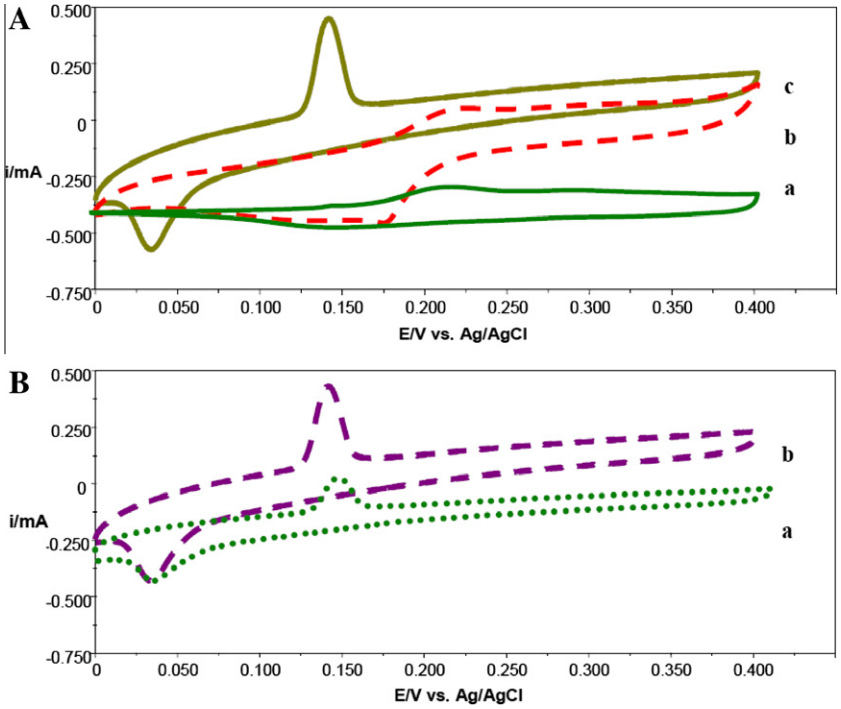


Fig.3. (A) Cyclic voltammograms of GCE (a), c-MWCNT/ZrO_2NPs film modified electrode (b), and AChE–ChO/c-MWCNTs/ZrO_2NPs nanocomposite film modified GCE. Supporting electrolyte: 0.1 M KCl containing 5 mM $Fe(CN)_6^{3-/4-}$; scan rate: 50 mV s^{-1}. (B) CV curves of AChE–ChO/c-MWCNTs/ZrO_2NPs/GCE in 0.1 M sodium phosphate buffer (pH 7.4) without (a) and with (b) 0.1 mM ChCl solution. Supporting electrolyte: 0.1 M KCl solution; scan rate: 50 mV s^{-1}.

were carried out at pH 7.4 and 25 °C. The biosensor showed an optimum response within 4 s.

Effect of substrate concentration

Fig. 3B shows cyclic voltammograms of the AChE–ChO/c-MWCNT/ZrO_2NPs/GCE in an unstirred 0.1M KCl solution (20 ml) and 0.1 M sodium phosphate buffer (pH 7.4, 5 ml) without (curve a) and with (curve b) 0.1 mM ChCl solution at a scan rate of 50 mV s^{-1}. When 0.1 mM ChCl was added, the well-defined oxidation (0.15 V) and reduction (0.035 V) peaks (curve b) were observed and clearly indicate the catalytic properties of the modified electrode.

A well-defined increasing trend in the current response of the biosensor in ChCl concentrations ranging from 0.05 to 200 μM was observed. However, there was no significant improvement in current after 200 μM ChCl concentration. The electrode had reached its saturation level at 220 μM Fig. 4). In the absence of ChCl, the peak responses were negligible. The detection limit of the electrode was calculated as a concentration that gave a signal equal to three times the standard deviation of the blank signal and was found to be 0.01 μM.

Evaluation of biosensor

The mean analytic recoveries of added choline chloride at 5.0 and 10.0 μM (final concentration in serum) as determined by the current biosensor were 90.0 ± 0.3 and 98.0 ± 0.2%, respectively. To test the reproducibility and reliability of the current choline biosensor, choline content in six serum samples was determined on

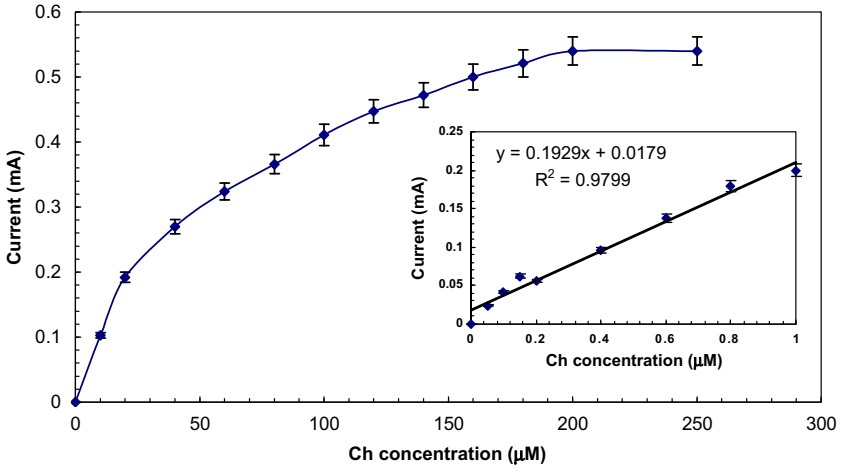


Fig.4. Calibration graph for ChCl obtained at pH 7.4 and 25 °C. The inset shows the response to 0.05 to 1.0 μM ChCl.

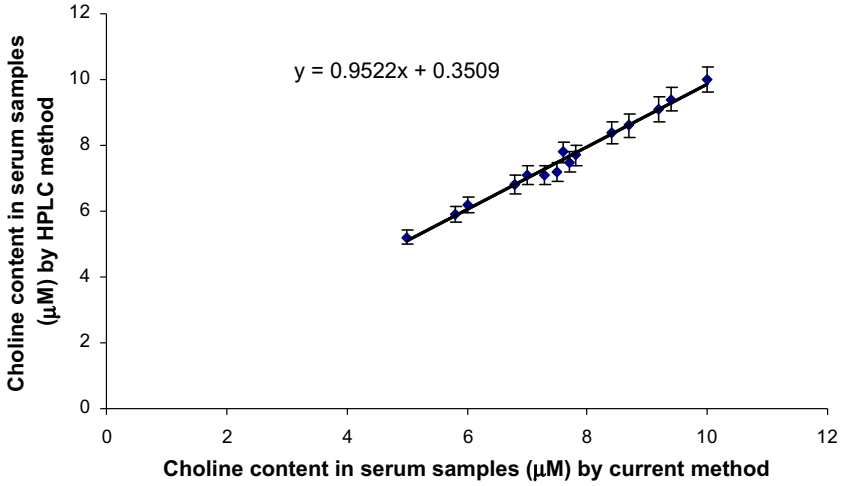


Fig.5. Correlation between choline values as determined by current biosensor (*x* axis) based on AChE–ChO/c-MWCNTs/ZrO_2NPs/GCE and standard HPLC method (*y* axis).

a single day (within batch) five times and again after storage at –20 °C for 1 week (between batch). The results showed that determinations were consistent and that within and between coefficients of variation (CVs) were 1.35% and 2.97%, respectively, and bias was 4.88%. These results indicated the reproducibility and consistency of the current method. The choline values of our method were in good agreement with those by the standard high-performance liquid chromatography (HPLC) method (on a commercial basis) with a good regression coefficient ($r = 0.994$) (Fig. 5). The *F* test values for both methods was 1.044, which is less than the critical value, indicating no significance difference. Similarly, the *t* value was 0.402 ($P > 0.05$), showing a nonsignificant difference.

Practically no interference was observed during measurements of choline by the current biosensor in the presence of ascorbic acid, uric acid, dopamine, lactic acid, heparin sodium, $CuSO_4$, KCl, NaCl, and $MgCl_2$, each at 1.0 mM, whereas the previously reported choline biosensors showed a maximum decrease in their activity in the presence of ascorbic acid [8,13].

Application

The choline value in serum of apparently healthy individuals (children, adults, and older adults, $n = 100$), as measured by the current biosensor, was in the range of 9.0 to 12.8 μmol/L (with a mean of 10.81 μmol/L), which is in the normal established range (9.73–13.13 μmol/L) [22]. The choline value in serum of Alzheimer's patients ($n = 100$) was in the range of 5.0 to 8.4 μmol/L (with a mean of 6.53 μmol/L), which is significantly lower ($P < 0.01$) than that in healthy individuals (Table 1).

Table 1
Choline levels in sera of apparently healthy persons and Alzheimer's patients, as determined by biosensor based on AChE–ChO/c-MWCNT/ZrO_2NPs/GCE.

Age group (years)	Sex	Choline level in apparently healthy persons (μmol/L)	Choline level in Alzheimer's patients (μmol/L)
Children (1–20 years)	Male	9.0 ± 1.0 ($n = 10$)	7.0 ± 1.0 ($n = 10$)
	Female	11.0 ± 0.7 ($n = 10$)	6.0 ± 0.7 ($n = 10$)
Adults (21–45 years)	Male	12.8 ± 0.4 ($n = 15$)	5.8 ± 0.4 ($n = 15$)
	Female	10.8 ± 0.4 ($n = 15$)	7.8 ± 0.4 ($n = 15$)
Older adults (46 years and above)	Male	10.0 ± 0.7 ($n = 25$)	5.0 ± 0.7 ($n = 25$)
	Female	11.3 ± 0.3 ($n = 25$)	8.4 ± 0.3 ($n = 25$)

Note. Values are means ± standard deviations ($n = 100$ for each group).

Table 2
Comparison of various analytical performances of amperometric choline biosensors.

Electrode material/Immobilization matrix	Immobilization method	Detection Limit (μM)	Linearity (μM)	Applied voltage (V)	Response time	Storage stability percentage loss (days)[a]	References
PVA–SbQ and covered with Nafion (perfluorosulfonated membrane)	Entrapped	50	50–5000	1.20	3 min	50 (45)	[11]
Poly(ethylene glycol)-modified choline oxidase in a poly(vinyl alcohol) cryogel membrane	Physical immobilized	2.3	5–200	1.65	2 min	50 (30)	[12]
Polyvinylferrocenium Perchlorate-coated Pt electrode	Electrostatic interaction	1.2	0.0–1.2	0.70	30–50 s	50 (42)	[8]
Poly-5,2′:5′,2″-terthiophene-3-carboxylic acid modified electrodes	Covalent immobilization	0.1	1.0–80.0	0.20	5 s	20 (55)	[9]
Poly(pyrrole)/poly(2-naphthol) bilayer membrane	Glutaraldehyde co-crosslinking	0.1	50	0.70	1 min	60 (60)	[13]
cMWCNT/ZrO_2NPs/GCE	Covalent immobilization	0.01	0.01–200	0.20	4 s	50 (60)	Current biosensor

[a] Days are in parentheses.

Stability and reusability

The enzyme electrode lost 50% of its initial activity after its 100 uses over a period of 60 days, when stored at 4 °C. This storage stability of the current biosensor is higher than that of previously reported choline biosensors [8,9,11,12].

Table 2 summarizes a comparison of various analytical properties of previously reported amperometric choline biosensors with the current biosensor.

Conclusion

The use of a c-MWCNT/ZrO_2NPs modified GCE resulted in improved analytical performance of the choline biosensor in terms of low response time (4 s), wider working range (0.05–200 μM), higher storage stability (60 days), and no interference by serum substances compared with previously reported biosensors. There was a significant decrease ($P < 0.01$) in the serum choline level in persons suffering from Alzheimer's disease compared with apparently healthy persons as measured by the current biosensor.

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