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Highly sensitive and rapid detection of acetylcholine using platinum-graphene nanoparticles modified ITO plate

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Abstract

The determination of acetylcholine (ACh) and choline (Ch) are clinically important. ACh is a neurotransmitter which plays a key link in communication between neurons in the spiral chord and in nerve skeletal junctions in vertebrates, an import role in transmitting signals in the brain. A bienzymatic sensor for the determination of ACh was prepared by co-immobilizing choline oxidase (ChO) and acetylcholinesterase (AChE) on graphene matrix/platinum nanoparticles, electrodeposited on ITO coated glass plate. Graphene nanoparticles were decorated with platinum nanoparticles and were electrodeposited on modified ITO coated glass plate to form a modified electrode. The modified electrode was characterized by using scanning electron microscopy (SEM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) studies. The optimum response of the enzyme electrode was obtained at pH 7.0 and 35 ^{o}C. The response time of ACh sensing system was recorded by 4 s. The linear range for ACh was 0.005-700 μM. This biosensor exhibits excellent anti-interferential property and good stability, retaining 50% of its original current value even after 4 months. It has been applied for the determination of ACh level in human serum samples.

Keywords: Choline oxidase; Acetylcholinesterase; Graphene nanoparticles

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Introduction

Acetylcholine (ACh) is one of the major neurotransmitter in autonomic nervous system [1]. ACh functions as a neuromodulator in the peripheral nervous system (PNS) and central nervous system (CNS). In the PNS, ACh binds to acetylcholine receptors (AChR) and regulates muscle contraction where as in the CNS, it plays a crucial role in the processes related to behavioral activities, arousal, attention, learning and memory [2]. Acetylcholine-producing cholinergic system in the brain was shown to be associated with one of the chronic neurodegenerative disease known as Alzheimer's disease [3]. Therefore the determination of ACh content in human blood samples is of great importance for the prediction of these nervous diseases [4]. The ACh concentration in a healthy human blood is approximately 8.66±1.02 nM [5].

In order to diagnose the nervous diseases, it is important to measure the ACh concentration in patient's blood by simple, fast, inexpensive and accurate method. Common techniques for ACh detection are radio-labeling [6], high-performance liquid chromatography (HPLC) method on microdialysis samples [7], gas chromatography–mass spectrometry (MS) [8] and capillary zone electrophoresis [9]. These methods are complicated and require time consuming sample pretreatment and expensive equipment with highly trained persons to operate. For these reasons, there is a great interest in developing faster and simpler method. A variety of biosensors systems have been developed for ACh and Ch detection [10-13]. The fast detection and the cost effectiveness of these sensors are the main advantages of this approach.

Nanostructured materials have recently increased research interest among multidisciplinary researches due to their large surface area and unique structural and electromechanical properties, good biocompatibility, easy preparation and renewal of their surface [14,15]. In comparison with the bulk materials, nanostructured materials exhibit remarkable properties by improving the analytical performances of biosensors and electrochemical devices [16].

Graphene is one of the most novel nanostructure of carbon which is formed by a two-dimensional honeycomb crystalline single layer of carbon lattice [17]. It has a large

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specific surface area, extraordinary electrical and thermal conductivity [18,19], high mechanical stiffness and good biocompatibility. Graphene oxide nanoparticles (GrONPs) play an important role in the process of charge transfer. Therefore, in the electrochemistry field, GrONPs has shown a great potential as membrane material for modifying electrodes [20,21].

Platinum nanoparticles (PtNPs) provide biocompatible surface for immobilization of biomolecules, high surface area and good conductivity between electrode and biomolecules. PtNPs possess similar properties of other noble metal nanoparticles. They show excellent electrocatalytic property for oxidation/reduction towards H_2O_2 [22,23]. These metal nanoparticles in combination with other nanoparticles exhibit cumulative effects to increase the surface area, ability to promote electron transfer and good electrocatalytic activity for H_2O_2.

In our study the fabrication, characterization and analytical performance of acetylcholinesterase (AChE)–choline oxidase (ChO) bienzymatic system on the GrONPs/PtNPs modified ITO coated glass plate was described.

Experimental

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, sp.* 10 U/mg solid) and acetycholine chloride (AChCl) were purchased from Sigma Chemical Co. USA. Hexachloroplatinic (IV) acid hydrate ($H_2PtCl_6 \cdot xH_2O$) from SISCO Research Laboratory, Mumbai, India, was used. ITO-coated glass plates were a gift from Dr. Suman, Amity Institute of Advanced Research Studies, Amity University, (Noida, India). All other chemicals were of analytical reagent grade. Double distilled water (DW) was used throughout the experiments. All the buffers used in this experiment were oxygen saturated. Electrochemical measurements were conducted using a potentiostat/galvanostat (model: Autolab AUT83785, Ecochemie, the Netherlands).

Preparation of graphene oxide nanoparticles (GrONPs)

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GrONPs were prepared according to a modified Hummers–Offeman method [24]. 0.5 g of graphite powder was initially dispersed in 23 mL of H_2SO_4 at 4°C, and 0.5 g of $NaNO_3$ followed by 10 mL of $KMnO_4$ (2 mM) was then dropwise added. The well-mixed slurry was stirred for 1 h at a 35°C water bath. Following that, 140 mL of H_2O was added in the mixture, and the temperature was raised to 90 °C. Afterwards, 3 mL of H_2O_2 (30 wt%) was injected, and the mixture changed to a light brown color. Consequently, GrONPs was obtained by filtering, washing and centrifugation at 4000 rpm.

Decoration of GrONPs with PtNPs

The GrONPs were decorated with PtNPs by in situ reduction of H_2PtCl_6 in aqueous solution. Typically, 5 mL of solution of GrONPs was mixed well with $H_2PtCl_6 \cdot 6H_2O$ (1 mM), and the resulting suspension was kept for 1 h at room temperature. To this mixture 0.2 mL of 0.08% $NaBH_4$ was added, and the mixture was stirred in room temperature for 12 h. The resulting GrONPs/PtNPs hybrid material was collected by centrifuging with an ultracentrifuge. The GrONPs/PtNPs hybrid material was then washed well with water and dried at room temperature under vacuum.

Preparation of GrONPs/PtNPs/ITO coated glass plate

Prior to the surface modification, ITO coated glass plate was cleaned using piranha solution (H_2SO_4:H_2O_2=3:1) for 20 min, then rinsed thoroughly with DW. GrONPs/PtNPs were electrochemically deposited onto ITO coated glass plate by cyclic voltammetry (CV) by immersing it into a mixture of 23 mL 2.5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) and 2 mL of GrONPs solution and then applying potential between -0.2 to +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 Vs^{-1}. GrONPs/PtNPs/ITO coated glass plate was rinsed with DW and then dried at room temperature. The electrode was rinsed with DW and dried in air. Amine-terminated self-assembled monolayer (SAM) of cysteamine was then formed on the surface of GrONPs/PtNPs/ITO coated glass plate by immersing it in 1 mM ethanol solution of cysteamine for 10 h at room temperature. The sulfur atoms of the molecules bind to the metallic nanoparticles surface, while the amino groups can be employed for the attachment of other groups to the SAM.

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Preparation of working electrode (AChE-ChO/GrONPs/PtNPs/ITO coated glass plate)

The modified ITO electrode was activated with glutaraldehyde (GA) by exposing its surface to 5% GA solution for 1 h. Then the GA-activated cysteamine monolayer of the electrode was rinsed with DW and dried in air. In the next step, an amide bond was formed between free $-NH_2$ groups on the surface of enzymes molecule and the free –CHO group of GA by dipping the electrode in 2.5 mL solution of phosphate buffer (0.1 M, pH 7.0) containing 10 µL of AChE solution (2 mg/mL) and 10 µL of ChO (10 mg/mL) of enzyme for 24 h. The prepared enzyme electrode was rinsed with DW clearly, dried, and stored at 4^{o}C until use (Scheme 1).

Electrochemical measurement

Electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the AChE-ChO/GrONPs/PtNPs/ITO coated glass plate as the working electrode, a Pt wire as the auxiliary electrode and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode. The electrode system was pre-equilibrated at +0.2 V for 15 s before each run of detection and the steady current readings were obtained at +0.2 V (vs. Ag/AgCl). After 100 µL of AChCl (0.05 M) solution was injected into the cell containing 20 mL electrolyte [2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1)], a low voltage of +0.2 V was applied. All electroanalytical measurements were carried out at room temperature.

The determination of ACh and Ch are based on the following enzyme reactions:

$$\mathrm{ACh} + \mathrm{H_2O} \xrightarrow{\mathrm{AChE}} \mathrm{acetate} + \mathrm{Ch}$$

$$\mathrm{Ch} + \mathrm{O_2} \xrightarrow{\mathrm{ChO}} \mathrm{betaine\ aldehyde} + 2\mathrm{H_2O_2}$$

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The current generated by the oxidation of the hydrogen peroxide is linearly related to Ch, and ACh is converted to Ch enzymatically using enzyme AChE, the oxidation current is in turn proportional to ACh.

Optimization of electrode

Optimization of ACh measurements with AChE-ChO/GrONPs/PtNPs modified ITO coated glass plate was accomplished by testing at different pH in two buffers: phosphate buffer (pH 5.5-7.5) and borate buffer (pH 8.0-10.0) each at 0.1 M. Every buffer contained 0.1 M potassium chloride. To determine optimum temperature, the reaction mixture was incubated at 20 ^{o}C to 55 ^{o}C at an interval of 5 ^{o}C. To study the effect of substrate concentration, different AChCl concentrations, ranging from 0.005 to 700 μM were tested by chronoamperometry (applied potential +0.2 V vs. Ag/AgCl reference). The amperometric response was also measured in presence of interfering species viz. ascorbic acid, 4-acetamidophenol, glucose and uric acid each at 0.1 mM.

Stability and reproducibility of the biosensor

To reuse the working electrode, it was washed by dipping it in 5 mL of 0.1 M phosphate buffer pH 7.0. The long-term storage and stability of the biosensor was investigated over 4-months, when AChE-ChO/GrONPs/PtNPs modified ITO coated glass plate was stored dry in a refrigerator at 4 ^{o}C. The activity of enzyme electrode was measured once in a week.

Results and discussion

TEM image of GrONPs and PtNPs

The morphological characterization of both nanoparticles was carried out by Transmission Electron Microscope (TEM) at AIRF, JNU, New Delhi. TEM of GrONPs and PtNPs nanoparticles (Fig. 1) revealed that both nanoparticles were very fine with diameter in the range 30-60 nm and 13-42 nm, respectively.

SEM study of modified electrode

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The changes in surface morphology of ITO coated glass plate studied by SEM are shown in Fig. 2. After modification by deposition of GrNPs/PtNPs and then immobilization of AChE-ChO onto it was carried out in the chronological order. The bare ITO coated glass plate showed a uniform surface (a), while the surface of GrONPs modified ITO coated glass plate exhibited formation of almost uniform layer of graphene oxide on the surface of ITO coated glass plate (b). The SEM of AChE-ChO/GrNPs/PtNPs/ITO coated glass plate (c) clearly showed a regular globular structural morphology indicating the successful immobilization of the enzymes on the surface of modified electrode.

Cyclic Voltammerty study

To evaluate the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry using potassium ferricyanide-potassium ferrocyanide solution as electrolyte was employed. Cyclic voltamogramms (Fig. 3A) were recorded in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ [(1:1)] and 0.1 M phosphate buffer (pH 7.0). No peak was observed for bare ITO coated glass plate (curve a). An oxidation peak at 0.2 V (vs Ag/AgCl) (curve b) was identified by cyclic voltammogram of GrONPs@PtNPs/ITO coated glass plate. The electrodeposition of GrONPs/PtNPs onto ITO coated glass plate surface led to increase in current intensity.

Response towards AChCl at the AChE-ChO/GrNPs@PtNPs/ITO coated glass plate

To evaluate the catalytic activity of AChE-ChO at the GrONPs@PtNPs/ITO coated glass plate, the modified electrode was characterized by a cyclic voltammogram in the presence of AChCl in the potential range from -0.2 V to +0.4 V. Fig. 3B shows CV of the AChE-ChO/GrNPs/PtNPs/ITO coated glass plate in 0.1 M phosphate buffer (pH 7.0) without (curve a) and with (curve b) AChCl solution at scan rate 20 mVs^{-1}. It was observed that with the addition of 0.5 mM AChCl, oxidation current was increased, revealing the improved catalytic properties of modified electrode to the oxidation of AChCl. A well defined oxidation peak (0.2 V vs Ag/AgCl) was observed, which clearly indicate the catalytic properties of modified electrode.

Electrochemical impedance measurements

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An electrochemical impedance spectra (EIS) of (a) bare ITO coated glass plate (b) GrONPs/PtNPs/ITO coated glass plate (c) AChE-ChO/GrONPs/PtNPs/ITO coated glass plate in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1)] at a polarization potential of 0.2 V in the frequency range of 0.1-10^4 Hz are shown in Fig. 4.

Nyquist plot of GrONPs/PtNPs/ITO coated glass plate (curve b) gave an R_{CT} value of 160 Ω, implying that the PtNPs coated GrONPs exhibited a good electron conducting materials and a good electron transfer between the redox probe and the electrode was noticed. When compared to the nanocomposite modified electrode, the semicircle diameter was increased by the immobilization of AChE-ChO on GrONPs/PtNPs modified ITO coated glass plate, showing an increased R_{CT} value of 215 Ω (curve c). The change in the R_{CT} value reflecting the increase or decrease in the diameter of the semicircle at high frequencies in the impedance spectra is associated with the blocking behavior of the electrode surface for the charge transfer to the redox probe. These results confirm the successful assembly of the enzyme electrode.

Optimization of experimental conditions of biosensor

CV of AChE-ChO/GrNPs/PtNPs/ITO coated glass plate was recorded in the potential range of +0.0 to +0.6 V at a scan rate of 20 mVs^{-1} versus Ag/AgCl as reference electrode in 15 mL of 0.1 M phosphate buffer (pH 7.0) containing 1.0 mL of 0.5 mM AChCl. The maximum response was observed at +0.2 V (Supplementary Fig. 1a); hence, subsequent studies were carried out at this voltage. The biosensor showed a maximum response at pH 7.0 (Supplementary Fig. 1b), which is slightly lower than that of the free enzyme (pH 8.0) [25]. The optimum temperature of biosensor was 35 ºC (Supplementary Fig. 1c). This optimum temperature of the present biosensor AChE-ChO/GrNPs/PtNPs/ITO coated glass plate is higher than that of the free enzyme (30 ^oC) [25] and earlier biosensors (30 ^oC) [13,26]. These minor changes in the kinetic properties of the enzymes after immobilization might be due to the change in confirmation and microenvironment of enzymes after immobilization.

Hence, the subsequent experiments were carried out at pH 7.0 and 35 ^oC. The biosensor showed an optimum response within 4 s.

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Evaluation of biosensor

A typical current–time plot was plotted against concentrations of AChCl (Fig. 5). Voltammetric measurements were performed after each addition of the AChCl up to a maximum concentration of 700 μM. No significant current improvement after 700 μM of AChCl concentration was observed. The electrode had reached its saturation level at 700 μM. The time required to attain the 95% of the steady state response was within 4 s, which indicated a very fast diffusion process.

The difference between the baseline and the maximum value of current reached on the plateau at 40 s which was used to build the calibration curve of the biosensor. A linear relationship between current (μA) and AChCl concentration ranging from 0.005-700 μM was observed (inset of Fig. 5). Km for ACh was noticed by 2.5 μM. The detection limit of the present sensor was 0.005 μM (S/N=3), which is lower/better than that of enzyme electrode based on multiwalled carbon nanotubes/zirconium oxide electrodeposited on modified glassy carbon electrode [12], chitosan/gold-coatedferric oxide nanoparticles modified Au electrode [13] and phenyl carboxylic acid-grafted multiwalled carbon nanotube modified screen printed electrode [4]. The mean analytic recoveries of added AChCl at 5.0 and 10.0 μM (final concentration in serum) (spiked samples) as determined by the current biosensor were 96.6±0.2 and 98.8±0.4%, respectively. To test the reproducibility and reliability of the current ACh biosensor, ACh content in six serum samples (real samples) was determined on a single day (within batch) five times and again after storage at 4 ^{o}C for 1 week (between batch). The determinations were consistent and that within and between coefficients of variation (CVs) were observed by 3.56% and 2.75% respectively. These results indicated the reproducibility and consistency of the current method. The good stability, repeatability and reproducibility observed for the proposed biosensor could be attributed to the excellent immobilization of AChE-ChO onto GrONPs/PtNPs/ITO coated glass plate.

The ACh 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. 6). Practically no interference was observed during measurements of ACh by this biosensor in the

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presence of ascorbic acid, 4-acetamidophenol, glucose and uric acid. Efforts were made to study the effects of individual interferants at 0.1 mM concentration. The value of I_{max} did not vary significantly in the presence of interferants indicating non-influence of the individual interferants (Fig. 7). The earlier ACh biosensors showed maximum decrease in their activity by ascorbic acid [25, 27].

Determination of ACh in serum samples

The ACh value in serum of apparently healthy individuals (n=10) was measured in the range of 9.0 to 12.8 nM (with a mean of 9.24 nM). This concentration is reported in normal established level (i.e. 8.66±1.02 nM) [5]. However in Alzheimer's patients (n=10) (Table 1), a concentration of 5.0–8.4 nM (with a mean of 6.93 nM) was reported which is significantly lower ($p<0.01$) than those in healthy individuals. It was reported earlier that the level of neurotransmitter ACh is decreased in Alzheimer's patients [28].

Stability and reusability of biosensor

The enzyme electrode lost 50% of its initial activity after using 100 times within the span of 4 months and stored at 4 ^{o}C. This is much better than earlier biosensors e.g. multiwalled carbon nanotubes/zirconium oxide electrodeposited on modified glassy carbon electrode (2 months) [12] and chitosan/gold-coated ferric oxide nanoparticles modified Au electrode (3 months) [13]. The enzyme electrode was constructed thrice and the effect of storage at 4◦C was studied in response to these three similarly constructed electrodes. The results showed practically no variation in storage stability of the three electrodes. The better shelf-life of present enzyme electrode over previously reported sensors [12,13] may be attributed to the presence of GrONPs/PtNPs with a large surface area and strong affinity to bind with the enzyme.

Conclusions

The use of GrONPs and PtNPs composite film in the construction of biosensor for ACh has led to its improved analytical performance in terms of low working potential (0.2 V), short response time (4 s), low detection limit (0.005 μM) and high storage stability (4

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months). Based on these observations, this composite could be employed for the improvement of other biosensors.

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Captions to Figures

Scheme 1. Chemical sequence involved in the preparation of working electrode.

Fig. 1. TEM image of (A) GrONPs and (B) PtNPs.

Fig. 2. Scanning electron micrographs of (A) bare ITO coated glass plate, (B) PtNPs/GrONPs/ITO coated glass plate and (C) AChE-ChO/PtNPs/GrONPs/ITO coated glass plate.

Fig. 3. **(A)** CV curve of (a) bare ITO coated glass plate and (b) GrONPs/PtNPs/ITO coated glass plate. **(B)** AChE-ChO/PtNPs/GrONPs/ITO coated glass plate without (curve a) and with 0.5 mM AChCl (curve b) solution (100 μL) in 0.1 M sodium phosphate buffer pH 7.0; Scan rate: 20 mV s^{-1}.

Fig. 4. Nyquist plot of Electrochemical impedance spectrum of (a) bare ITO coated glass plate, (b) GrONPs/PtNPs/ITO coated glass plate and (c) AChE-ChO/PtNPs/GrONPs/ITO coated glass plate at 0.1 M phosphate buffer (pH 7.0) containing 0.5 mM $[Fe(CN)_6]^{3-}$ + 0.5 mM $[Fe(CN)_6]^{4-}$ and 0.5 mM AChCl. Frequency range: 0.01Hz to 10 KHz. Inset shows the equivalent circuit for 'mixed kinetic and diffusion control.

Fig. 5. Current-time response curve of the enzyme electrode on increasing the AChCl concentration by 60 μM in each step in phosphate buffer (0.1 M, pH 7.0) at an applied potential of +0.2 V vs. Ag/AgCl. The inset shows the response to 100 to 700 μM AChCl (for higher substrate concentration).

Fig. 6. Correlation between serum ACh values as determined by standard HPLC method (y-axis) and present biosensor (x-axis) based on AChE-ChO/PtNPs/GrONPs/ITO coated glass plate.

Fig. 7. Effect of interferences (each at 0.1 mM) on the activity of AChE-ChO/PtNPs/GrONPs/ITO coated glass plate. Eap = +0.2 V vs. Ag/AgCl, phosphate buffer pH 7.0 and AChCl concentration 0.5 mM.

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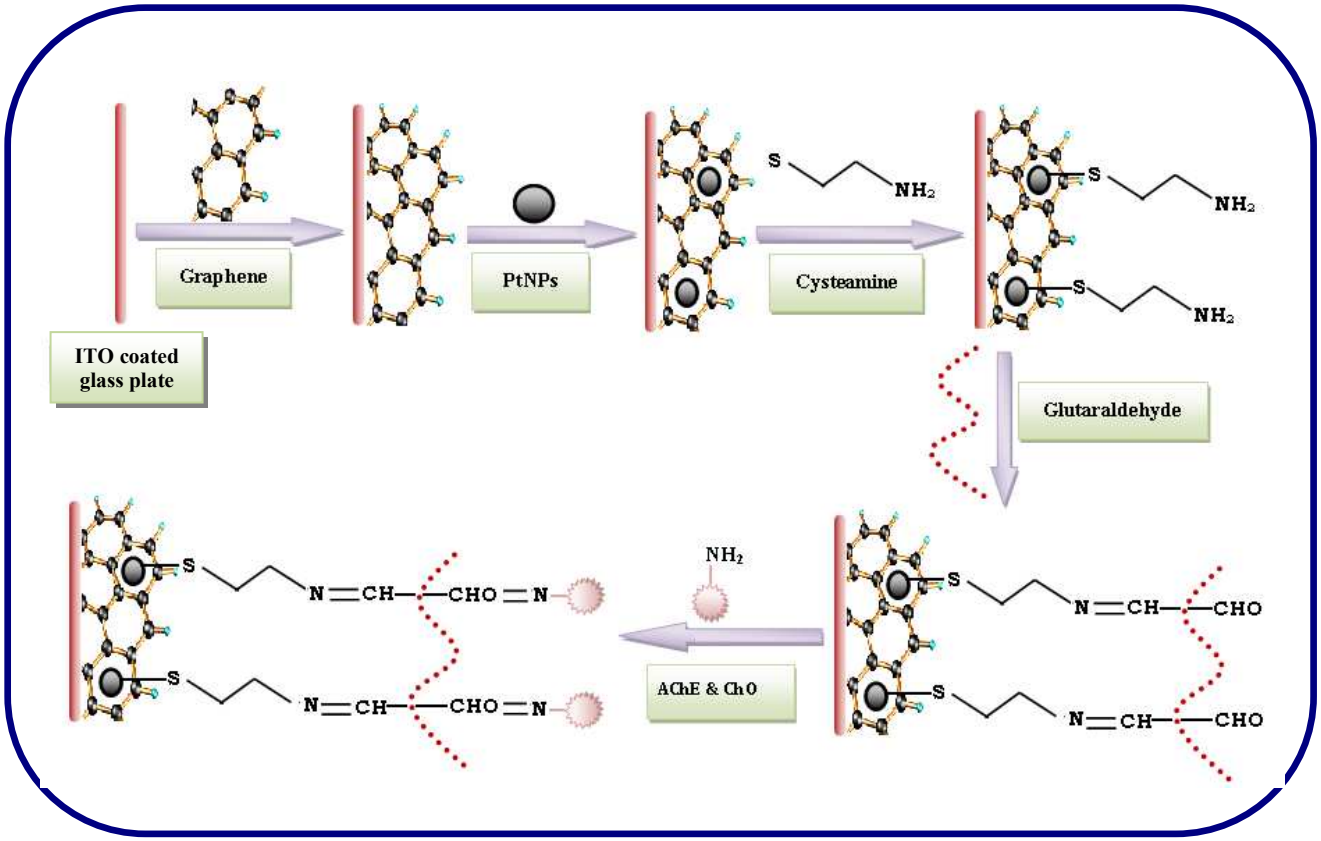

Scheme 1.

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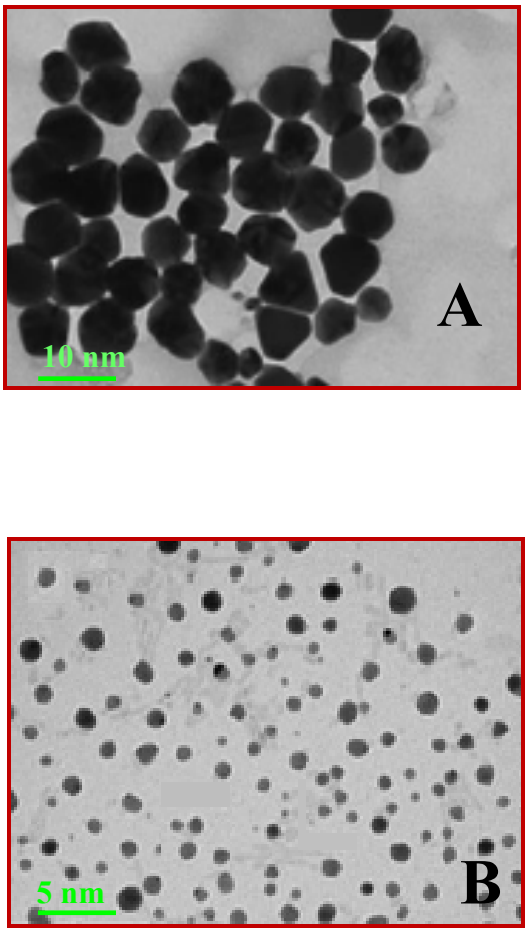

Fig. 1.

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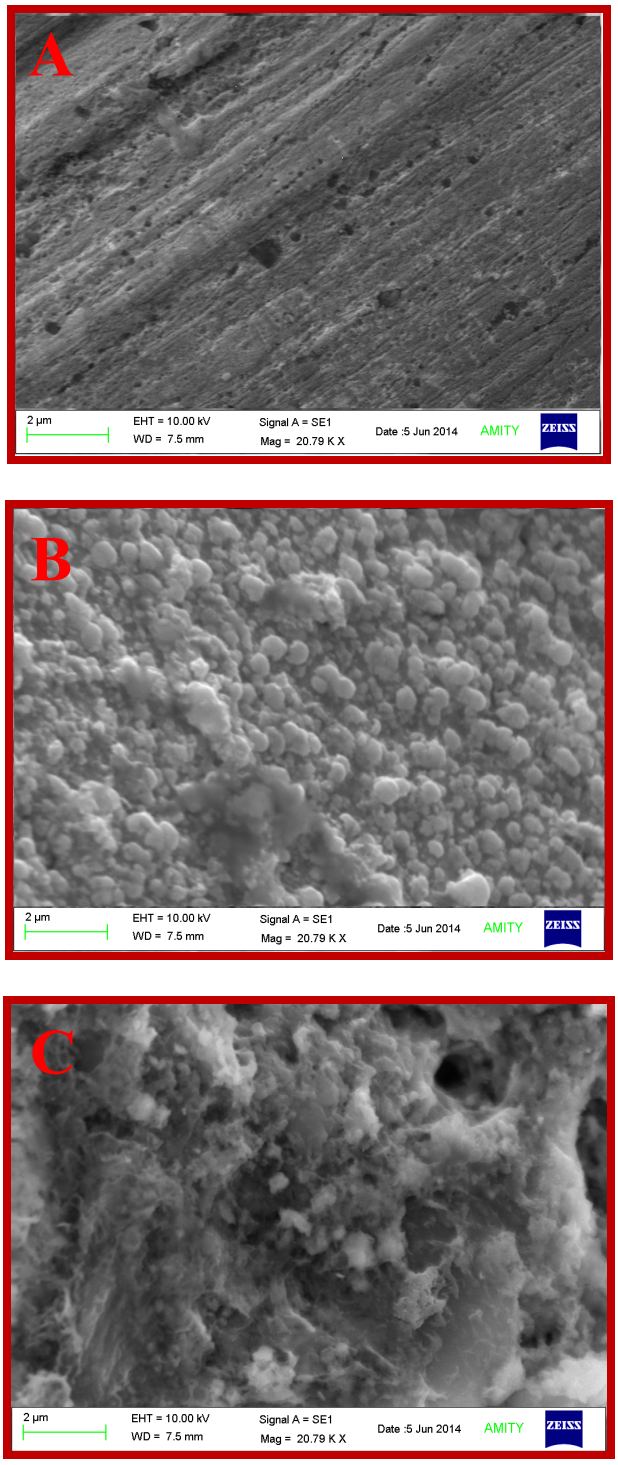

Fig. 2.

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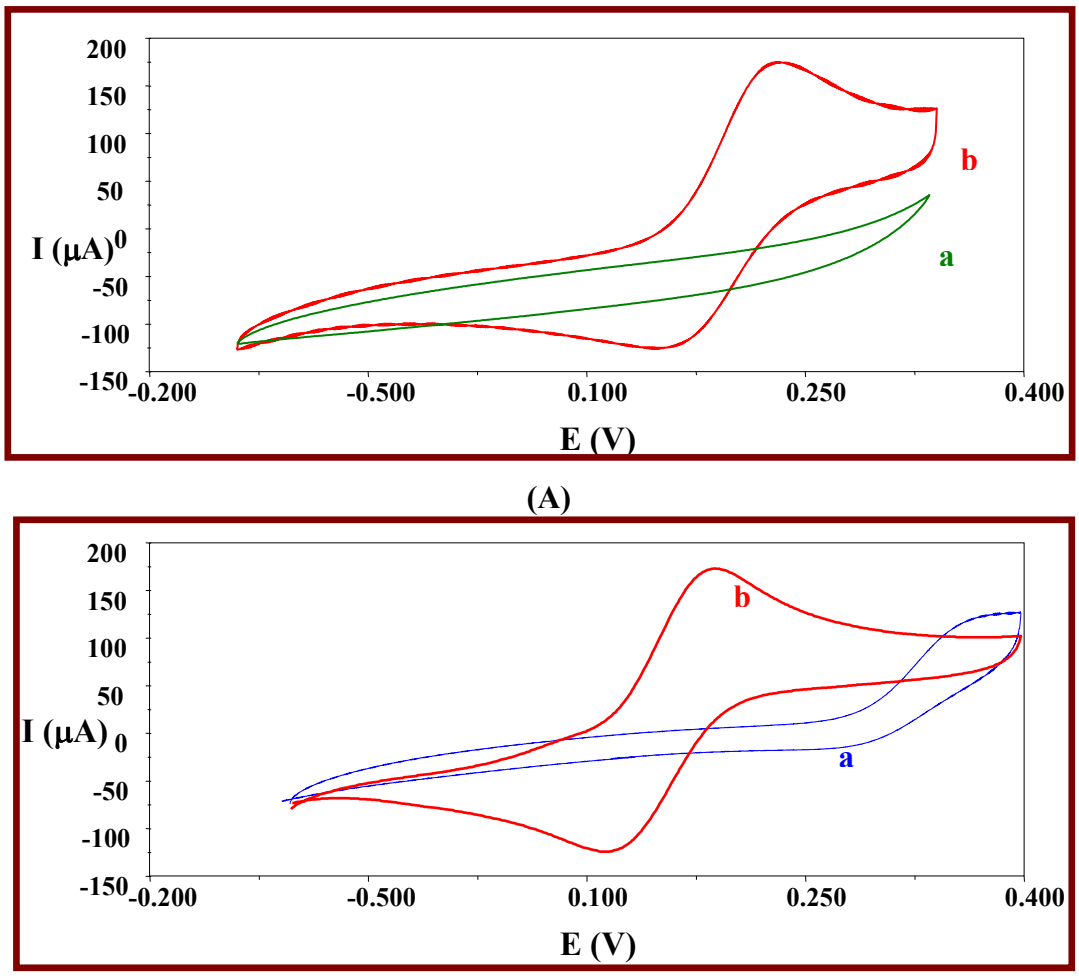

Fig. 3.

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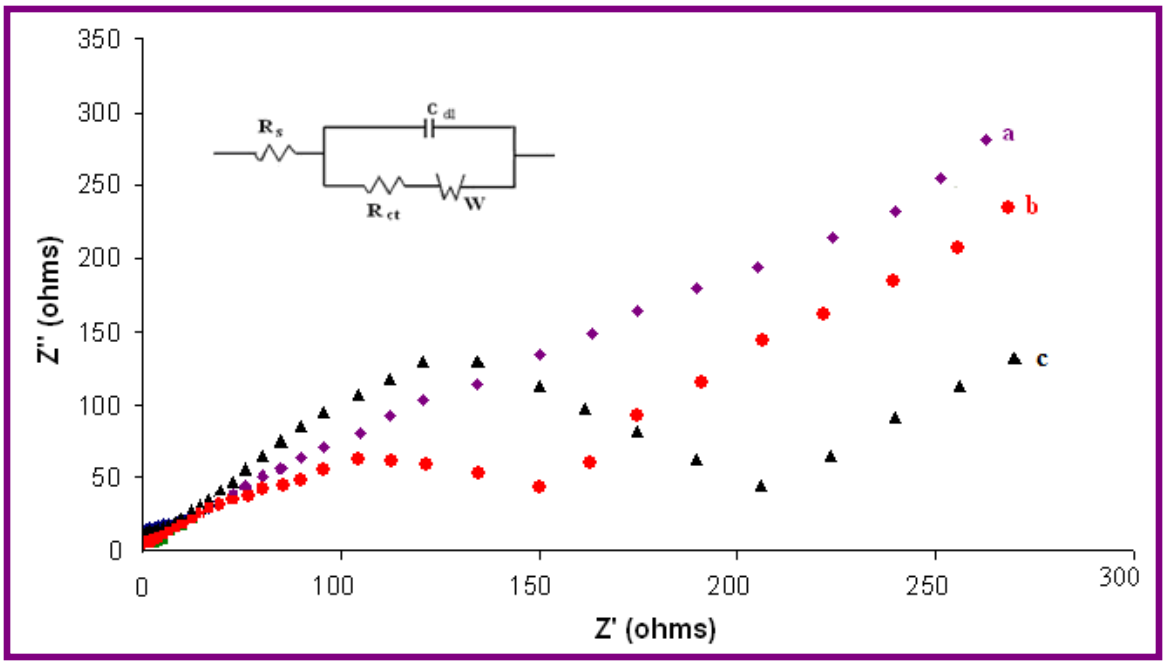

Fig. 4.

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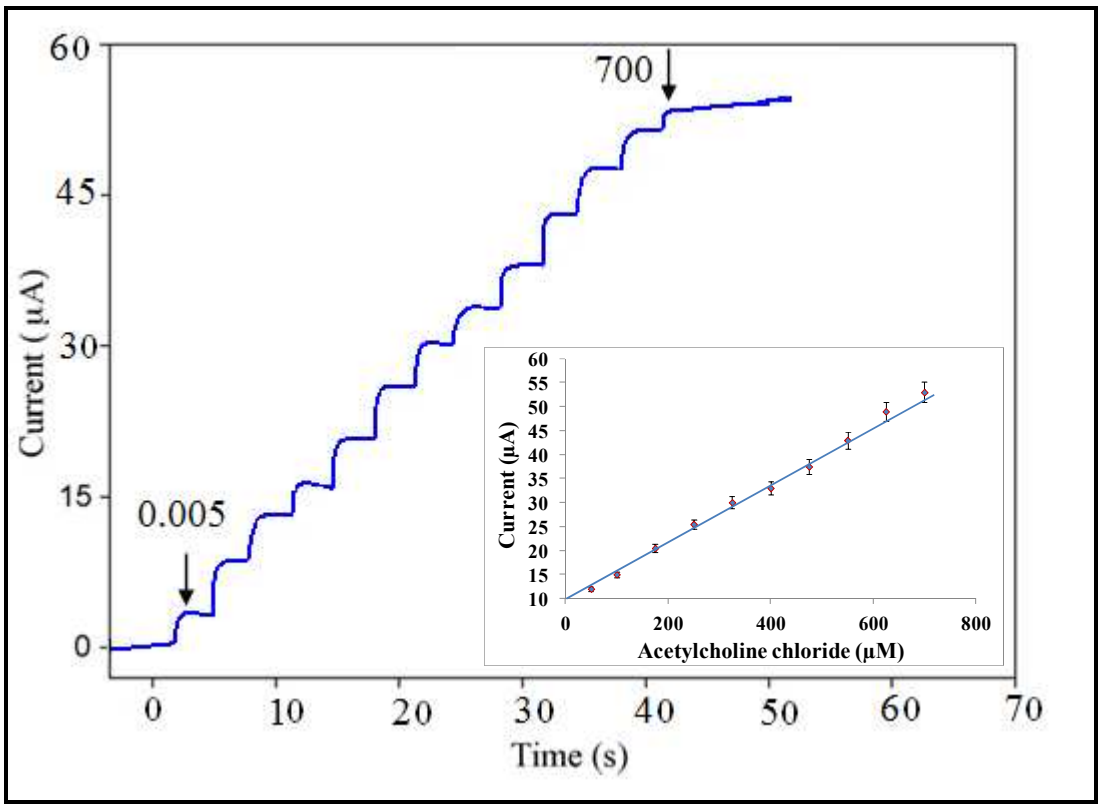

Fig. 5.

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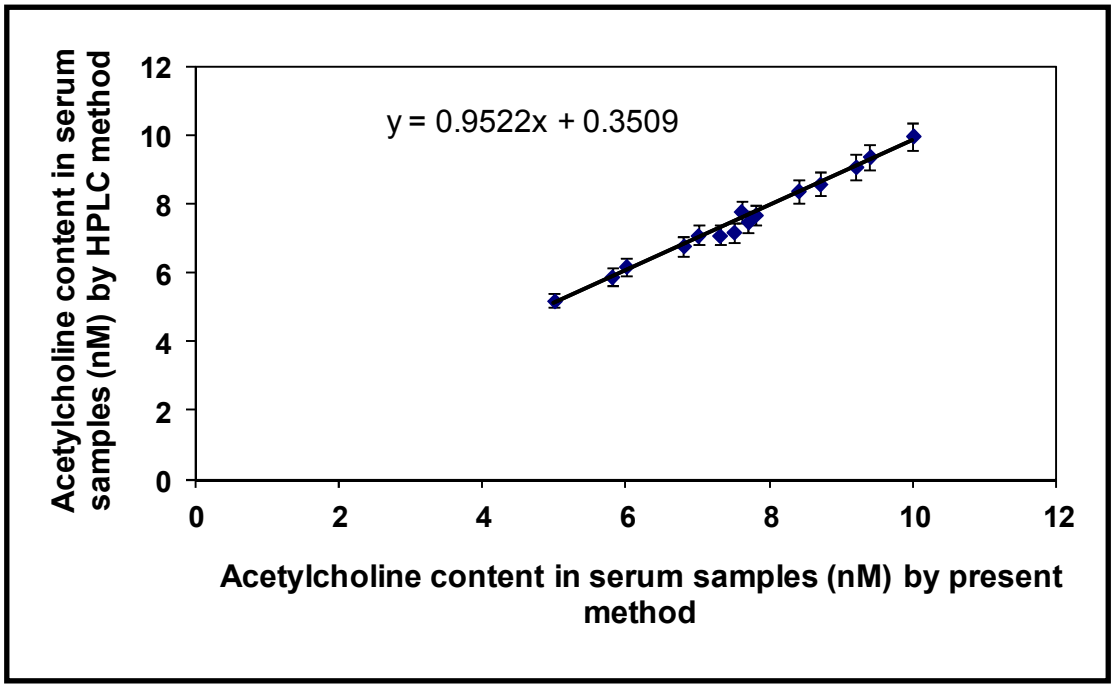

Fig. 6.

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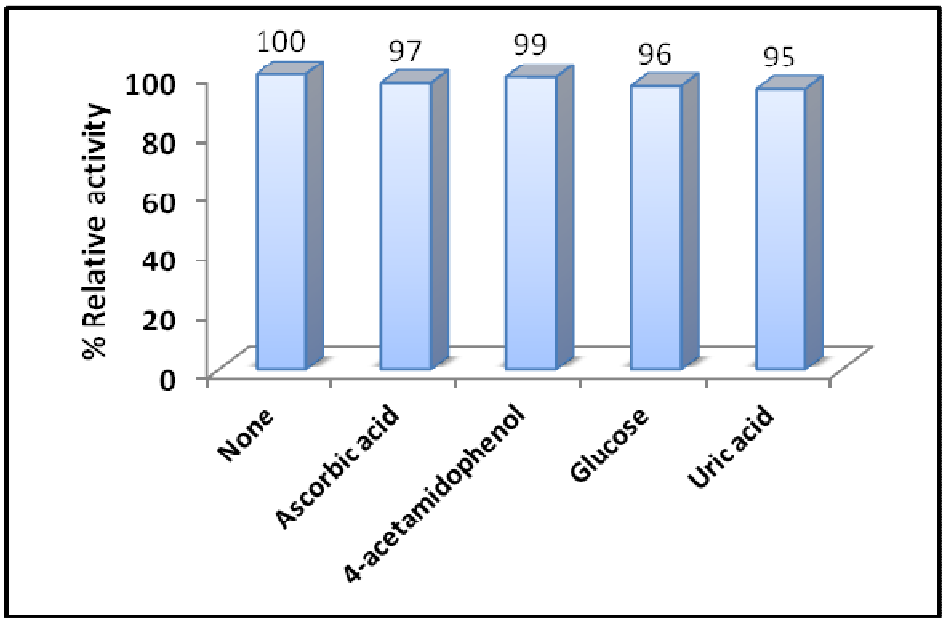

Fig. 7.

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Table 1. Acetylcholine levels in sera of Alzheimer patients and apparently healthy adults, as determined by biosensor based on AChE-ChO/PtNPs/GrONPs/ITO coated glass plate.

Serum sample	Acetylcholine (nM) Mean±S.D	
	Alzheimer patients	Apparently healthy adults
S1	7.0±1.0	9.0±1.0
S2	6.0±0.7	8.1±0.7
S3	5.8±0.4	9.1±0.4
S4	7.8±0.4	10.2±0.4
S5	5.0±0.7	10.0±0.7
S6	7.3±0.3	11.3±0.3
S7	7.5±0.4	8.5±0.4
S8	7.7±0.4	8.7±0.4
S9	6.8±0.8	8.1±0.8
S10	8.4±0.9	9.4±0.9