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Immobilization of rat brain acetylcholinesterase on porous gold-nanoparticle–$CaCO_3$ hybrid material modified Au electrode for detection of organophosphorous insecticides

Nidhi Chauhan, Jagriti Narang, C.S. Pundir*

Department of Biochemistry, M.D. University, Rohtak 124 001, Haryana, India

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ABSTRACT

An acetylcholinesterase (AChE) purified from rat brain was immobilized onto gold nanoparticles (AuNPs) assembled on the surface of porous calcium carbonate ($CaCO_3$) microsphere. The resulting AChE–AuNPs–$CaCO_3$ bioconjugate was mounted on the surface of Au electrode with the help of silica sol–gel matrix to prepare the working electrode. This electrode was connected to Ag/AgCl (3 M/saturated KCl) as standard and Pt wire as an auxiliary electrode through a potentiostat to construct an organophosphorus (OP) biosensor. The biosensor was based on inhibition of AChE by OP compounds/insecticides. The biosensor showed optimum response at pH 7.0, 30 °C, when polarized at +0.2 V. Two OP compounds, malathion and chlorpyrifos could be detected in the range of 0.1–100 nM and 0.1–70 nM, respectively at 2.0–3.0% inhibition level of AChE. The sensor was reactivated by immersing it in 0.1 mM 2-pyridine aldoxime for 10 min. The detection limit of the sensor was 0.1 nM for both malathion and chlorpyrifos. The biosensor exhibited good reusability (50 times without considerable loss) and storage stability (50% within 60 days, when stored at 4 °C).

1. Introduction

The measurement of an analyte by biosensors can be carried out in two ways: (i) if the enzyme metabolizes the analyte, the analyte can be determined by measuring the enzymatic product and (ii) if the analyte inhibits the enzyme, the decrease of the enzymatic product formation can be measured and correlated to the analyte concentration. In the latter case, the biosensor is called "biosensor based on enzyme inhibition". Organophosphorus (OP) and carbamate insecticides are widely used in agriculture. Their presence in water, food and animal feedstuffs presents a potential hazard due to their high mammalian toxicity [1]. Due to their relatively low environmental persistence in comparison to organochlorine pesticides, OP pesticides are applied in vast amounts in many countries. Such an extensive use led to several problems with regards to environmental and food safety. Existing methods for OP pesticide monitoring include conventional chromatographic techniques (mainly gas chromatography-GC and high performance liquid chromatography- HPLC, coupled with different kinds of detectors) [2–11]. Although these techniques are rationally selective and have low limits of detection, these are often time-consuming, quite expensive and require trained person to operate. Therefore, these are not quite suitable for screening large number of samples for potential pesticide traces.

As a good alternative and with the improvement of being quick and consistent, several bioanalytical techniques based on inhibition of acetylcholinesterase (AChE) and coupled with simple detectors have been developed recently [12–19]. Biosensors are considered a viable alternative to the chromatographic methods for pesticide determination for on-site analysis. Besides being specific and sensitive, they are portable, less expensive and do not require tedious sample pretreatment [20]. Various nanoparticles based enzyme (AChE) electrodes have been reported for determination of pesticides such as ZrO_2NPs-modified screen printed electrode [21], multiwalled carbon nanotube (MWCNT) modified glassy carbon (GC) electrode [22], AuNPs–polypyrrole nanowires composite film modified GC electrode [23] and prussian blue modified GC electrode [24]. However these electrodes suffer from leakage of enzyme resulting into low stability of enzyme electrode and require several operations involving pretreatment of the sample. In electrochemical studies, electrodes made up of commonly used solid materials, such as graphite, carbon paste and GC, exhibit common disadvantages e.g. the electrode area and the activity of the metal deposited electrically change continuously and oxide layers affecting the reproducibility of the measurements [25]. If gold nanoparticles

* Corresponding author.
E-mail addresses: pundircs@rediffmail.com, pundircs@yahoo.com (C.S. Pundir).

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(AuNPs) are assembled on porous $CaCO_3$, the AuNPs–$CaCO_3$ composite will couple the advantages of both the nanoparticles and minerals. This material is expected to retain the porous structure and inherit the advantage from its parent materials such as satisfying biocompatibility, good stability and dispersibilty in water [26]. The hybrid material will therefore provide a native environment and increase the enzyme loading [27]. It is known that carboxyl group can interact with calcium ions, so the citrate groups around AuNPs could interact with calcium ions of vaterite. Therefore, the negatively charged AuNPs could assemble on positively charged vaterite surface via electrostatic interaction and other supramolecular interactions [28]. We describe herein construction of AuNPs–$CaCO_3$ microspheres composite modified Au electrode and then immobilization of AChE on this modified Au electrode to construct an amperometric biosensor for detection of pesticides in environmental samples.

2. Experimental methods

2.1. Materials

Acetylcholinesterase. (AChE) (EC 3.1.1.7) was purified from adult rat brain. Acetylthiocholine chloride and 2-pyridine aldoxime methiodide [2-PAM] from Sigma Aldrich Chemical Co. USA were used. α-Methyl-D-mannoside, Triton X-100, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), $CaCl_2{\cdot}2H_2O$, Na_2CO_3, $HAuCl_4{\cdot}4H_2O$ and tetraethyl orthosilicate (TEOS) were purchased from Sisco Research Laboratory, Mumbai, India. The pesticides (malathion and chlorpyrifos) were from Lentrek North America (San Francisco, CA). Gold wire (1.5 cm × 0.05 cm) (23 carat) was purchased from local market. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used throughout the experiments.

2.2. Apparatus and methods

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/Galvanostat (Autolab, Eco Chemie, The Netherlands. Model: AUT83785) with a three electrode system consisting of a Pt wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and modified Au wire as a working electrode. All the electrochemical experiments were performed at an ambient temperature (25 °C). Fourier transform infrared (FTIR) spectroscopy was performed on FTIR spectrometer (Make: iS10, Thermoelectron, USA). Scanning electron microscopy (SEM) measurements were carried out at Department of Chemistry, M. D. University, Rohtak. Transmission electron microscopy (TEM) was performed at Punjab University, Chandigarh. Ultrasonication was performed on Misonix Ultrasonic Liquid Processors (mode XL-2000 series).

2.3. Preparation of rat brain acetylcholinesterase

All operations were carried out at 4–10 °C. The brains of 7 adult albino rats (6.7 g) were homogenized in 60 ml of saline buffer (12.5 mM sodium phosphate buffer, pH 7.2 containing 0.4 M NaC1) in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 25,000 × *g* for 2 h. The supernatant was discarded and the pellet was suspended into 60 ml of DW. The suspension was added slowly into 100 ml of saline buffer (13.75 mM phosphate buffer, pH 7.2 containing 0.44 M NaC1 and 0.55% Triton X-100) and stirred for 30 min. The suspension was then centrifuged as above. The pellet was discarded and the supernatant was treated as crude enzyme and tested for AChE activity and protein [29].

2.3.1. Purification of crude acetylcholinesterase

2.3.1.1. Gel filteration on Sephadex G-100 column. The crude enzyme was applied to Sephadex G-100 (40 × 2 cm) column previously equilibrated with saline 13.75 mM phosphate buffer pH 7.2 containing 0.5% Triton X-100. The column was eluted with 1–3 void vol. of the column with same buffer containing 0.5 M α-methyl-D-mannoside at flow rate of 70 ml/h. The activity and protein in fractions (3 ml each) were measured. The fractions containing high specific activity were pooled.

2.3.1.2. Ion exchange chromatography on DEAE-Sephacel column. The pooled fractions of Sephadex G-100 gel filtration were loaded on to DEAE-Sephacel column (16 × 1 cm) along with its side wall. The column was washed thoroughly with 0.01 M Tris–HCl buffer pH 8.5 until no protein was detected in eluting buffer. The enzyme was eluted in a linear gradient of KCl (0.1–0.6 M) using 100 ml of 0.01 M Tris–HCl buffer pH 8.5 containing 0.1 M KCl in mixer and 100 ml of same buffer containing 0.6 M KCl in reservoir. The buffer in mixing aspirator was stirred with the help of a magnetic stirrer. The flow rate was maintained at 0.5 ml per min. The fractions (3 ml each) were collected and monitored for enzyme activity and protein content. The fractions with high specific activity were pooled and treated as purified enzyme. The purified enzyme was dialysed against 0.05% Triton X-100 and stored at −20 °C.

2.3.2. Assay of acetylcholinesterase

AChE preparation (100 μl) was added to 150 μl of DW. After pre-incubation for 10 min at 30 °C, 250 μl of 12.5 mM acetylthiocholine chloride in 100 mM sodium phosphate buffer, pH 7.0, was added to diluted enzyme and incubated further for 120 min at 30 °C. After incubation, 300 μl of the reaction mixture was transferred to a new vial and then 1425 μl of 100 mM sodium phosphate buffer, pH 7.0 and 75 μl of DTNB in 100 mM sodium phosphate, pH 7.0, was added to this vial. The A_{412} was read after 1 min. The control was run in the same manner as described above except that the enzyme was replaced by DW [30]. The following chemical reactions occurred during the assay of enzyme-

$$\text{Acetylthiocholine chloride} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Acetic acid}$$

$$\text{Thiocholine} + \text{DTNB} \rightarrow \underset{\text{Yellow colour } (\lambda_{max}=412)}{\text{Thionitrobenzoic acid}}$$

$$+\text{Thionitrobenzoic acid–thiocholine}$$

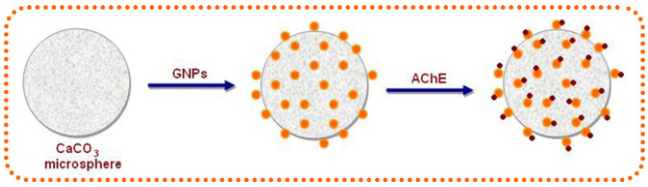
One enzyme unit is defined as 1 μmol of thiol group appearance/min/ml. The enzyme activity was calculated using the following formula-

$$R = 5.74 \times 10^{-4} \times \frac{A}{\text{CO}}$$

where, *R* = rate in moles of substrate hydrolyzed/min/g tissue; *A* = change in absorbance/min; CO = original protein concentration of the tissue (mg/ml).

2.4. Preparation of gold nanoparticles (AuNPs) and $CaCO_3$ microspheres

AuNPs were prepared according to the literature [31,32] by adding a sodium citrate solution to a boiling $HAuCl_4$ solution. The molar ratio of $HAuCl_4$:sodium citrate used for preparation of the colloid was 0.75. The diameter of the prepared AuNPs was ca. 25 nm, as measured using transmission electron microscopy (TEM). $CaCO_3$ microspheres were prepared by rapidly pouring a 0.33 M Na_2CO_3 solution into an equal volume of a 0.33 M solution of $CaCl_2$ at room



Scheme 1. Formation of AChE–AuNP–$CaCO_3$ bioconjugates.

temperature on a magnetic stirrer. The precipitate was centrifuged, washed with pure water and dried.

2.5. Preparation of AuNPs–$CaCO_3$ hybrid material

$CaCO_3$ (0.5 g) was dispersed into 50 ml of Au colloid solution (pH 7) and sonicated for 20 min. After centrifugation, the light purple AuNPs–$CaCO_3$ composites were obtained; the supernatant liquor was colorless. The composites were further washed with DW three times and dried [26].

2.6. Preparation of AChE–AuNPs–$CaCO_3$ bioconjugates

The hybrid material (10 mg) prepared above was dispersed in 1 ml solution of AChE (1 mg/ml, pH 7) and shaken for 1 h at 4 °C for enzyme absorption. The bioconjugates were then centrifuged and washed with DW three times. The UV–vis spectrum was used to monitor the concentrations of the gold colloid and AChE solutions before and after the assembly process. The decreases in absorbance of the two solutions were applied to quantify the loading amount of AuNPs and AChE bound to the $CaCO_3$ microspheres; the bands used were 527 and 402 nm, respectively (Scheme 1).

2.7. Preparation of silica sol

Silica sol was prepared according to the literature [33] by mixing 600 μl of ethanol, 50 μl of TEOS, 10 μl of 5 mM NaOH and 60 μl of H_2O in a small test tube and sonicated for 20 min at room temperature. The sol so formed was stored at 4 °C.

2.8. Fabrication of the AChE–AuNPs–$CaCO_3$ modified Au electrode

Au electrode was polished successively with 1.0 μm alumina powder and rinsed thoroughly with DW between each polishing step. The polished electrode was sonicated in acetone and DW and was then allowed to dry at room temperature. The AChE–AuNPs–$CaCO_3$ bioconjugates obtained above were resuspended in 0.5 ml of water and 10 μl of this suspension was smeared on the surface of the pretreated Au electrode and then left at room temperature to dry. Silica sol (10 μl) was then added on surface of the electrode for encapsulation of the bioconjugates. The electrode was then left to dry and was stored for at least 24 h at 4 °C. The electrode was stored at 4 °C, when not in use.

2.9. Electrochemical measurement

Electrochemical measurements were carried out in a potentiostat with a conventional three-electrode system comprising Pt wire as auxiliary electrode, Ag/AgCl electrode (3 M KCl) as reference and AChE immobilized on nanocomposites modified Au electrode (AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE) as working 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 ATCl (0.05 M) solution was injected into the cell containing 15 ml phosphate buffer (0.1 M, 7.0), a low voltage of +0.2 V was applied. The substrate (ATCl) was enzymatically hydrolyzed to thiocholine, which was then subjected to electrocatalytic oxidative dimerisation at +0.2 V vs. Ag/AgCl reference electrode to give the disulphide compound. This response, due to the oxidation of the thiocholine at the working electrode, was correlated to the activity of AChE.

$$\text{Acetylthiocholine chloride} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} + Cl^-$$

$$2\text{Thiocholine} \rightarrow \text{dithio-bis-choline} + 2H^+ + 2e^-$$

2.10. Optimization of working electrode

Optimization of acetylcholine measurements with AChE–AuNPs–$CaCO_3$/silica sol–gel-modified Au electrode was accomplished by testing at different pH in two buffers: phosphate buffer (pH 5.5, 6.0, 6.5, 7.0 and 7.5) and borate buffer (pH 8.0, 8.5, 9.0 and 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 °C to 55 °C at an interval of 5 °C. To study the effect of substrate concentration, different ATCl concentrations, ranging from 0.1 to 500 μM were tested by cyclic voltammetry from −0.1 to +0.55 V at a scan rate of 20 mV s^{-1}. The amperometric response was also measured in presence of interfering species viz. ascorbic acid, 4-acetamidophenol and uric acid each at 0.1 mM.

2.11. Inhibition measurement

Malathion is a broad-spectrum insecticide and chosen as a model compound, because it has been one of the most commonly applied OPs since 1956 [34]. Hence, in the present work it was chosen as standard pesticide for the AChE inhibition. AChE-immobilized AuNPs–$CaCO_3$/silica-sol gel modified Au electrode was immersed into 15 ml of 0.1 M phosphate buffer pH 7.5. This was followed by the addition of substrate ATCl to the phosphate buffer, so that the final concentration of substrate reaches 2 mM and the current was measured. This value of current, before introduction of malathion, corresponds to I_0. Now, a known concentration of malathion is added onto the electrode, left for incubation for nearly 10 min, followed by washing with buffer solution. After washing, current response was measured again and this value corresponds to I_i, inhibited current. Inhibition (%) was measured using the following equation:

$$\text{Inhibition}\,(\%) = 100 \times \frac{I_0 - I_i}{I_0}$$

2.12. 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 7.5. The long-term storage and stability of the biosensor was investigated over 3-months, when AChE–AuNPs–$CaCO_3$ modified Au electrode was stored dry in a refrigerator at 4 °C. The activity of enzyme electrode was measured once in a week.

3. Results and discussions

3.1. Characterization of the AuNPs–$CaCO_3$ hybrid material

Porous $CaCO_3$ microspheres were used as template for enzyme immobilization. The presence of nanometer-sized pores and channels in $CaCO_3$ particles offered a great opportunity for capturing

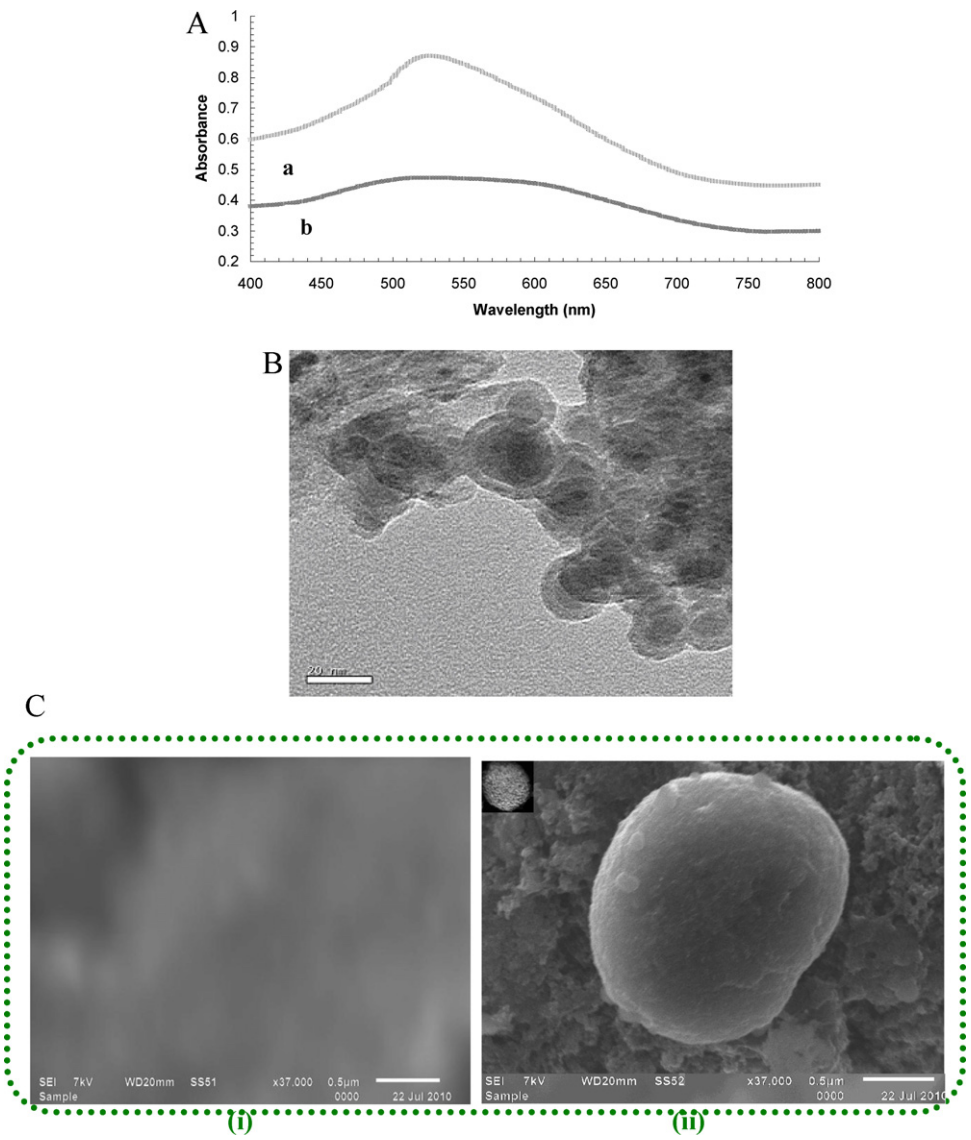


Fig. 1. (A) UV–vis spectra of (a) Au colloid solution and (b) the gold solution after sonication with $CaCO_3$ for 20 min. (B) TEM images of AuNP–$CaCO_3$ microspheres. (C) Scanning electron microscopic image of the bare Au electrode (i) and AChE–AuNP–$CaCO_3$/silica sol–gel/Au electrode (ii) inset: AChE–AuNP–$CaCO_3$ hybrid material.

biomacromolecules such as proteins via physical adsorption/pore diffusion, thus enabling very high substrate loading [35]. With the isoelectric point at pH 8.5 [35] $CaCO_3$ microspheres were positively charged at pH 7.0, whereas AuNPs were negatively charged at the same pH. The electrostatic interaction caused the assembly of AuNPs on the surface of the oppositely charged $CaCO_3$ microspheres; the unique structure of $CaCO_3$ microspheres greatly facilitated this assembly process.

The process was monitored by UV-vis spectroscopy. Fig. 1(A) shows the UV–vis spectra of the prepared colloidal gold solution (a) and the gold solution after being sonicated with $CaCO_3$ for 20 min and centrifugation (b). A great loss in the intensity of the AuNPs–$CaCO_3$ could be found at 527 nm after the AuNPs were assembled on $CaCO_3$ microsphere, which indicated the absorption of the AuNPs on the $CaCO_3$ surface.

Fig. 1(B) shows TEM images of the ultramicrotome spheres were used to obtain information about the inner structures of the hybrid material and the presence and distribution of the AuNPs in the $CaCO_3$ microsphere. The AuNPs decorates the surface of the $CaCO_3$ microspheres.

Fig. 1(C) shows the typical SEM image of bare AuE (i) and AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE (ii). The nanocomposite and enzyme mixture were prepared on the AuE via physical adsorption/pore diffusion between enzyme and nanocomposite. To investigate the microstructure of AChE–AuNPs–$CaCO_3$, the morphology of AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE was examined by SEM as shown in Fig. 1C (ii), which shows that the enzyme was immobilized in the nanocomposites structure and formed a regular array on the surface of the AuE.

3.2. Electrochemical characteristics of acetylthiocholine

The CV characteristics of enzymatically produced thiocholine were studied using the nanocomposite-modified Au electrode as the working electrode in the potential range of −0.1 to +0.6 V and at the scan rate 20 mV s^{-1} in 0.1 M phosphate buffer pH 7.5 (Fig. 2). A bare Au electrode, AuNPs–$CaCO_3$/silica sol–gel-modified AuE and AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE were studied for the comparative study of thiocholine oxidation. The bare AuE fails to give any peak, which confirms that it does not play

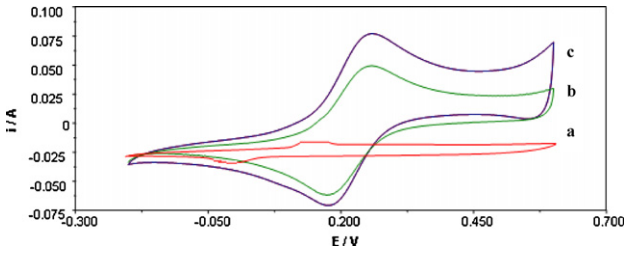


Fig. 2. Cyclic voltammograms of bare AuE (a); AuNP–$CaCO_3$/silica sol–gel-modified AuE (b) and AChE–AuNP–$CaCO_3$/silica sol–gel/AuE (c) in pH 7.0 phosphate buffer containing 100 μl of ATCl (0.05 M) scan rate: 20 mV s^{-1}.

any role in catalyzing thiocholine oxidation (Fig. 2a). An oxidation peak with small oxidative current is obtained in the case of the AuNPs–$CaCO_3$/silica sol–gel-modified AuE (Fig. 2b), which confirms catalysed oxidation of thiocholine by AuNPs–$CaCO_3$/silica sol–gel. The AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE exhibited a redox peak at +0.275 (ox.) and +0.192 (red.) V, which was related to the oxidation and reduction of the immobilized AChE (Fig. 2c).

Electrochemical impedance spectroscopy (EIS) was employed to characterize the interface properties of the modified electrodes. In a typical Nyquist plot, the semicircle portion correspond to the electron-transfer resistance (Rct) at higher frequency range, while a linear part at lower frequency range represents the diffusion limited process. In this report, EIS study of the modified electrodes was carried out in 1.0 mM Fe $(CN)_6^{3-/4-}$ containing 0.1 M KCl solution with a frequency range of 0.01 Hz–10 kHz. The amplitude of the applied potential was set at +0.2 V. As shown in Fig. 3, the Rct value ~11 Ω correspond to the bare Au (curve i). After modification with AuNPs–$CaCO_3$/silica sol–gel, the EIS of the resulting layer shows semicircle domain with Rct ~25 ((Fig. 3, curve ii) suggesting that AuNPs–$CaCO_3$/silica sol–gel layer blocked the redox probe to diffuse towards the electrode surface. However, the Rct value further increases (~45.9 Ω, curve iii) after the immobilization of AChE onto AuNPs–$CaCO_3$/silica sol–gel AuE due to the electron transport between the enzyme and electrode. These results were supported by cyclic voltammogram data also (Fig. 2).

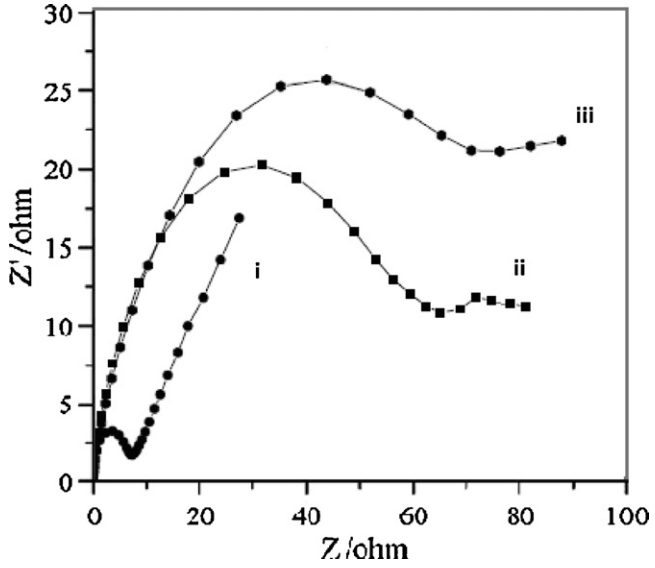


Fig. 3. Nyquist plot of EIS in a solution containing 1 mM $Fe(CN)_6^{3-/4-}$ with 0.1 M KCl at 0.20 mV s^{-1} (frequency range of 0.01 Hz–10 kHz) for bare Au electrode (i), AuNP–$CaCO_3$/silica sol–gel-modified AuE (ii) and AChE–AuNP–$CaCO_3$/silica sol–gel-modified AuE (iii).

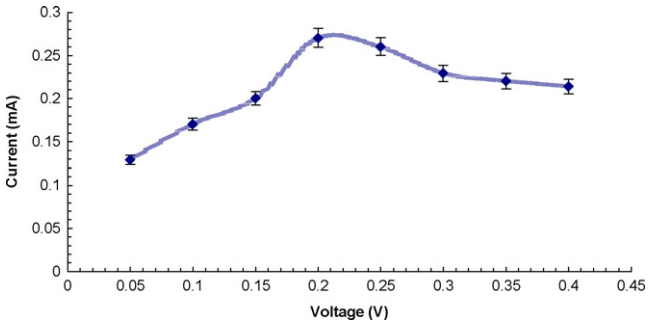


Fig. 4. Effect of applied potential on the response current in pH 7.0, 0.1 M phosphate buffer contains 100 μl ATCl (0.05 M).

3.3. Amperometric response

The determination of the optimum potential for the amperometric detection was measured by linear sweep voltammetry. This study has been carried out using 1 ml acetylthiocholine chloride (0.05 M) +15 ml phosphate buffer pH 7.5 with the working electrode at the desired potential and transient currents were allowed to decay to a steady value. The response of the plain buffer solution was subtracted from the response in the presence of thiocholine. As shown in Fig. 4, significant values of current enhancement were observed up to +0.2 V and it levels off after +0.25 V. The operation of the OP sensor at low electropotential is desirable to reduce the electrochemical interferences for high selectivity; hence a low potential of +0.2 V was selected for sensor operation.

3.4. Optimization of experimental parameters

The pH dependence of the biosensor response in 0.1 M phosphate buffer (pH 5.5, 6.0, 6.5, 7.0 and 7.5) and borate buffer (pH 8.0, 8.5, 9.0 and 10.0) in the range 5.5–10.0 was studied. The response current changed slightly between pH 5.0 and pH 7.0 but the maximum current was observed at pH 7.0. However, the enzyme lost activity irreversibly at the lower or higher pH values. Therefore, phosphate buffer of pH 7.0 was selected for subsequent experiments. The effect of the incubation temperature on the biosensor response from 20 to 50 °C was also investigated. No significant change in current response was observed during this temperature range, but slightly peaked at 30 °C. Hence, this optimum temperature (30 °C) was selected for subsequent experiments. The microenvironment provided by nanocomposite around the enzyme make it thermally stable, protects it from denaturation and significantly stabilizes its biological activity. The concentration of ATCl was optimized by studying cyclic voltammetry study of AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE in the cell solution containing ATCl substrate under optimum conditions. Fig. 5 shows well defined increasing trend in the current response of the biosensor from 0.1 to 500 μM ATCl concentrations. In the absence of ATCl, the peak responses were negligible. Voltammetric measurements were performed after each addition of the ATCl up to a maximum concentration of 500 μM. There was no significant current improvement after 500 μM of ATCl concentration and this revealed that the electrode had reached its saturation level. A good linear relationship of response current with substrate concentration from 0.1 to 500 μM is evident from cyclic voltammetric study. Over a concentration range of 0.1–500 μM, the electrode provided a linear response to ATCl with a sensitivity of 0.39 mA/(μM) (Inset Fig. 5). From these results, 500 μM of ATCl was selected as optimum concentration for further experiments.

The general problem in the electrochemical detection of acetylcholine is the interference from physiological species such as ascorbic acid, uric acid and 4-acetamidophenol. We have studied

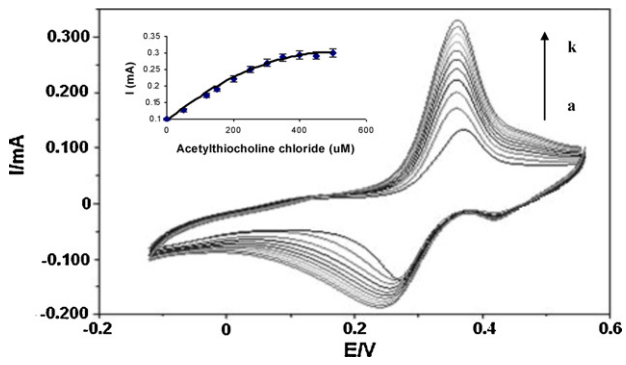


Fig. 5. Cyclic voltammograms measured with AChE–AuNP–$CaCO_3$/silica sol–gel/AuE containing (a) 0.1, (b) 50, (c) 100, (d) 150, (e) 200, (f) 250, (g) 300, (h) 350, (i) 400, (j) 450, (k) and 500 μM of ATCl in a solution with 0.1 M KCl; scan rate 20 mV s^{-1}. Inset shows plot of acetylthiocholine chloride concentration (μM) vs. peak height in mA.

the selectivity of the present acetylcholine biosensor against these possible interfering species through measuring the amperometric response to 0.5 mM acetylthiocholine chloride and successive addition of physiological levels of various interfering species (0.1 mM) at the potential of +0.2 V vs. Ag/AgCl (data not shown). The current of the three interfering substances was only about 0.21 μA, 0.17 μA and 0.14 μA, respectively, indicating that no noticeable changes in current were detected for the interferences in acetylcholine solution. Compared to the high sensitivity of the resulting acetylcholine biosensor, the interference current can be negligible. The high selectivity of the biosensor could be mainly attributed to the relative low working potential for detection, minimizing the responses of common electroactive interferences.

3.5. Pesticide detection

The biosensor was then employed for the detection of malathion and chlorpyrifos analyte under substrate saturation conditions. Thus, current response depends only on the enzyme–inhibitor reaction and not on the mass transfer of substrate or inhibitor from the electrolyte solution to the electrode–electrolyte interface. Fig. 6 is the inhibition curve, which has been obtained by plotting inhibition percentage (*I*%) vs. malathion and chlorpyrifos concentrations. This inhibition plot of AChE shows high linearity in the range measured from 0.1 to 100 nM for malathion and from 0.1 to 70 nM for chlorpyrifos. If the detection limit is defined as 2.0–3.0% inhibition of the enzyme, then in the present case, the limit of detection obtained was 0.1 nM for both the pesticides (Fig. 6 inset). This value is better than other reported values [36,37]. In this case, high sensitivity can be attributed to the presence of AuNPs on the surface of $CaCO_3$/silica

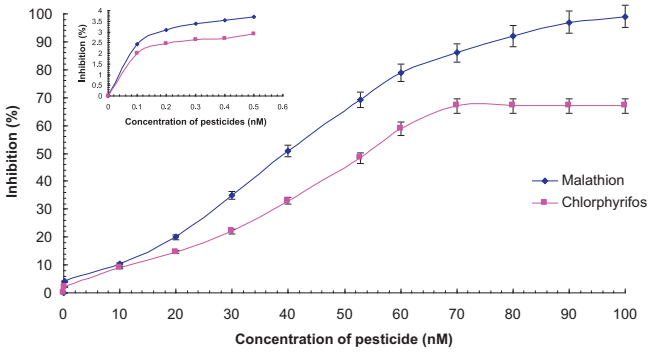


Fig. 6. Inhibition plot of immobilized AChE by malathion and chlorphyrifos after 10 min incubation with 0.05 M acetylthiocholine chloride. Inset, shows the magnification of inhibition plot in the lower concentration range of malathion.

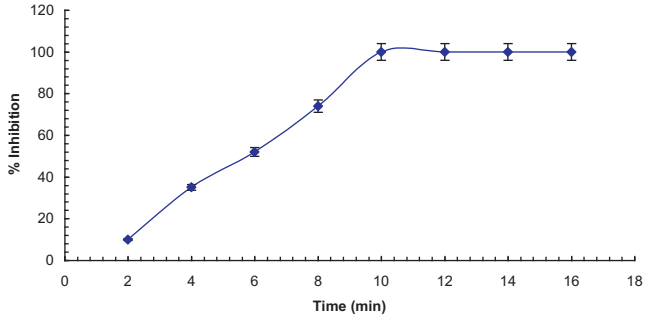


Fig. 7. The effect of incubation time on inhibition. Response of the pesticide biosensor to 0.05 M acetylthiocholine chloride in the presence of acetylcholinesterase and 1.0 nM malathion. Buffer: 0.1 M phosphate buffer, pH 7.0, with 0.1 M KCl. Applied potential +200 mV vs. Ag/AgCl.

sol–gel-modified AuE, which provide high anchoring sites for the enzymes, as well as a good electron transfer coefficient.

3.6. Effect of inhibition time on the response of AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE

For insecticide analysis, one of the most influential parameters is the inhibition time. With an increase of inhibition time in the malathion solution, the peak current of ATCl on the AChE–AuNPs–$CaCO_3$/silica sol–gel-modified AuE was decreased significantly. When the inhibition time was longer than 10 min, the curve tends to a stable value (Fig. 7), indicating the binding interaction between malathion and active target groups in the enzyme reached saturation.

3.7. Precision and accuracy

The precision and accuracy of the present method were determined over seven days. Each day seven replicate quality control samples were analyzed for acetylcholine content. The relative standard deviation of the calculated concentrations was used as the index of precision. The accuracy of the method was determined by comparing the calculated concentrations with their nominal values. The repeatability and inter-day precision were less than 10% and the accuracy within 95–100%. This demonstrates that the present method is precise and accurate for determination of acetylcholine within the range of 0.5–40.0 μM. The results are summarized in Table 1. The analytic recovery of known amount of malathion and chlorpyrifos in river water samples/spiked samples under optimum conditions was determined by the present biosensor. The mean analytic recoveries were in the range of 95–110% (Table 2).

3.8. Reactivation of the enzyme electrode with 2-pyridine aldoxime methiodide

Experiments show that after coming into contact with a pesticide solution, it is difficult for the enzyme activity to recover due to the irreversible inhibition of organophosphate to AChE. However, the enzyme membrane can be reactivated by immersion in a solution of cholinesterase reactivator (2-pyridine aldoxime methiodide, 2-PAM) (0.1 mM) to recover the activity of AChE after the determination of pesticide [1,30]. The activity of AChE could be recovered to 98% of its original value. This takes only about 10–15 min.

3.9. Repeatability and lifetime

The stability and lifetime of the sensor were investigated by measuring the biosensor response with 0.1 nM of malathion over a 3-month period. The response current of the sensor decreased to

Table 1
Precision and accuracy of the present method.

Added concentration (μM)	Mean ± SD measured concentration (μM)	Repeatability (RSD%)	Inter-day precision	Accuracy (%)
0.50	0.49 ± 0.02	4.0	3.2	99.0
1.50	1.55 ± 0.05	3.2	0.80	98.0
6.50	6.52 ± 0.05	0.76	2.25	99.2
11.0	11.8 ± 0.10	0.84	1.40	99.6
25.0	25.4 ± 0.09	0.35	2.10	98.5
40.0	42.5 ± 0.21	0.49	2.29	97.0

Table 2
Recovery studies of malathion and chlorpyrifos in river water samples under optimum conditions.

Pesticides	Added concentration (nM)	Concentration found (nM)	% Recovery	SD ($n = 3$)
Malathion	15	15.2	101.33	2.2
	20	19.3	96.5	1.7
	25	24.8	99.20	1.8
Chlorpyrifos	10	10.95	109.00	1.4
	25	24.9	99.60	1.21
	30	30.3	101.0	2.32

50% after 60 days. After 90 days of testing, the biosensor retained about 40% of its original response, which is much better than earlier electrodes [38–40]. The electrode can be used to determine pesticides at least 50 times. When not in use, the electrode was stored dry at 4 °C in a refrigerator.

4. Conclusion

A multifunctional AuNPs–$CaCO_3$ hybrid material was used for AChE assembly and biosensor fabrication to evaluate the feasibility of using this material in bioelectroanalysis. A highly sensitive, low-cost, simple and fast fabrication of the sensor makes it superior to other techniques. The sensor possesses high sensitivity and has been employed to measure concentrations up to 0.1 nM. This inhibition biosensor is reusable because the enzyme activity could be recovered through being treated with cholinesterase reactivator (2-pyridine aldoxime methiodide) just 10 min after coming into contact with the inhibitor. The developed sensor can also be employed for the detection of other OP compounds.

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