

Immobilization of Acetylcholinesterase and Cholineoxidase on Mercapto-Carboxylic Acid Self-Assembled Monolayer on the Gold Electrode

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Abstract An electrochemical enzyme biosensor based on self-assembly on the polycrystalline gold electrode is constructed. The Acetylcholinesterase (AChE) and Cholineoxidase (ChO) are two enzymes that covalently co-immobilized on the mercaptopropionic acid self-assembled monolayer on the polycrystalline gold electrode (Au-MPA SAM). AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine to acetate and choline that is oxidized by ChO and benzoquinone to yield the respective betaine and hydroquinone. The molecular dimension distances between two immobilized enzymes on Au-MPA SAM result in preconcentration of choline, in the ChO nano-environment. Hydroquinone, which is formed in the catalytic cycle, was measured amperometrically at a rotating disk electrode (RDE). The regeneration of benzoquinone from hydroquinone takes place at a fixed potential (0.35 V versus Ag/AgCl). The resulting current is a measure of the enzymatic activity. Carbaryl was chosen as a model toxin and AChE inhibition characteristics were utilized for the toxin detection. The biosensor fabrication steps are presented and discussed from which the biosensor is applied for carbaryl determination based on the Relative Inhibition (RI) of chronoamperometry responses by the addition of toxin.

Keywords Electrochemical Biosensor, Carbaryl, Acetylcholinesterase Inhibition, Self-Assembled Monolayer, Electrochemical Impedance Spectroscopy, Chronoamperometry

1. Introduction

With recent advances in nanotechnology, electrochemical biosensors, in combination with nanomaterials have become simple, efficient tools to measure the concentration of analytes and the response time functions of target biomolecules to the drugs or the toxic reagents [1]. Carbaryl (N-methyl-1-naphthylcarbamate; Sevin) is a carbamate nerve toxin that presents a potential teratogenic capability [2]. There are limited analytical methods available for the determination of carbaryl such as spectrophotometry [3], spectrofluorimetry [4], infrared spectroscopy [5], photothermal [6], chemiluminescence [7], quartz crystal microbalance [8], mass spectrometry [9] and chromatography. However, these techniques require expensive equipment as well as complicated and time-consuming solvent cleanup steps. Recently, some novel concepts to overcome these obstacles have been suggested. A promising alternative approach involves the use of enzyme-linked immunoassay (ELISA) [10]. While they have

often demonstrated the required sensitivity and acceptable specificity, these assays need specific antibodies and, indirectly, the use of animals in order to produce these "receptors" [11].

Great efforts have been devoted to the fabrication and characterization of a large variety of amperometric enzyme biosensors [12, 13]. The enzyme dissolved in electrolyte solution or immobilized on a solid electrode serves as a redox centre, and reacts selectively with biological species. The reaction product may be used directly or by mediation of a reversible redox couple for the determination of the species of interest. However, mediated electron transfer is the most efficient process and typically used for biosensors construction [14]. Immobilization of the enzyme on a solid electrode will decrease the distance between conducting substrate and enzyme redox centre, therefore, the electron transfer will be carried out near or inside the diffusion layer, which in turn, increases the sensitivity and selectivity of the biosensor [15]. The enzyme may be immobilized in a thin layer at the sensor surface in different ways; such as using polymers [16], carbon paste [17], monolayers [18], and multilayer self-assemblies [19], among them, immobilization via covalent attachment of enzyme to the functionalized self-assembled monolayers (SAMs) [20] is specially useful where miniaturization of the sensor in

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nanometre scales is required [21]. The functionalized SAMs formed on gold surface are ordered molecular assemblies, which are widely used for the immobilization of proteins and enzymes in biosensors fabrication [22].

The biosensors usually contain two basic components connected in series: (i) A biochemical recognition system, which translates information from the biochemical domain into a chemical or physical output signal, and (ii) the transducer, which serves to transfer the sensor signal from the output domain of the recognition system to mostly the electrical domain. An electrochemical biosensor is a biosensor with an electrochemical transducer [23]. Most of the biosensor electrochemical transducers are based on potentiometric [24] or amperometric [25] detections. The amperometric detections are based on measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species, which is (i) a biocatalytic product (*e.g.* hydrogen peroxide), or (ii) a redox couple mediating redox enzymes and the electrode support at a constant potential. The earlier study [26] was shown that carbaryl inhibits the acetylcholinesterase (AChE), a key enzyme in the transmission of nerve impulses. On the basis of this effect, a method was developed to quantify carbaryl by measuring the decrease in enzyme activity, determined using modified Ellman's spectrophotometric method. The carbaryl inhibition effect on AChE extracted from different sources was studied and the implications in terms of kinetic mechanism and toxicity discussed [27, 28].

In this work, AChE and Cholineoxidase (ChO) are immobilized covalently on the topside of the gold Mercaptopropionic acid self-assembled monolayer (Au-MPA SAM) to produce Au-MPA-AChE/ChO SAMs as a working electrode (biosensor). Next, the biosensor is used to determine the carbaryl in the presence of parabenzoquinone (PBQ) mediator using chronoamperometry. The basis of the recognition system in this work is the diffusion of the carbaryl to the biosensor and inhibition of choline oxidation that resulted from acetylcholine hydrolysis. The data are presented and discussed from which a new method is proposed for carbaryl determination based on the chronoamperometry measurements.

2. Experimental

2.1. Chemicals

Acetylcholine chloride and AChE from *electric eel* (500 U/mg, EC 3.1.1.7) ChO from *Alcaligenes sp.* (50 U/mg, EC 1.1.3.17), carbaryl, pralidoxime iodide (2-PAM), 3-mercaptopropionic acid (MPA), Bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), parabenzoquinone (PBQ), 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC), and other chemicals were of commercial sources (Merck or Sigma) and used as supplied without further purification except parabenzoquinone that was recrystallized from hot solution of n-hexane. All

solutions were prepared with double-distilled water. Phosphate buffer solutions (PBS) contained 0.05 M KCl, 0.05 M K_2HPO_4/KH_2PO_4 were used and the pH was adjusted with NaOH or H_3PO_4 dilute solutions. The biosensor was then kept in dry condition at 4 °C until used.

2.2. Electrode Modification

The polycrystalline gold working electrode (0.0314 cm², Azar electrode Co. Urmia, IRAN) was polished using aqueous slurries of alumina (0.3 to 0.05 μ m), sonicated in three steps: in water, chloroform and water each for 5 min, and then cleaned electrochemically by cycling the electrode potential between 0.000 and +1.500 V vs. Ag/AgCl in 0.5 M sulfuric acid until reproducible voltammograms were observed [29]. A roughness factor of 1.8 ± 0.1 was obtained from ratio of the real to geometric surface area of the electrode [30], and attempted to maintain it constant in all experiments. The cyclic voltammograms obtained on the electrode in the presence of reversible marker, $Fe(CN)_6^{3-}$, showed a peak separation that confirms the safety of the system ($\Delta E_p \approx 60$ mV). Immediately before modification, the electrode was thoroughly rinsed with distilled water. Cleaned gold electrode was modified by placing into a 25:75 (v/v) water/ethanol solution containing 70 mM MPA for 12 h to form Au-MPA SAM electrode. The modified electrode was washed with the same ethanolic solution, dried in argon stream, and activated in PBS (pH = 5.5) containing 0.002 M EDC and 0.005 M NHS for 2 h. Then, the electrode was rinsed with the same PBS and immediately an aliquot of 50 μ l of 2 mg/ml AChE and 100 mg/ml ChO enzymes in PBS (pH 7) was dropped onto it for at least 1.5 h to fabricate Au-MPA-AChE/ChO SAMs electrode, washed with PBS, and used for electrochemical measurements.

2.3. Electrochemical Measurements

A conventional three-electrode cell, consisting of Au-MPA-AChE/ChO SAMs modified electrode as working, Ag/AgCl, KCl (sat'd) as reference, and a platinum foil with large surface area as auxiliary electrode, was used for electrochemical measurements. The measurements were carried out using Potentiostat/Galvanostat Autolab 12 equipped with Frequency Response Analyzer (FRA 4.9), interfaced with a personal computer, and controlled by GPES 4.9 and FRA 4.9 softwares (Eco Chemie BV, Utrecht, The Netherlands).

The electrochemical characterization of Au-MPA SAMs electrode was performed in the presence of 0.5 mM $K_3Fe(CN)_6$ redox probe using EIS and CV. Quantitative determination of carbaryl was performed in the presence of 5 mM PBQ as a mediator by chronoamperometry. For the characterization of the modified electrode, the DC potential was formal potential of the redox couple, (*i.e.* $E^0 = 0.200$ V vs. Ag/AgCl for $[Fe(CN)_6]^{3-/4-}$ and $E^0 = 0.080$ V vs. Ag/AgCl for PBQ/ H_2Q). Quantitative determination of carbaryl was performed in different DC potentials. Other experimental conditions are described in the respective figures.

2.4. Analytical Procedure

The Au-MPA- AChE/ChO SAMs modified electrode was allowed to settle to a stable current over a period of at least 0.5 h in the background solution. Background solution was the 10 ml of PBS (pH 7.0) containing 2.0 mmol l⁻¹ acetylcholine as the enzyme substrate and 5 mM PBQ as the electron mediator. Prior to each experiment, the solutions were bubbled with high-purity argon gas for at least 20 min and blanketed with the same gas during the experiments to eliminate oxygen interference. All experiments were carried out at room temperature. Once the electrode background current was stable, additions of carbaryl were made from the stock solution using micropipette. The degree of inhibition, in percentage, was calculated as the relative decay of the biosensor response after the contact with the carbaryl.

3. Results and Discussion

3.1. Fabrication and Characterization of Au-MPA SAMs Electrode

3.1.1. Formation of SAMs

For enzyme electrode a short alkyl chain alkanethiol,

usually three carbons long, is used so that the grafted redox centre (*e.g.* an enzyme) being as close as possible to the electrode. An additional advantage of the short alkyl chain is that a relatively disordered SAM is formed which means the underlying metal is still electrochemically accessible. In this study MPA was selected as a short alkyl chain to bind AChE/ChO into the Au electrode surface, and form Au-MPA-AChE/ChO SAMs biosensor. The schematic diagram of biosensor preparation is shown in Fig. 1. The formation of SAMs was traced by CV and EIS.

The complex plane plots obtained on clean Au, and Au-MPA electrodes are shown in Fig. 2. The data were approximated using CPE model from which the R_{ct} and other model parameters were extracted.

The bare gold electrode shows a very small semicircle (curve a, $R_{ct}^0 = 1114 \Omega$ from the high frequency range semicircle) followed by straight line indicating domination of mass diffusion limiting effect on the electron transfer process (curve a, in the low frequency range). The respective semicircle diameter at the high frequencies range, corresponding to the charge transfer resistance at the electrode surface, increases upon the MPA SAMs formation on the gold electrode surface (curve b, $R_{ct} = 3730 \Omega$).

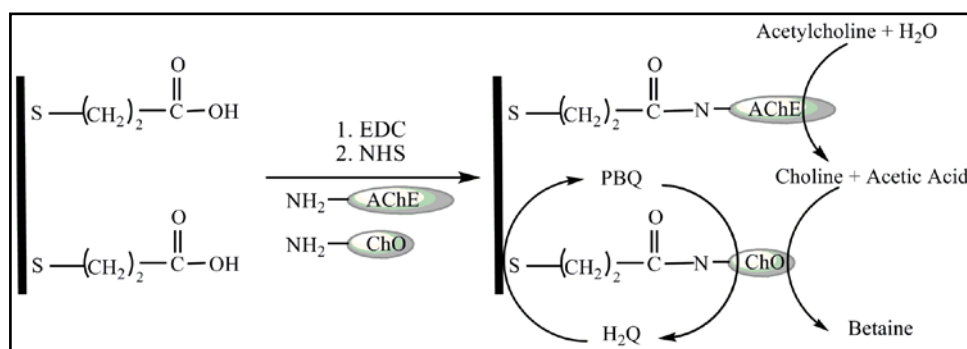


Figure 1. Schematic illustration of the immobilization of AChE and ChO on gold electrode using a SAM of MPA and EDC-NHS as coupling agents

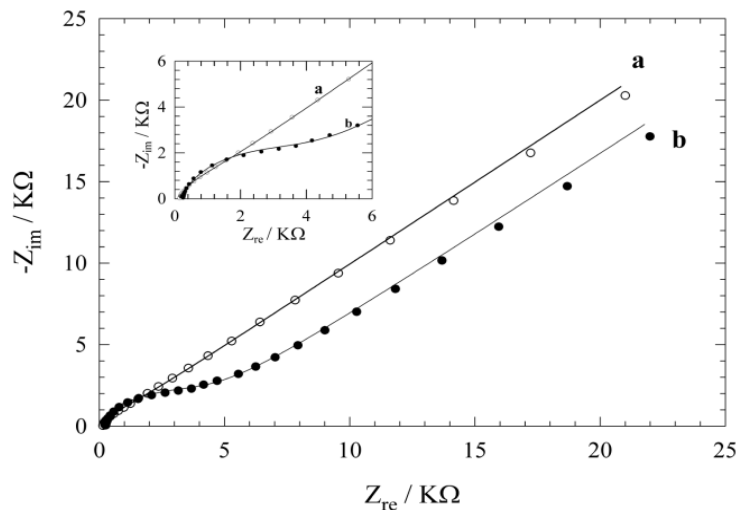


Figure 2. The complex plane plots obtained on (a) bare gold and (b) Au-MPA SAMs electrodes in 0.1 M PBS in the presence of 0.5 mM $\text{Fe}(\text{CN})_6^{3-}$, pH 5.5, coating time in 20 mM MPA ethanolic solution is 12 h, $E_{DC} = +0.200 \text{ V}$ (vs. Ag/AgCl), $E_{ac} = 5 \text{ mV}$, and frequency range; 10 kHz to 100 mHz. The inset shows expansion the high frequency range

The formation of MPA SAMs on the electrode surface has produced a larger barrier to the interfacial charge transfer, which is revealed by the increased diameter of the semicircle in the spectrum. However, the charge transfer resistance is not infinitive and there is still diffusion line (Warburg impedance). This means that the surface is not completely blocked and the charges can penetrate to the gold surface producing faradaic current. Since the EIS measurements are made at formal potential where no overpotential acting on the electrodes, the EIS results particularly R_{ct} , indicate the information of surface coverage [31].

3.1.2. Determination of Surface Coverage of Au-MPA SAMs

The Total surface coverage, Γ of the MPA SAMs on gold, may be evaluated by integrating the cathodic peak associated with desorption process obtained in NaOH solution by CV [32]. The charges consumed for the MPA desorption, measured from the first cyclic voltammograms (corrected for background) was $53 \mu\text{C}/\text{cm}^2$. Assuming one-electron reduction process ($\text{Au-SR} + e^- \rightarrow \text{Au} + \text{RS}^-$) the charge of $53 \mu\text{C}/\text{cm}^2$ was further converted to a surface concentration of $5.55 \times 10^{-10} \text{ mol}/\text{cm}^2$. Comparing this value with ideal value of $7.68 \times 10^{-10} \text{ mol}/\text{cm}^2$ for the packing arrangement close to $(\sqrt{3} \times \sqrt{3})$ R30 [33] shows that almost $73 \pm 7\%$ of the gold electrode surface is covered by MPA. It should be emphasized that Au-MPA SAMs is stable in the applied potential range between +0.800 and -0.700 V vs. Ag/AgCl, which is a suitable potential window to study most of biological redox species.

The partial surface coverage (θ) was also estimated using $R_{ct}^0 = 1114 \Omega$ and $R_{ct} = 3730 \Omega$ for uncovered and covered gold electrodes (Fig. 2, curve a and b), and the relation $\theta = (1 - R_{ct}^0 / R_{ct})$ [34], as $70 \pm 5\%$. This value is in good agreement with the value obtained by CV. However, it should be mentioned that it is difficult to find a perfect baseline in CV plots.

3.2. Enzymes Immobilization

The two enzymes AChE and ChO can be co-immobilized onto the surface of the working Au electrode in the same or separate immobilization matrices or, one can be immobilized and the other can be added in solution. The co-immobilization of the two enzymes generally allowed detection of analytes in a lower concentration range [35]. To bind enzyme molecules tightly onto the Au electrode surface, the amine groups of the AChE and ChO were coupled to the acidic head groups of Au-MPA SAMs through the formation of imide groups using EDC and NHS, according to the literature [36]. Therefore, the immobilized enzymes on the gold surface and the solubilized carbaryl in the electrolyte solution could react near the surface of the gold electrode. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine to acetate and choline that is oxidized by ChO to yield the respective betaine. However, when choline reduces the ChO enzyme-bound FAD, FADH_2 forms, which cannot directly go to the electrode surface and oxidize, because of fixation of FAD to the enzyme molecule. To solve this problem an organic electron mediator such as parabenzoquinone (PBQ) was chosen as a mediating agent between ChO and electrode.

Cyclic voltammetry is a simple and easy tool to show the changes of electrode behavior after each assembly step, because the electron transfer between the solution species and the electrode must occur by tunneling through either the barrier or the defects in the barrier. Fig. 3A shows the cyclic voltammograms obtained on the bare Au (curve a), Au-MPA SAMs (curve b), and Au-MPA-AChE/ChO SAMs (curve c) electrodes in the presence of 5 mM PBQ redox mediator. While the redox probe exhibits quasi-reversible electrochemical features at the bare Au electrode, immobilization of the MPA SAMs onto the electrode results in a peak separation.

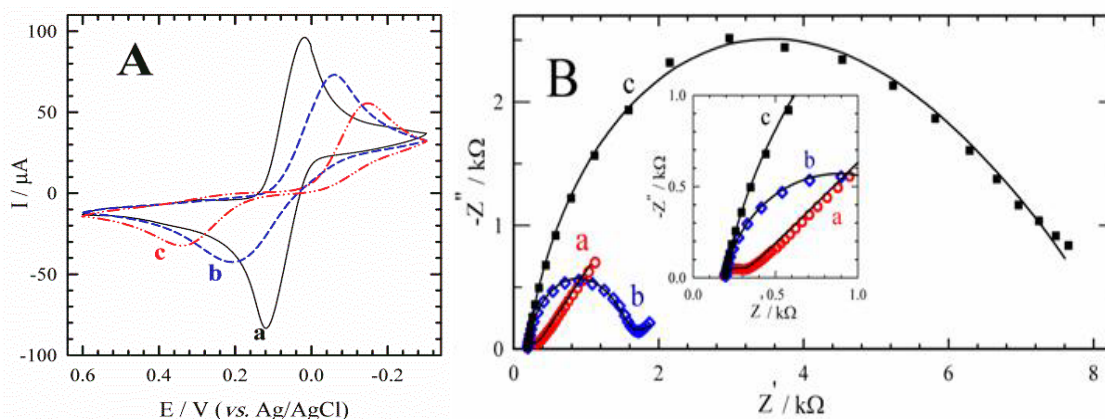
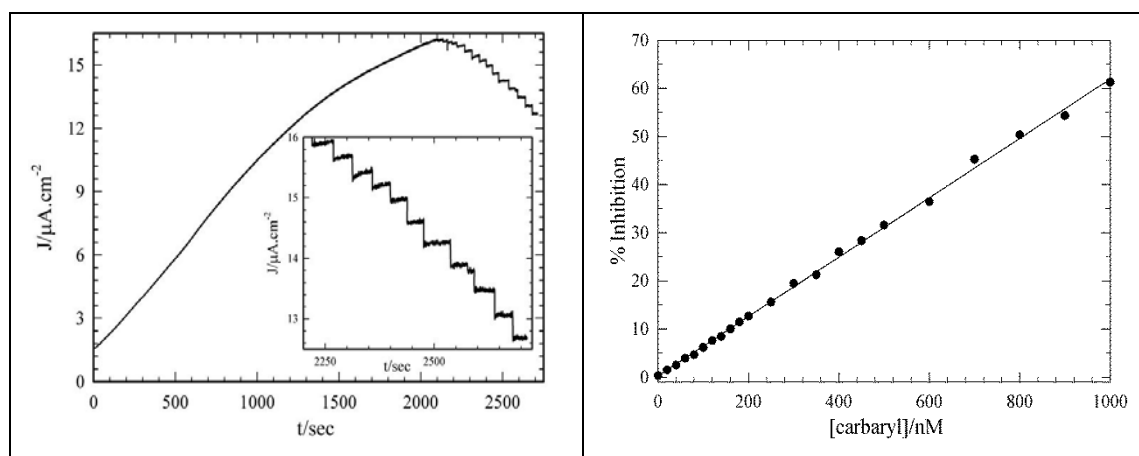


Figure 3. Cyclic voltammograms (A) and complex plane plots (B) obtained on (a) bare gold, (b) Au-MPA SAMs, and (c) Au-MPA-AChE/ChO SAMs electrodes in PBS, pH 7, in the presence of 5 mM PBQ. Scan rate is 100 mV s^{-1} ; $E_{dc} = +0.080 \text{ V}$ (vs. Ag/AgCl), $E_{ac} = 5 \text{ mV}$, and frequency range 10 kHz–100 mHz. The inset shows expansion of the high frequency range

Table 1. Electrochemical parameters extracted from the cyclic voltammograms (Fig. 3A) and complex plane plots (Fig. 3B)

| Electrode | CV | | EIS | | |
|-----------------|-------------------|----------------------|-------------------|-----------------|--------------------------------------|
| | ΔE_p (mV) | $I_{p,a}$ (μ A) | $R_{ct}/k\Omega$ | $C_{dl}/\mu F$ | k_{app} ($cm\ s^{-1})\times 10^3$ |
| Au | 104 \pm 3 | 86 \pm 1 | 0.118 \pm 0.005 | 1.38 \pm 0.02 | 6.41 \pm 0.01 |
| Au-MPA | 253 \pm 6 | 44 \pm 2 | 1.262 \pm 0.011 | 0.46 \pm 0.01 | 0.60 \pm 0.01 |
| Au-MPA-AChE/ChO | 471 \pm 5 | 35 \pm 2 | 5.128 \pm 0.020 | 0.27 \pm 0.01 | 0.15 \pm 0.01 |

**Figure 4.** (left) Long time chronoamperometric response of rotating Au-MPA-AChE/ChO SAMs modified electrode to 2.0 mmol l^{-1} acetylcholine in 10 ml PBS 0.1 mol l^{-1} , pH 7.0 deoxygenated solution containing 5 mmol l^{-1} PBQ, at a potential of 0.380 V (vs. Ag/AgCl). Each stages after plateau region that also magnified as inset plot, is related to the addition of 100 nmol l^{-1} carbaryl to the cell solution. Electrode rotation speed is 2000 rpm. (right) Relationship between enzyme inhibition rate and [carbaryl]

Upon the assembly of the enzymes on the Au-MPA SAMs, the interfacial charge transfer between the redox probe and the electrode is further blocked and maximum peak separation occurs. Quantitative parameters extracted from CV data are summarized in Table 1. Also, according to EIS data (Fig. 3B), the complex plane plots obtained on the clean bare Au, Au-MPA and Au-MPA-AChE/ChO SAMs electrodes at the same solution conditions. The bare gold electrode shows a very small semicircle (curve a, from the high frequency range semicircles) followed by straight line indicating domination of the mass diffusion limiting effect on the electron transfer process (curve a, in low frequency range). The layer by layer assembly of thiol (curve b) and enzymes (curve c) on the gold electrode impedes the electrode surface and produce a larger barrier to the interfacial charge transfer, which is reveal by the increased diameter of the semicircle (R_{ct}) in the spectra and decreased apparent rate constant (k_{app}) (Table 1).

These results are consistent with the fact that immobilization of enzyme on the electrode retards the interfacial electron transfer kinetics of the redox probe. It is also apparent (Fig. 3, curve c) that the anodic diffusional current is located in the potentials region after +0.350 V vs. Ag/AgCl. Therefore, the DC potentials in this region (e.g. +0.380 V) were selected for chronoamperometric experiments to ensure that measurements were carried out at the limiting anodic diffusional currents and maximum sensitivity was obtained.

3.3. Chronoamperometric Response

Reversible inhibitors can bind to enzymes through weak non-covalent interactions such as ionic bonds, hydrophobic interactions, and hydrogen bonds. Because reversible inhibitors do not form any chemical bonds or reactions with the enzyme, they bind rapidly and can be easily removed; thus the enzyme and inhibitor complex is rapidly dissociated in contrast to irreversible inhibition. Carbaryl is known to involve in the reversible inhibition on AChE [37]. Therefore, the immobilized AChE enzyme activities diminish to its substrate, acetylcholine in the presence of carbaryl. Due to carbamylation of the serine residue at the active site of the AChE enzyme by carbaryl, the production of the choline from acetylcholine decreases and so diminishes the oxidation current related to enzymatic reaction product through the ChO enzyme and mediator which in turn PBQ is oxidized at Au electrode surface in diffusion layer. A simple hydrodynamic chronoamperometry method for the determination of carbaryl was established due to remarkable current decreasing in the amperometric signals of the Au-MPA-AChE/ChO SAM electrode by successive carbaryl addition to the cell solution containing acetylcholine substrate and PBQ redox mediator (Fig. 4).

As is shown in Table 1, the double layer capacitance (C_{dl}) of the bare gold electrode is decreased by MPA SAM formation on Au and also enzyme immobilization on the Au-MPA SAM electrode. Therefore, the charging current

has been dramatically reduces which lead to increasing in sensitivity, as well as the enhancing in reproducibility and robustness that can be achieved by using such an immobilization method. However, biosensors employing amperometric transducers often suffer from high initial background currents and long equilibration times (the time required to reach the steady state) [38]. The Relative inhibition percentage (I%) obtained from $100 \times (I_0 - I_i) / I_0$ where, I_0 and I_i represent the steady state oxidation current responses in the absence and presence of carbaryl respectively (Fig. 4). In order to ensure a linear relationship between the amperometric response and the analyte concentration, the enzymes loading, the immobilization method of two enzymes onto the working electrode surface, the pH, and the substrate concentration must be optimized accordingly. Generally, the AChE hydrolysis must be carried out under kinetically controlled conditions and the ChO catalyzed reaction under diffusion-controlled conditions [39]. Typically, the ChO is used in excess and the AChE amount is optimized in order to ensure the kinetically controlled conditions. The effect of pH on the reactions of the immobilized enzymes was examined with the use of PBS over the pH range 4.0–9.0 by checking the biosensor response (data are not shown). The optimum pH for this biosensor was 7.0, very close to the free AChE and ChO [40]. Similar pH profiles have been observed in others enzyme immobilization systems [41]. The inhibition measurements were performed in conditions corresponded to the saturation of the enzymatic layer with the substrate, *i.e.* at the acetylcholine concentration of 2 mmol l^{-1} . In saturation conditions, the shift in the biosensor response after the contact with the carbaryl solution is related only to the enzyme–inhibitor interactions and does not depend on the mass transfer of the substrate/inhibitor from the bulk solution to the enzyme layer. This makes it possible to reach the maximum sensitivity of the carbaryl determination. If the concentration of the substrate chosen is lower, the decay in the enzyme activity due to the inhibition is partially compensated by the enzyme active sites which were free in the response measurement prior to the incubation in an inhibitor solution. This effect of substrate concentration is often observed in inhibition measurement with immobilized enzymes [42]. Experiments showed that after coming into contact with a pesticide solution, it was difficult to recover the activity of immobilized AChE due to the reversible or irreversible inhibition of AChE by pesticides. However, the immobilized enzyme could be reactivated following pesticide determination by immersion in a solution containing cholinesterase reactivator (2-PAM, 4.0 mmol l^{-1}) [43]. The activity of AChE was restored to 98% of its original value by 5 mM treatment for about 12–15 min. The selectivity of the biosensor relative to various potentially electroactive species, such as ascorbic acid, uric acid, acetaminophen, and dopamine was tested in PBS. No noticeable changes in current were detected through measuring the amperometric response to 2 mM acetylcholine on successive addition of $1 \text{ }\mu\text{M}$ ascorbic acid, $1 \text{ }\mu\text{M}$

acetaminophen, $0.5 \text{ }\mu\text{M}$ uric acid, and $0.1 \text{ }\mu\text{M}$ dopamine at the potential of 0.380 V vs. Ag/AgCl. The acceptable selectivity of the biosensor could be mainly attributed to the high sensitivity of the resulting biosensor and the relative low working potential for detection. Moreover, it may be due to the MPA SAM, which has the good anti-fouling properties [44].

4. Conclusions

We first studied the formation of a surface with carboxylic acid functional groups that in molecular dimension close to the electrode surface. Then the surface carboxylic acid functional groups are activated by some activator agents to link with amine groups of a few enzyme amino acids to form amide bonds and by the way the enzymes were immobilized covalently, close to the gold surface. The resulting AChE/ChO biosensor in the presence of acetylcholine substrate and appropriate redox mediator (PBQ) exhibited high sensitivity, good reproducibility, long-term stability and low-cost processes, which provided a new promising tool for the characterization of enzyme inhibitors and pesticide analysis. These results suggest that the fabricated bienzymatic self-assembled monolayer biosensor can be successfully used for the electrochemical determination of carbaryl in real samples.

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