A new changeable bioreactor for detection of organophosphate in a flow-through system

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Abstract
A flow-through biosensor consisting of a fixed bed bioreactor was employed to detect the insecticide paraoxon. Based on the inhibition of organophosphorous insecticide to the enzymatic activity of acetylcholinesterase (AChE), using paraoxon as a model compound, the condition for detection of the insecticide were optimized. The influence of enzyme loading on the packing surface was studied and AChE loading was set at 0.36 U/cm^2 for subsequent studies. Maximum value of absorbance response occurred at the residence time in bioreactor of 5 min, that was chosen as the optimal residence time. This flow-through system gave a linear response (R^2 = 0.9869) to acetylthiocholine iodide (ATChI) at concentrations of 0.050 to 1 mM. Under appropriate conditions, the inhibition of paraoxon was proportional to its concentration in two ranges, from 0.25 to 25 mg/l and 25 to 60 mg/l and the detection limit for paraoxon was 7.3×10^{-8} M. The incubation time was 14 min. These results demonstrate that silicate-multiwall carbon nanotube (MWCNT) sol film is very efficient for retaining the activity of AChE with a good long-term stability.
Keywords: Flow-through system; Biosensor; Acetylcholinesterase; Bioreactor; Silicate sol-gel; Multiwall Carbon Nanotube

INTRODUCTION

Organophosphates (OP) compounds, as one group of the most commonly applied pesticides in agriculture, are typical examples exhibiting fairly high toxicity. Therefor, rapid determination and reliable quantification of trace level of OP compounds are significant to healthiness and environment (Solna *et al*., 2005). Toxic action of OP compounds is due to their ability to irreversibly modify the catalytic serine residue in acetylcholinesterases (AChE) and subsequent inhibition of the AChE effectively prevents nerve transmission by blocking breakdown of the transmitter choline (Massoulie *et al*., 1993) Biosensors based on the inhibition to AChE have been widely used for the detection of OP and carbamates pesticides. Comparing with other analytical techniques such as gas and liquid chromatography, enzyme based among the fabrication of biosensor, immobilization of enzyme to solid surface is a crucial step for the design of the biosensor (Amine *et al*., 2006; Wissiack *et al*., 2000; Puig *et al*., 1997; Corcia *et al*., 1991). A key requirement of enzyme immobilization is attachment without the bioactivity being sacrificed (Gill *et al*., 2000).

There are many advantages of using sol-gel as a membrane material for biosensors. The most important property of sol-gel is its ability to entrap large amount of enzymes, but other properties such as its thermal and chemical stability, simplicity of preparation and flexibility in controlling pore size and geometry also are desirable features (Doong *et al*., 2001).

Since discovered in 1991 (Iijima *et al*., 1991), carbon nanotubes (CNTs) have attracted much attention due to their unique mechanical and electrical properties (Baughrman *et al*., 2002; Odom *et al*., 2000; Ajayan *et al*. 1999). Because of the advantages in the sensing field: small size with larger surface, easy immobilization of protein with retention of activity (Huang *et al*., 2003) and particularly the ability to facilitate electron transfer when being used as electrode (Wang *et al*., 2002; Davis *et al*., 1997), such materials have been widely used for preparation of

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biosensors and the study of the properties of biomolecules (Pedano *et al*., 2004; Valentini *et al*., 2004).

Gas chromatography, liquid chromatography, thin film chromatography and various spectroscopic techniques (Aprea *et al*., 2002), coupled to selective detectors such as mass spectrometry; involve compound extraction, preconcentration and clean-up steps, which make them tedious, time-consuming, expensive and not suitable for in-field analysis. Moreover, highly trained technicians and specialized laboratories are required. Another drawback is the fact that they do not measure the toxicological effect of the pesticides (Diehl-Faxon *et al*., 1996). Despite their clear advantages, the development of a successful biosensor has encountered several problems, such as low response stability (due to biomolecule leaking or deactivation), low mechanical stability, high diffusion resistance of the substrate/biocomponent assembly, interfering signals arising from other compounds present in real samples and, especially for cholinesterase-based biosensors, multi-stage procedures. Thus, the biosensor design is a key factor to overcome these drawbacks.

The combination of biosensors with flow-injection analysis (FIA) techniques offers the possibility to control the whole procedure, simplifying the sequence of steps and allowing an easier optimization of the reaction conditions (Schmidt *et al*., 1993; Schmid *et al*., 1990). In the past 25 years, FIA has been the most widely proposed method of automation, due to its efficiency and versatility (Gorton *et al*., 1991). Compared to batch systems, flow-through based biosensing systems present the advantages of the reduction in analysis time, which allows a high sample throughput, and the possibility to work with small volumes of substrate and sample (Tran-Minh *et al*., 1996).

In this work, a new changeable fixed bed reactor in flow-through system based on acetylcholine esterase (AChE) is described. The system was prepared by mixing AChE and silicate-multiwall carbon nanotube (MWCNT) and encapsulating this mixture in sol-gel derived from tetraethylorthosilicate (TEOS) on the ceramic cylinder packing before transferring it into a reactor. The details for the construction of this system is explained.

MATERIALS AND METHODS

Reagents: Acetylthiocholine iodide (ATChI) with 99% purity was purchased from Fluka (UK), multiwall carbon nanotubes, with 95% purity (MWCNTs, length 1-10 μm, external diameter 37 nm, and wall No. 3-15), were obtained from Plasma Chem. GmbH Company (Germany). Modified acetylcholinesterase (AChE, 4300 U/ml) from *Drosophila melanogaster* was kindly supplied by New Idea Research Institute (Iran). 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) with 99% purity and TEOS with 99% purity were purchased from Merck (Germany). Dimethilformamide (DMF) was from Sigma (USA). Ceramic packing (average length 1.8 mm, external diameter 1.9 mm and internal diameter 1 mm) were obtained from Tailor China Shop (Iran). All solutions were prepared with distilled water. All other chemicals and reagents were of analytical grade.

Preparation of silicate-MWCNT sol-gel: MWCNTs were sonicated in water bath for 20 minutes and functionalized by sonicating in a mixture of concentrated HNO_3 and H_2SO_4 for 4 h followed by extensive washing in water until the filtrate was neutral. Then, MWCNTs were sonicated in ethanol for another 30 min, and dried at 80ºC for 30 min (Vivek *et al*., 2006). The MWCNT mixture was prepared by adding 3 mg dried MWCNTs to DMF solution (2 mg MWCNT/1 ml DMF solution) (Beatriz *et al*., 2006; Guodong *et al*., 2005) and then cooling to 4ºC. Then homogeneous silicate sol solution was prepared by dissolving 0.5 ml of TEOS in 0.6 ml of water, 0.1 ml of 5 mM NaOH was added and the solution was stirred 20 min at room temperature. 6 ml of ethanol was added to this solution which was stirred regularly for another 10 min. This stock was immediately cooled to 4ºC after mixing (Du *et al*., 2007; Quanlin *et al*., 2004). 0.59 ml of MWCNT mixture was blended with 2.84 ml silicate sol solution for preparing silicate-MWCNT sol mixture.

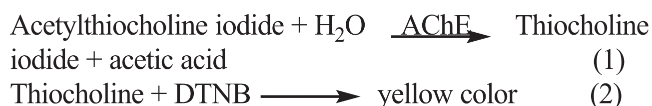
Preparation of AChE solution: The AChE solution was prepared by mixing 5 μl of enzyme solution (4300 Unit/ml) with 200 μl of 0.1 M phosphate buffer solution (pH 7.4) containing 5 μl glycerol. The small amount of glycerol in the enzyme stock solution serves as an emollient so that AChE can spread easily on the ceramic cylinder and does not curl up on the surface after drying.

Enzyme immobilization: 5.3 g of ceramic packing were added to the 630 μl of enzyme solution and manually mixed until all of the ceramic packing was coated by enzyme. Then, 3.43 ml of silicate sol-MWCNT mixture was added and mixed for 3 min. Silicate-sol was used to encapsulate enzyme and to provide a reasonable environment for processing. The final mixture was allowed to polymerize for 1 h at room tempera-

ture. Then, ceramic cylinder packing were transferred on to a clean surface and allowed to dry for 72 h at 4ºC in refrigerator. The mechanism of enzyme binding was hydrogen bound with carboxyl group on functionalized MWCNT (Andreescu *et al*., 2002; Li *et al*., 2001; Lin *et al*., 2006). Finally the packing was immersed in PBS (pH 7.4) and kept at 4ºC in a refrigerator in order to remove the excess materials from the immobilized surface.

Measurement setup: All the experiments were carried out with a flow-through system set up (Fig. 1) with the sol-gel-MWCNT immobilized enzyme fixed bed bioreactor. Fixed bed bioreactor was constructed using a serum tube with 4.79 cm length, and external and internal diameters of 1.41 and 1.19 cm, respectively. All of the coated ceramic packing was carefully packed into the bioreactor (Fig. 1). Then the fixed bed bioreactor was connected to the flow line after peristaltic pump as shown in Figure 1. Bioreactor feed was prepared by dissolving 0.0116 g of ATChI in 38.7 ml phosphate buffer (0.1 M and pH 7.4) and 1.3 ml DTNB standard solution (40 ml). The absorbance was measured at 412 nm using UV-visible spectrophotometer.

Absorbance measurement of the colorimetric biosensor: Ellman's spectrophotometrical method is based on the fact that thiocholine (one of the products of enzymatic hydrolysis of ATCh) reacts immediately, quantitatively and irreversibly with 5, 5-dithiobis-2-nitrobenzoic acid (Ellman's reagent, DTNB) forming yellow product 5-mercapto-2-nitrobenzoic acid and its dissociated forms. The dependence of absorbance on time was measured at 25ºC spectrophotometer (Cary 50, Varian, Australia). The absorbance of the final yellowish solution was measured at 412 nm (Ellman *et al*., 1958; Ellman *et al*., 1959):



Acetylthiocholine iodide + H_2O $\xrightarrow{AChE}$ Thiocholine iodide + acetic acid (1)

Thiocholine + DTNB $\longrightarrow$ yellow color (2)

Enzyme inhibition: The degree of irreversible inhibition (I %) of the OP insecticide on the enzymatic activity of immobilized AChE was measured as a relative decrease in absorbance following a contact of the biosensor with paraoxon. The absorbance response I_0 of 1 mM ATChI (flow rate 0.4 ml/min) was first measured. After the system was washed with carrier buffer

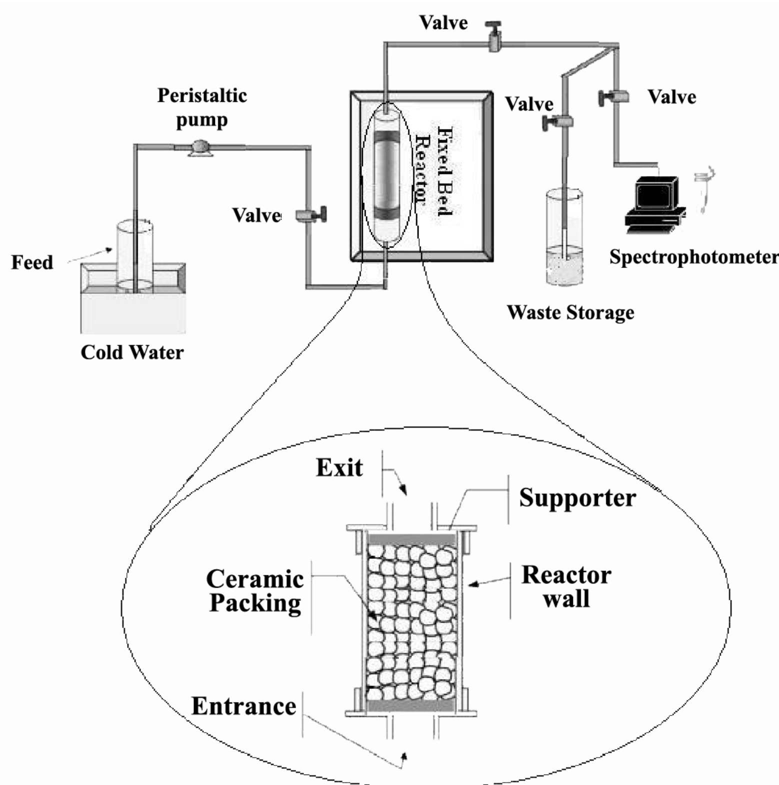


Figure 1. Schematic diagram of flow-through system and fixed bed bioreactor.

for 10 min, 0.1 ml of paraoxon solution was injected and stopped in the packed bed bioreactor for 14 min (as an appropriate incubation time) followed by washing with the carrier buffer for 15 min. The response of 1 mM ATChI was measured (I_f) again and the inhibition (I %) was calculated with Equation (3) and reported.

$$I\ (\%) = 100 \times (I_0 - I_f)/I_0 \quad (3)$$

RESULTS

Effect of enzyme level on absorbance response: The influence of enzyme loading on the packing surface was studied and the responses of the flow-through biosensor in presence of a 1 mM substrate concentration are shown in Figure 2. The response of the system increases with increasing the amount of the enzyme, first rapidly and then slowly. For a loading of 0.36 unit of the enzyme per cm^2 of packing surface, into the silicate-MWCNT sol the absorbance response intensity was almost as good as for 2.9 U/cm^2. Thus, AChE loading was set at 0.36 U/cm^2 for subsequent studies to conserve the enzyme while retaining comparable sensitivity.

Residence time in bioreactor: One of the main factors that affect the analytical performance of this flow-through system based on fixed bed bioreactor is its residence time in bioreactor. This work examined the effect of residence time in bioreactor on absorbance response in the range of 1.5 to 16 minutes. As shown in Figure 3 residence time in bioreactor has a direct effect on the reaction. With the increasing residence time in bioreactor the absorbance response increased in the range of 1.5 to 5 min and then levels off. Therefore, optimum value of absorbance occurred at the residence time in bioreactor of 5 min, and was chosen as appropriated value.

At lower residence time in bioreactor the time for substrate diffusion into the pores of the immobilized surface was not sufficient so the reaction was restricted to only surface layers of enzyme. Therefore the difference in behavior of the system towards different residence time in bioreactor can be explained by a diffusion rate of the substrate to the immobilized layer especially at low residence time in bioreactor and also possible slow reaction when less substrate is available.

The response of the flow-through biosensor to ATChI concentrations: Figure 4 represents the effect of ATChI concentration on absorbance response. In the immobilized layer of AChE and silicate-MWCNT sol-gel film, the enzyme catalyzes the hydrolysis of ATChI to thiocholine and acetic acid. Thus, ATChI was used as a substrate for the characterization of the enzyme (Andres *et al*., 1997). The ATChI concentration of 1 mM demonstrates the largest absorbance change when compared with 1.25 or 1.5 mM ATChI, therefore

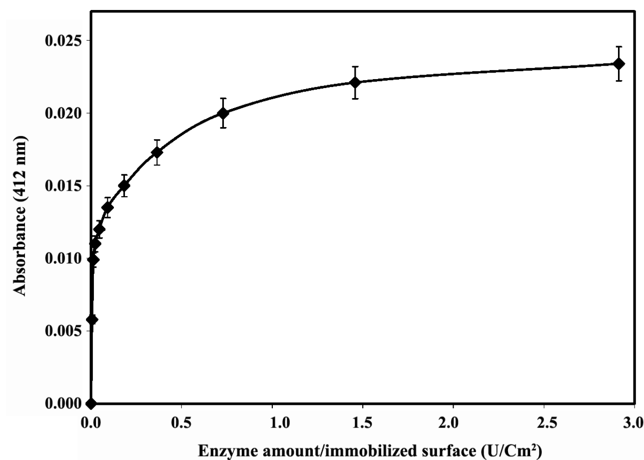


Figure 2. Effect of enzyme loading into silicate-MWCNT sol matrix on the absorbance response of the system.

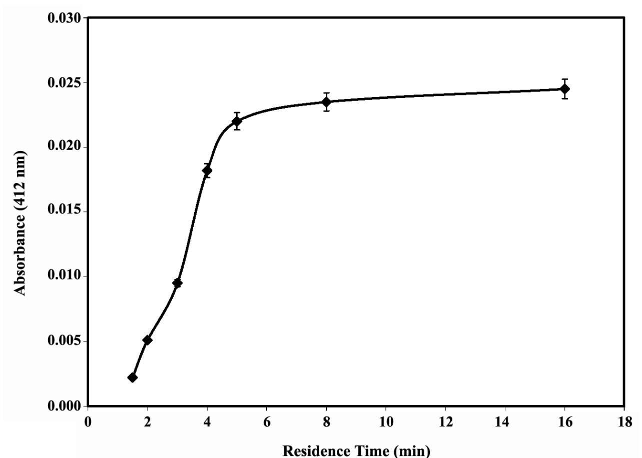


Figure 3. Effect of residence time in bioreactor on absorbance response of the flow-through system (ATChI concentration: 1 mM, Feed pH: 7.4).

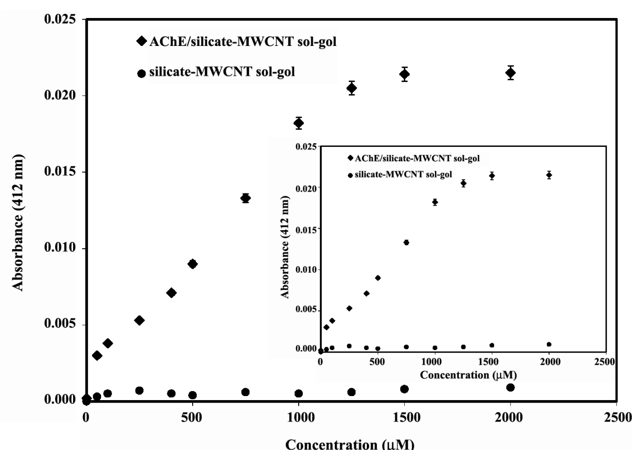


Figure 4. Response of the flow-through biosensor towards changes of ATChI substrate concentrations. (Residence time in bioreactor: 5 min, Feed pH: 7.4).

ATChI at 1 mM was selected for obtaining the maximum response. This leads to a highest change in the absorbance response as detected by the immobilized layer of AChE and silicate-MWCNT sol-gel film. In the absence of the AChE, the immobilized layer of silicate-MWCNT sol-gel film did not give obvious response to the changes of ATChI (Fig. 4). But when AChE is immobilized, the biosensor based on this flow-through system gave a linear response (R^2 = 0.9869) to ATChI at concentrations of 0.050 to 1 mM with a sensitivity slope of 2×10^{-5} absorbance change per micromole of ATChI (Fig. 4). In above 1.5 mM, the value of enzyme in reaction (1) will be saturate and with increase in ATChI concentration no further changes in absorbance can be observed. The difference in behavior of the biosensor towards different concentrations of substrate can be explained by a slower diffusion of the substrate to the sol-gel layer especially at low substrate concentrations (Jin *et al*., 2002) and also possible slow reaction kinetics in the alkoxysilane derived matrices when less substrate is available (Shen *et al*., 1997).

SEM data: The results of morphological studies conducted on enzyme/silicate-MWCNT sol are shown in Figure 5. The SEM of immobilized surface indicates that the film has a porous structure. This structure provided a significant increase of effective surface for enzymatic reaction in fixed bed bioreactor.

DISCUSSION

Reproducibility and stability of the flow-through system: At a concentration of 1 mM ATChI, the flow-through biosensor showed absorbance 0.0175 as a response and a quite good reproducibility. The flow-through biosensor prepared in the same batch demonstrated a standard deviation of 1.88% at 30 min after process initiation for the three successive assays. These values display better results compared with that reported by Vivek *et al*., as they managed to cut the result variance at the substrate concentration of 0.5 mM, i.e. the appropriate value for their work was reported to be 3.9%. Dan Du *et al*. (2007) reported a reproducibility of 4.1%. Mianhong Shi *et al*., making use of a flow injection system, reported a reproducibility of 4.92 %. The relative standard deviation (RSD) of assay were reported 3.3% by Dan Du *et al*. (2010).

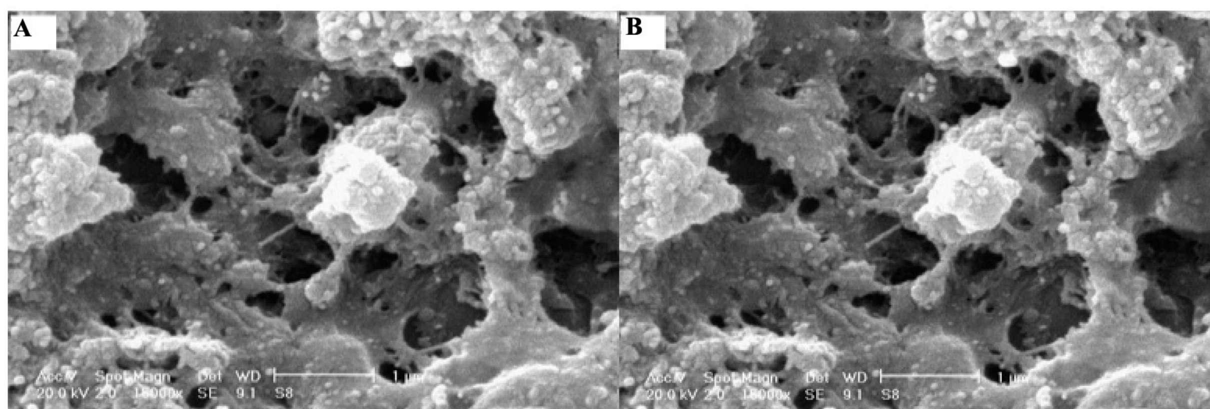


Figure 5. SEM images of enzyme encapsulated in silicate-MWCNT sol-gel on ceramic packing surface.

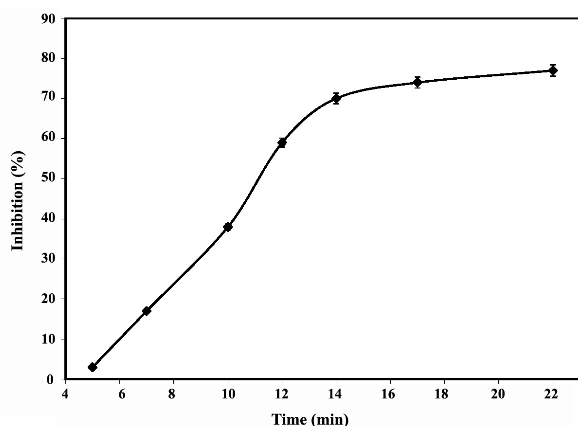


Figure 6. Effect of incubation time on inhibition of paraoxon.

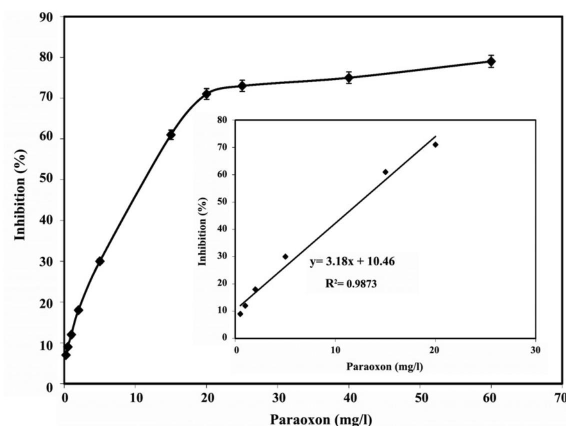


Figure 7. Inhibition profile of the flow- through system biosensor with various concentrations of paraoxon; A linear response range was obtained from 2 to 25 mg/l paraoxon (Residence time in bioreactor: 5 min, ATChI concentration: 1 mM, Feed pH: 7.4, Incubation time: 14 min).

When the packing of the flow-through system with immobilized enzyme was stored in the refrigerator at 4ºC, its response was stable in a 7 day-storage period, and then decreased to 89% after 25 days. When the flow-through system was preserved until 90 days it retained 79% of its initial absorbance response. The response decreased to 74% after 4 months, a stability that should be satisfactory for most applications. These results demonstrate that silicate-MWCNT sol film is very efficient for retaining the activity of AChE with a good long-term stability. This is attributed to the fact that the method provides a mild immobilization process. Specifically, the method does not involve any additive that results in chemical modification and fouling of the enzyme molecules. This allows the enzyme to maintain its activity to a large extent. Also, the sol-gel contains numerous hydroxyl groups which can form strong hydrogen bonds with acetylcholinesterase and silicate-MWCNT sol-gel cages for enzyme loading. As one of the best work, Dan Du *et al*. (2010) were reported 90% of its initial current response after 30 days storage period.

Determination of paraoxon using the flow-through system

Effect of inhibition time on response: One of the most influential parameters in pesticide analysis is inhibition time. With increase in incubation time in paraoxon solution, the absorbance response decreased greatly. Paraoxon displayed an increasing inhibition with increasing time. As shown in Figure 6, when the incubation time was longer than 14 min the curve trended to a stable value, indicating that binding interactions with active target groups in the enzyme were reaching saturation. This tendency of the absorbance response to decrease reflects a change in enzymatic activity, which decreases interactions with the substrate. Therefore, the change observed in the system can be used as an indicator for quantitative measurement of paraoxon pesticide. However, the maximum value of inhibition of paraoxon was not 100%, which is attributable to the binding equilibrium between pesticide and binding sites in the enzyme.

Inhibition curve: In order to show the usefulness of the AChE based flow-through system biosensor for the determination of paraoxon, the flow-through system biosensor was exposed to paraoxon concentration of 0.25 to 60 mg/l. Following an incubation time of 14 min, the calibration plot of inhibition degree versus pesticide concentration is shown in Figure 7. The inhibition effect of paraoxon on the immobilized enzyme was studied by recording absorbance response at constant conditions during a typical inhibition experiment. The response decreased with an increasing paraoxon concentration. The inhibition percent (I %) increased by 30% after inhibition with 5 mg/l paraoxon. At higher paraoxon concentration (60 mg/l), I % increased by up to 78%. The increase in I% increased with the increasing concentration of paraoxon. This was because paraoxon as one of the OP pesticides exhibited fairly high acute toxicity and involved in the irreversible inhibition action to AChE, thus reduced the enzymatic activity.

The inhibition of paraoxon was proportional to its concentration from 0.25 to 25 mg/l paraoxon. The linearization equation was inhibition (%) = $3.18x + 10.46$ (%), with the correlation coefficients of 0.9873 (Fig. 7). Measurements carried out for small analyte concentrations allow the estimation of the detection limit C_{DL},

$$C_{DL} = 3S_b / m \quad (4)$$

where S_b is the standard deviation and m is the sensitivity of the method (a change in the inhibition percent divided by the change in concentration) near the detection limit (Norouzi *et al*., 2007). The obtained detection limit was calculated to be 20 μg/l (7.3×10^{-8} M) for paraoxon. A further improvement of the lower limit can be achieved by increasing the inhibition time or reducing the AChE loading during the preparation of the packed bed bioreactor. Dan Du *et al*. (2010) were reported the detection limit 0.6 ng/ml for malathion.

CONCLUSION

Keeping in view of the performance of the biosensor, it can be reported that AChE has been successfully immobilized through a simple sol-gel technology which is taking minimal preparation time. Addition of MWCNT enhanced specific surface was observed. SEM images revealed a porous surface comprised of AChE encapsulated in silicate-MWCNT sol. The enzyme was well immobilized with in silicate-MWCNT sol matrix, retained satisfactory enzymatic catalytic activities. The present study suggest that the simple encapsulation procedure for the development of flow-through biosensor and changeable bioreactor is useful for OP detection. The developed biosensor showed rather stable and good responses towards substrate and can be applied to monitoring of other OPs compounds. The results showed that the detection limit for paraoxon was 7.3×10^{-8} M.

Acknowledgements

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References

Ajayan PM (1999). Nanotubes from carbon. *Chem Rev.* 99: 1787-1800.

Amine A, Mohammadi H, Bourais I, Palleschi G (2006). Biosensors for food safety and environmental monitoring. *Biosens Bioelectron*. 21: 1405-1423.

Andreescu S, Barthelmebs L, Marty JL (2002). Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides. *Anal Chim Acta*. 464: 171-180.

Andres RT, Narayanaswamy R (1997). Fibre-optic pesticide biosensor based on covalently immobilized acetylcholinesterase and thymol blue. *Talanta* 44: 1335-1352.

Aprea C, Colosio C, Mammone T, Minoia C, Maroni M (2002). Biological monitoring of pesticide exposure: a review of analytical methods. *J Chromatogr B*. 769: 191-219.

Baughrman RH, Zakhidov AA, Heer WA (2002). Carbon nanotubes the route toward applications. *Science.* 297: 787-792.

Beatriz P, Campàs M, Andreescu S, Marty JL (2006). Trends in flow-based biosensing systems for pesticide assessment. *Sensors* 6: 1161-1186.

Corcia AD, Marchetti M (1991). Multiresidue method for pesticides in drinking water using a graphitized carbon black cartridge extraction and liquid chromatographic analysis. *Anal Chem*. 63: 580-585.

Davis JJ, Coles RJ, Hill HAO (1997). Protein electrochemistry at carbon nanotube electrodes. *J Electroanal Chem*. 440: 279-282.

Diehl-Faxon J, Ghindilis AL, Atanasov P, Wilkins E (1996). Direct electron transfer based trienzyme electrode for monitoring of organophosphorus pesticides. *Sens Actuat B*. 36: 448- 457.

Doong RA, Tsai HC (2001). Immobilization and characterization of sol-gel-encapsulated acetylcholinesterase fiber-optic biosensor. *Anal Chim Acta*. 434: 239-246.

Du D, Chen S, Cai J, Zhang A (2007). Immobilization of acetylcholinesterase on gold nanoparticles embedded in sol-gel film for amperometric detection of organophosphorous insecticide. *Biosens Bioelectron*. 23: 130-134.

Du D, Wang M, Qin JY, Zhang A (2010). One-step synthesis of multiwalled carbon nanotubes-gold nanocomposites for fabricating amperometric acetylcholinesterase biosensor. *Sens Actuators B*: *Chem*. 143: 524-529.

Ellman G (1958). A colorimetrie method for determining low concentrations of mercaptans. *Arch Biochem Biophys.* 74: 443-450.

Ellman G (1959). Tissue sulfhydryl groups. *Arch Biochem Biophys*. 82: 70-77.

Gill I, Ballesteros A (2000). Bioencapsulation within synthetic polymers (Part 1): Sol-gel encapsulated biologicals. *Trends Biotechnol*. 15: 282-296.

Gorton L, Csöregi E, Domínguez E, Emnéus J, Jönsson-Pettersson G, Marko-Varga G, Persson B (1991). Selective detection in flow analysis based on the combination of immobilized enzymes and chemically modified electrodes. *Anal Chim Acta*. 250: 203-248.

Guodong L, Shawn L, Riechers M, Consuelo M, Lin Y (2005). Sensitive electrochemical detection of enzymatically generated thiocholine at carbon nanotube modified glassy carbon electrode. *Electrochem Commun*. 7: 1163-1169.

Huang W, Hu W, Song J (2003). Adsorptive stripping voltammet-

ric determination of 4-aminophenol at a single-wall carbon nanotubes film coated electrode. *Talanta* 61: 411- 416.

Iijima S (1991). Helical microtubules of graphitic carbon. *Nature* 354: 56-58.

Jin W, Brennan JD (2002).Properties and applications of proteins encapsulated within sol-gel derived materials *Anal Chim Acta*. 461: 1-36.

Lin Y, Guodong L, Wang J (2006). Biosensor based on self-assembling acetylcholinesterase on carbon nanotubes for flow injection/amperometric detection of organophosphate pesticides and nerve agents. *Anal Chem*. 78: 835-842.

Massoulie J, Pezzementi L, Bon S, Krejci E, Vallette FM (1993). Molecular and cellular biology of cholinesterases. *Prog Neurobiol*. 41: 31-91.

Mianhong S, Jingjing X, Song Z, Baohong L, Jilie K (2006). A mediator-free screen-printed amperometric biosensor for screening of organophosphorus pesticides with flow-injection analysis (FIA) system. *Talanta* 68: 1089-1095.

Norouzi P, Ganjali MR, Daneshgar P, Alizadeh T, Mohammadi A (2007). Development of fast Fourier transformation continuous cyclic voltammetry as a highly sensitive detection system for ultra trace monitoring of penicillin V. *Anal Biochem*. 360:175-181.

Odom TW, Huang JL, Kim P, Lieber CM (2000). Structure and electronic properties of carbon nanotubes. *J Phys Chem B*. 104: 2794-2809.

Pedano ML, Rivas GA (2004). Adsorption and electrooxidation of nucleic acids at carbon nanotubes paste electrodes. *Electrochem Commun*. 6: 10-16.

Puig D, Barce´lo D (1997). Determination of LSD in blood by capillary electrophoresis with laser-induced fluorescence detection. *J Chromatogr A*. 778: 313-319.

Quanlin W, Gongxuan L, Baojun Y (2004). Hydrogen peroxide biosensor based on direct electrochemistry of hemoglobin immobilized on carbon paste electrode by a silica sol-gel film. *Sens Actuators B Chem*. 99: 50-57.

Schmid RD, Künnecke W (1990). Flow injection analysis (FIA) based on enzymes or antibodies-applications in the life sciences. *J Biotechnol*. 14: 3-31.

Schmidt HL (1993). Biosensors and flow injection analysis in bioprocess control. *J Biotechnol*. 31: v-vi.

Shen CY, Kostic MN (1997). Kinetics of photoinduced electron-transfer reactions within sol-gel silica glass doped with zinc cytochrome study of electrostatic effects in confined liquids. *J Am Chem Soc*. 119: 1304-1312.

Solna R, Sapelnikova S, Skladal P, Winther-Nielsen M, Carlsson C, Emneus J, Ruzgas T (2005). Multienzyme electrochemical array sensor for determination of phenols and pesticides. *Talanta*. 65: 349-357.

Tran-Minh C (1996). Biosensors in flow-injection systems for biomedical analysis process and environmental monitoring. *J Molec Recogn*. 9: 658-663.

Valentini F, Oralanducci S, Terranova ML, Amine A, Palleschi G (2004). Carbon nanotubes as electrode materials for the assembling of new electrochemical biosensors. *Sens Actuators B*. 100: 117-125.

Vivek B, Kandimalla L, Huangxian J (2006). Binding of acetylcholinesterase to multiwall carbon nanotube-cross-linked chitosan composite for flow-Injection amperometric detection of an organophosphorous insecticide. *Euro J Chem*. 12: 1074-1080.

Wang J, Li M, Shi Z, Li N, Gu Z (2002). Direct electrochemistry of cytochrome at a glassy carbon electrode modified with single-wall carbon nanotubes. *Anal Chem.* 74: 1993-1997.

Wissiack R, Rosenberg E, Grasserbauer M (2000). Comparison of different sorbent materials for on-line solid-phase extraction with liquid chromatography-atmospheric pressure chemical ionization mass spectrometry of phenols. *J Chromatogr A*. 896: 159-170.