Chlorpyrifos Detection from Blood Matrix using Gold Nanoparticle based AChE –MWCNT Modified Graphite Electrode for the Forensic Applications

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ABSTRACT: Chlorpyrifos detection by a nanomaterial based electrochemical method from blood matrix is presented. Amperometric detection was performed using a graphite electrode covered with gold nanoparticles, multiwall carbon nanotubes, and acetylcholine esterase enzyme (Au/MWCNT/AChE) for greater electrochemical activity towards the oxidation of thiocholine compared to other conventional electrodes. The gold electrodeposition on graphite electrode leads to the enhancement of electrical signal. Scanning electron microscopy was used for the study of the distribution of DMF, TEOS, PDDA and AChE treated MWCNT. The range of amperage of detection of the chlorpyrifos was 2.11-15.89 μA with a range of detection of 0.01-1.00 ng/mL. This method gives a scope for the detection of chlorpyrifos in the real samples such as blood for monitoring toxicity levels for the forensic applications in cases of poisoning due to suicide, homicide, accidents, food toxicity and chemical warfare.

Keywords: chlorpyrifos, MWCNT, gold nanoparticle, graphite electrode, acetylcholine esterase

Introduction

Organophosphorus have been misused for committing suicides, homicides and also been used as chemical warfare agents. For these reasons, these compounds have become very significant for forensic scientists. Low level detection of organophosphorus compounds in the real samples like blood, urine and saliva is much required for monitoring the toxicity levels and detoxification processes.

Chlorpyrifos (*O, O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) shown in Figure 1, belongs to the organophosphorus class of pesticides, serving as an insecticide and acaricide.

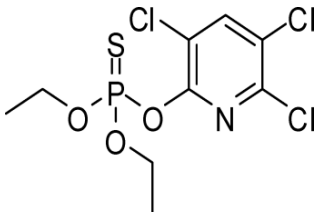

Figure 1: Structure of Chlorpyrifos (Chemical formula $C_9H_{11}C_{l3}NO_3PS$; Molecular weight 350.57)

The primary mechanism of action for the toxic effects of chlorpyrifos in human is related to the ability of the oxon metabolite of chlorpyrifos to bind to and irreversibly inhibit acetyl cholinesterase (AChE) in target tissues. The nervous system is the primary target because AChE and/or butlycholinesterase (BuChE) catabolyze the neurotransmitter acetylcholine, thereby terminating its synaptic function.

Like most organophosphorus pesticides, chlorpyrifos is oxidized to its oxon form, chlorpyrifos-oxon which is generally regarded as the principal toxic metabolite, and is responsible for inhibition of cholinesterases. Chlorpyrifos-oxon is either enzymatically or spontaneously hydrolyzed to form the diethylphosphate and 3,5,6-trichloro-2-pyridinol (TCPy). In addition to the formation of chlorpyrifos-oxon, chlorpyrifos is oxidized via cytochrome(s) P-450 to an unstable intermediate that spontaneously hydrolyses to form diethylthiophosphate and TCPy. The chlorpyrifos biotransformation is illustrated in Figure 2.These metabolites are excreted in the urine, or form glucuronide and sulfate conjugates, which are also excreted in the urine [1].

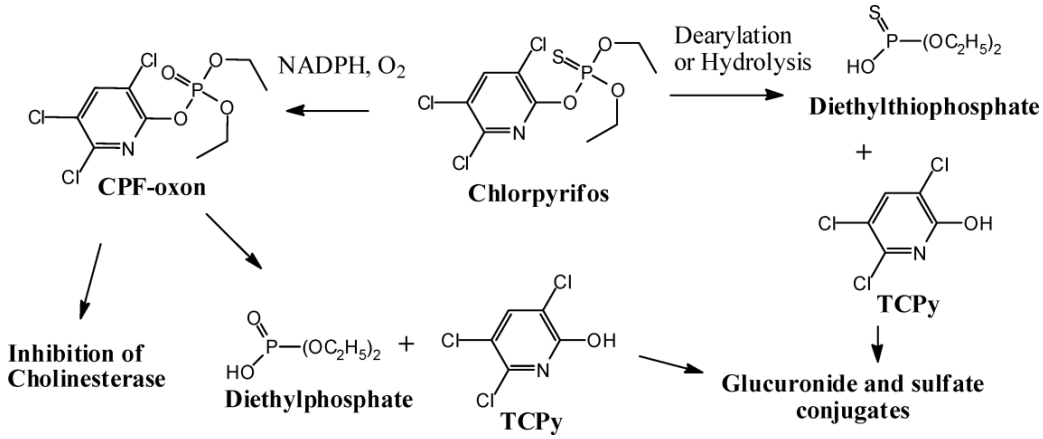

Figure 2: Biotransformation of chlorpyrifos in the body

Urine and blood (e.g., serum, plasma, blood cells) are the two primary matrices used to assess human exposure to pesticides [2, 3]. Blood samples are preferred because the measurements are usually more specific for pesticides because the parent chemical is generally measured, as opposed to its metabolite. Note that TCPy, a metabolic breakdown product of chlorpyrifos is an inadequate biomarker for chlorpyrifos exposure in non-occupational settings because chlorpyrifos-methyl also is converted to TCPy.

Chlorpyrifos appears to be relatively well absorbed from the intestine. In a study conducted using human volunteers, study involving a single oral dose of 0.5 mg/kg body weight, has recorded that peak blood chlorpyrifos levels were less than 30 ng/ml, and represented only a fraction of the levels of TCPy [4]. Only two studies have attempted to measure chlorpyrifos directly in blood following controlled exposures to humans [4, 5]. In a study carried out by Nolan et.al, six human volunteers were administered with 0.5 mg/kg of chlorpyrifos orally, and blood and urine samples were collected over a period of four days to obtain pharmacokinetic data [4]. The authors noted that chlorpyrifos concentrations in blood were "very low" (<30 ng/g, or 30,000 pg/g), despite high value of limit of detection (30 ppb), relative to today's methods.

In the second controlled human exposure study that measured chlorpyrifos in blood, Thimchalk *et. al.* administered single oral doses of chlorpyrifos of 0, 0.5, 1, or 2 mg/kg to six male and six female volunteers [5]. Blood samples were collected and analyzed for chlorpyrifos and TCPy at 2, 4, 8, 12, 24, 36, 48, 72, 96, 122, and 168 hour rate. Five out of 12 individuals had measurable chlorpyrifos concentrations in blood, but again, the limit of detection, although much better than that of [4] was quite high (3 mmol/L, which is equivalent to 1 ng/mL, or 1 ppb) and not adequate to measure CPF in the blood for more than 2 h following the highest dose. Thus, based on these two studies [4,5], it appears that the initial half-life of chlorpyrifos is quite short—probably only an hour or less—but the half-life of TCPy is much longer, on the order of 24–27 h.

At least at relatively high doses, chlorpyrifos is quickly metabolized to chlorpyrifos-oxon and TCPy, with an apparent half-life of 1 h or less, although some fraction of chlorpyrifos may have a relatively longer elimination rate due to extensive plasma protein binding and/or partitioning into lipids. Very few studies have attempted to measure chlorpyrifos in blood, and only one of the birth cohort studies, the Columbia cohort, has utilized blood chlorpyrifos as a biomarker of exposure. However, because of the limitations noted earlier in using TCPy as a specific biomarker of chlorpyrifos exposure, blood chlorpyrifos could be of potentially greater value. In contrast, the method developed by Barr *et al*. [6] is highly sensitive, with detection limit of more than 1000 times lower than that used by Thimchalk *et. al.* [5]. Thus, it is now possible to carefully evaluate the kinetics of chlorpyrifos in blood over time following repeated, relatively low-dose, exposures (e.g., μg/day seen from diet, vs. mg/day used in some studies [4, 5]). Chlorpyrifos has been measured in human whole blood, at concentrations as low as 10 ppb by Drevenkar *et al.* [7]. Blood chlorpyrifos levels were low (≤ 30 ng/mL;

limit of detection 5 ng/mL), and chlorpyrifos was not found in the urine following either route of exposure.

Detection of organiphosphorus using traditional methods, such as gas chromatography (GC) [8], liquid chromatography (LC) [9], gas chromatography–mass spectrometry (GC–MS) coupled method [10], ion mobility spectroscopy [11], atomic emission detection (AED) [12], Fourier transform infrared (FTIR) spectroscopy [13], Raman spectroscopy [14], and capillary electrophoresis (CE) [15], possess very high sensitivity, reliability, and precision. However these techniques pose significant challenges in the field application due to the need of expensive equipments, nonportable, require highly trained technicians, as well as time-consuming analytical processes. To meet the demand for on-site monitoring of organophosphorus compounds from body fluids, devices with low power consumption, low-cost, high efficiency, and portability are highly desirable.

In this article, multiwalled carbon annotates , MWCNT modified with PDDA , TEOS and AchE was used on a gold nanoparticle electro deposited graphite electrode for the determination of Chlorpyriphos .The performance of gold nanoparticle in combination with MWCNT ,AChE has shown high selectivity, linear range and good selectivity which is reported in the present paper. While the window of detection is short, hours to a few days due to the very nature of chlorpyrifos, sensitivity and metabolic conditions, direct analysis have significant advantages.

Experimental

Chemicals, reagents & samples

Acetyl choline Esterase (AchE), Acetyl choline chloride, Poly diallyl dimethyl ammonium chloride (PDDA), Chlorpyriphos , Gold (III) chloride trihydrate (HAuCl4.3H2O); Sodium citrate, Dimethyl formamide and, Tetraethyl orthosilicate (TEOS) were purchased from Sigma Aldrich and used with no further purification. Other chemicals used were Na2HPO4*12H2O, NaH2PO4*H2O, Ethanol. NaOH, and NaCl.

Chlorpyriphos (4.0, 3.5, 3, 2.5, 2, 1.5 and 1 μM) were prepared in PBS buffer.

1.0 mM Acetyl choline chloride in PBS pH-6.8 and 1 mg/mL PDDA (Poly diallyl dimethyl ammonium chloride) in 0.5M NaCl were prepared.

0.1 M Sodium phosphate buffer pH-6.8,1M NaOH was used to adjust the pH,0.5M NaCl.

Multiwalled carbon nanotubes (MWCNT) were synthesized in Amity Institute of Nanotechnology using Chemical Vapour Deposition (CVD) technique.

Instruments and measurements

In the electrochemical voltammetry, a homemade cell assembly with Graphite electrode, i.e., Ag/AgCl electrode as reference, platinum electrode (Counter) and a Teflon coated beaker was used. The assembly was connected to Electrometer (Model 6517 A-Keithly) and the amperage was set with a range of 2μA-200 μA. The potential was set with a range of 100mV-1000mV. All measurements were taken at room temperature. The model was used for amperage measurements for all the substrates at various concentrations.

UV-VIS Spectrophotometer (Schimadzu, Japan), Dynamic Light Scattering (DLS) and Scanning Electron Microscope (EVO 18, ZIESS) were used for the characterization of gold nanoparticles and MWCNTs.

Preparation of standard solutions and samples for analysis

The chlorpyrifos stock solution of 1000 μg/mL and 25 μg/mL concentration in acetonitrile was obtained. Gradual dilution of the stock solution was carried out to prepare the standard solutions of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250, 500 and 1000 ng/mL.

Left over infection free heparinized blood samples were collected from departmental pathology laboratories under requisition. Firstly, stock solution was prepared for chlorpyrifos. 20μL of the prepared standards was added to 1 mL serum and 1 ml of PBS containing 1.0mM acetylthiocholine chloride resulting in the following concentration series:0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.00 ng/mL chlorpyrifos in serum . A mixture of internal standard of chlorpyrifos was dissolved in acetonitrile at a concentration of approximately 0.25 ng/mL. This level was selected because chlorpyrifos is well detectable at this concentration. Blood

samples were analyzed for the concentration of chlorpyrifos.

Synthesis of Au Nano Particles

An aqueous HAuCl4.3H2O solution at 0.002 M concentration was prepared by dissolving 0.0787g of HAuCL4 in 100 ml of distilled water). The solution was stirred with magnetic beads in round bottom flask attached to a rotamantle for 15 minutes at 80°C.

Sodium citrate solution of 0.01 M concentration was prepared by dissolving 0.0294g Sodium citrate in100 ml of Distilled water. The solution was added to the HAuCl4.3H2O solutions and the mixture was stirred for 30 minutes at 80°C. The clear solution turned in to ruby red colour indicating the formation of gold naoparticles. The formation of gold nanoparticles were confirmed using UV-VIS Spectrophotometry and Dynamic Light Scattering analysis.

Functionalization of MWCNT

Multiwalled carbon nanotubes were prepared by catalyzed CVD method. The 10mg crude MWCNTs were stirred in 2ml of 3M Nitric Acid and refluxed for 24 Hrs at 60°C.They were suspended and again refluxed in 2ml of 5 M HCl solution for 6h at 120° C. After acid treatment, the samples were calcined in static air at 510°C for 60 min, and the pure MWNTs were obtained.

For fictionalization of MWCNT, following steps were followed:

i) Purified 2mg of Nanopowder of Multiwalled Carbon Nanotube was mixed in 1ml of Dimethyl formamide and stored at 4 degree celsious.
ii) 0.59ml MWCNT solution was mixed with 2.84 ml of Tetraethyl orthosilicate (TEOS).
iii) 0.5ml of the resulting solution was mixed in 0.6 ml of distilled water and 0.1ml 5mM.NaoH and stirred for 20 minutes at room temperature.
iv) 6ml of Ethanol was added to the resultant solution and stirred for 10 minutes and cooled to 4°C

Immobilsation of AChE on MWCNT

10 µL of 0.2 unit ml-1 AchE (Acetyl choline Esterase) in Phsphate buffer pH-6.8 was mixed with 10 µL of functionalized MWCNT (AchE: MWCNT; 1:1)

Electrodeposition of Au nanoparticles on graphite electrode

Electrodeposition of Au Nanoparticles was done by subjecting the graphite electrode in Au Np solution at -3.0V for 250 seconds.

Designing of MWCNT modified Graphite electrode

About 20 µL of the AChE immobilized MWCNTs were casted on the Graphite Electrode (GE) surface by drop wise on electrode and dried. Negative charges were introduced above this MWCNTs modified GE by dipping in 1 M NaOH solution for 5 min, followed by washing with distilled water twice.

This negatively charged AChE/MWCNTs/GE was dipped into an aqueous solution of 1mg·mL-1 PDDA (Poly diallyl dimethylammonium chloride) containing 0.5 M NaCl for 20 min, which lead to the adsorption of positively charged polycation layer. The layer-by-layer electrostatic self-assembly of Immobilized AChE on multiwalled carbon nanotubes (MWCNTs) modified GE was made. Above this polycation layer, a negatively charged AChE layer was absorbed by dipping the PDDA/MWCNTs/CE in 0.2 unit·mL-1 AChE in Sodium phosphate buffer solution pH 6.8. In order to prevent the leakage of AChE from the electrode surface, another PDDA layer was adsorbed above this AChE layer. This resulted in a sandwich-like structure of PDDA/AChE/PDDA/ on the AChE/MWCNT surface.Voltammetry was employed using Electrometer to study the electro oxidation of thiocholine and its inhibition by pesticides. The amperometric results were used to reveal the response of thiocholine at PDDA/AChE/PDDA/AChE/ MWCNT/AuNP/GE.

Amperometry in µA against different concentrations of chlorpyrifos

Before addition of chlorpyrifos; the electrode in presence of 1.0 mM acetylthiocholine chloride in PBS, pH 6.8 showed an oxidation peak at 800 mV which was resultant of the oxidation of thiocholine, hydrolysis product of acetylcholine chloride catalyzed by the immobilized AChE

Results and discussion

Samples for scanning electron microscopy were prepared by dropping a small amount of purified MWCNT solution on a mica substrate and allowed to dry overnight. Gold nanoparticles and MWCNTs observed under SEM were shown in Figures 3 and 4.

The formation of gold nanoparticles was confirmed by UV-VIS spectrophotometry analysis with the peak at 509 nm, Figure 5. Table 1 shows DLS readings.

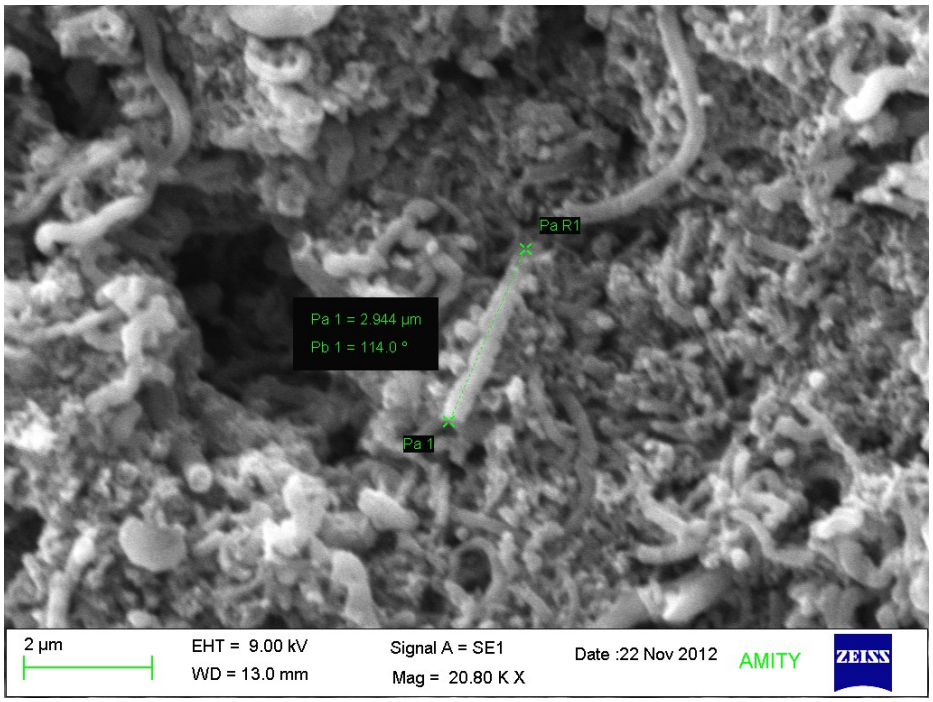

Figure 3: SEM Images of AChE immobilized DMF-TEOS treated MWCNT

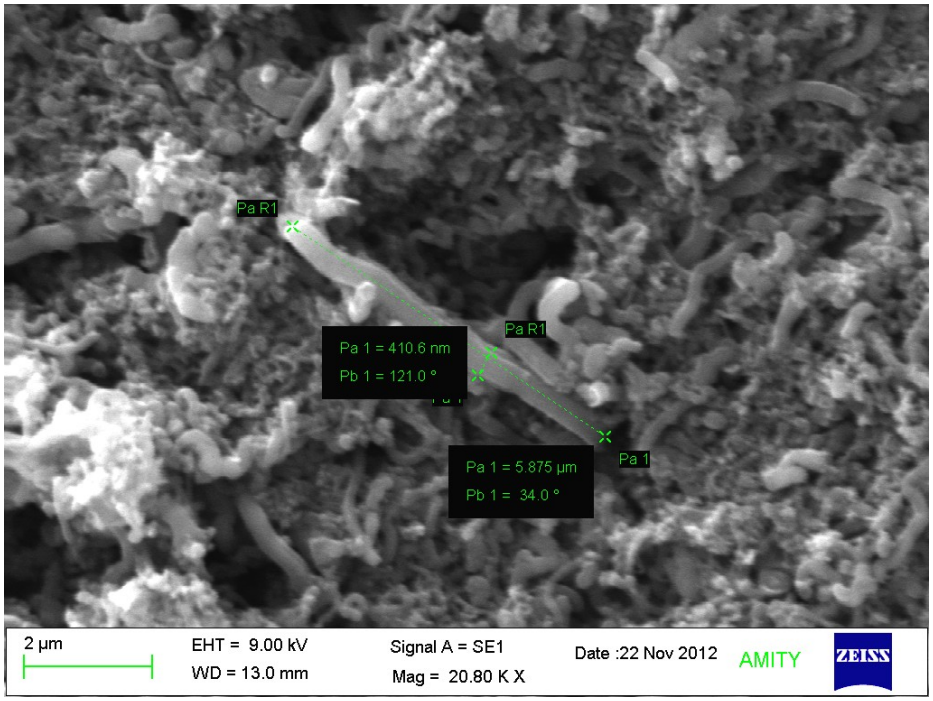

Figure 4: SEM Images of PDDA applied AChE/ MWCNT

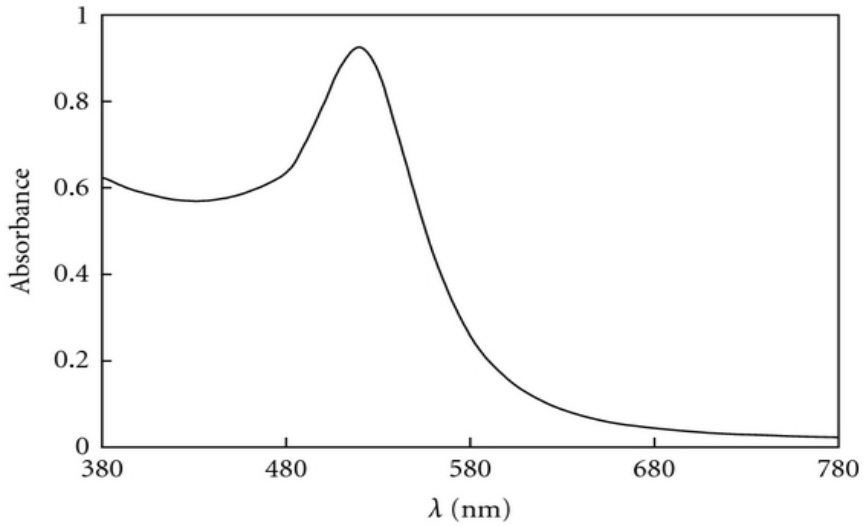

Figure 5: UV-Visible spectrum of colloidal Au nanoparticle solution (509nm)

Table 1: DLS readings (Particle size – 5037 nm (average of radius) Pdi-polydispersity Index=0.361)

Peak	Size	Intensity	Width
Peak 1	101.9nm	51.0%	33.52
Peak 2	16.99	42.5%	5.50
Peak 3	0.5665	2.9%	0.16

The current measurements (μA) for chlorpyriphos in blood ranging 2.11-15.89 μA, using PDDA/AChE/PDDA/MWCNT/AuNP/EC on added 20μL of standards in 1 mL of blood and 1 mL of PBS having 1.0mM acetylthiocholine chloride, pH 6.80 with the chlopyrifos concentration of .010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.00 ng/mL in the potential range of 100mV-1000mV are shown in Table 2. Graphical representation of current (μA) vs Chlorpyrifos in blood concentration (ng/ml) is shown in Figure 6.

Table 2: Current measured in μA for chlopyrifos of 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.00 ng/mL in the potential range of 100mV-1000mV

No	Potential/ mV	Current-μA /Concentration-ng/ml							Time/s
		1.00 ng/ml	0.500ng/ ml	0.250n g/ml	0.100n g/ml	0.050n g/ml	0.025ng/ ml	0.010ng /ml	
1	100	9.45	10.76	11.23	11.87	12.48	13.67	15.89	60
2	200	8.56	9.65	10.55	11.42	12.45	13.01	14.72	80
3	300	7.99	8.11	9.49	10.59	11.62	12.31	13.32	100
4	400	6.33	7.01	8.31	9.23	10.52	11.31	12.21	120
5	500	5.01	6.23	7.40	8.41	9.31	10.21	11.09	140
6	600	4.34	5.21	6.21	7.76	8.63	9.11	10.60	160
7	700	3.81	4.22	6.04	7.82	8.87	9.17	10.58	180
8	800	2.11	3.42	4.32	5.91	6.13	7.51	8.45	200

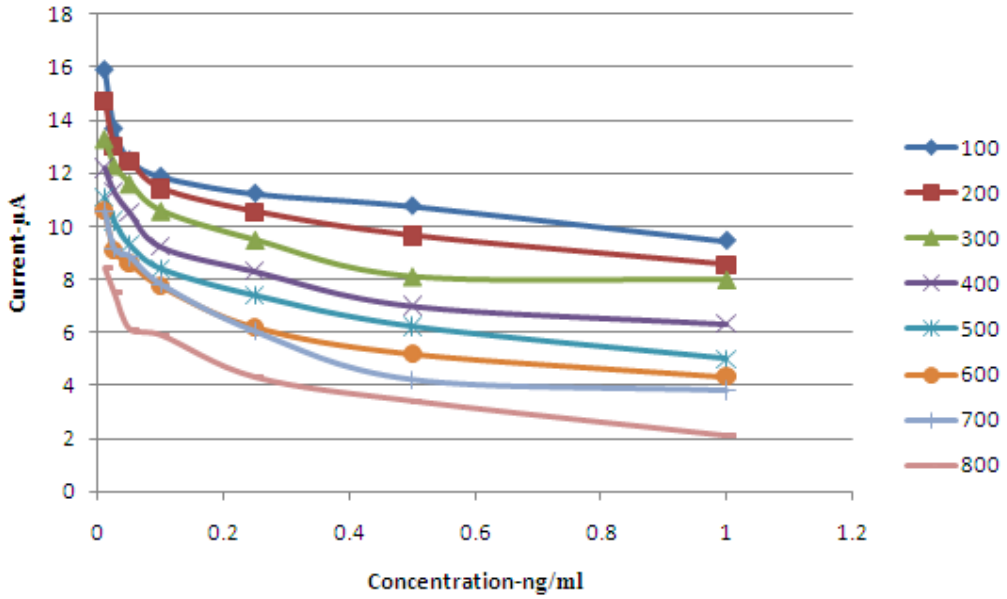

Figure 6: Graphical representation of current (µA) vs concentration (ng/ml) for Chlorpyriphos

PDDA/AChE/PDDA/AChE/MWCNT/AuNP/ GE exhibits a significant higher current that enables the detection of ng/ml concentrations of chlorpyrifos from blood. Seven different concentrations of chlorpyrifos were added in to the serum and PBS containing in different Teflon beakers. The current response peak for the increased levels of chlorpyrifos was distinctly visible. Well distinct current signal was observed for each of these concentrations (ng/mL) in the chlorpyrifos. The response current decreased linearly with the increase of the chlorpyrifos concentration. A comparison of the analytical parameters obtained at the modified electrode with those currently available methodologies for the detection of chlorpyrifos pesticide suggests the present approach has better sensitivity, stability for different ranges of concentrations.

Conclusions

A gold nanoparticle based electrochemical method is developed for the fast determination of chlorpyrifos in blood. PDDA/AChE/PDDA/AChE/MWCNT/AuNP/ GE modified electrode exhibits great electrochemical activity towards the oxidation of thiocholine. In addition PDDA/AChE/PDDA/AChE/MWCNT/AuNP/ GE modified electrode can be easily fabricated and used as a sensor for routine analysis. The enhancement of detection is mainly due to the greater surface area of the deposited MWCNT/AuNP. The advantage of the proposed method over existing technologies include its simplicity, low cost, low consumption of reagents, easy operation and transportable device combined with good sensitivity. It is a specific method. The compounds of interests are directly analyzed, on a regulated fluid, i.e the blood, so subsequent concentration correction was not necessary. This excludes the otherwise always present question of the source of the secondary biomarker, such as "is it exposure to chlorpyrifos or exposure to environmental degradants?" As a conclusion, this method provides a scope for the detection of chlorpyrifos in the real samples like blood, for monitoring the toxicity levels for the forensic applications in cases of poisoning due to suicide, homicide, accidents, food toxicity and chemical warfare.

References

1. David L. Eaton et.al. (2008). Review of the Toxicology of Chlorpyrifos with an Emphasis on Human Exposure and Neurodevelopment; *Critical Reviews in Toxicology,* Informa UK Ltd., S2,1–125.
2. Angerer J, Gundel J. (1996). Biomonitoring and occupational medicine. Possibilities and limitations. *Ann Ist Super Sanita*. 32,199–206.
3. Needham LL, Sexton K. (2000). Assessing children's exposure to hazardous environmental chemicals: an overview of selected research challenges

and complexities. *J Expo Anal Environ Epidemiol*.10, 611–629.
4. Nolan, R.J., Rick, D.L., Freshour, N.L., Saunders, J.H. (1984). Chlorpyrifos: Pharmacokinetics in human volunteers. *Toxicol. Appl.Pharmacol.* 73,8–15.
5. Timchalk, C., Nolan, R.J., Mendrala, A.L., Dittenber, D.A., Brzak,K.A., Mattsson, J.L. (2002b). A Physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the Organophosphate insecticide chlorpyrifos in rats and humans. *Toxicol. Sci.* 66,34–53.
6. Barr, D.B., Barr, J.R., Maggio,V.L., Whitehead, R.D., Sadowski, M.A., Whyatt, R.M., Needham, L.L. (2002). A multi-analyte method for the quantification of contemporary pesticides in human serum and plasma using high-resolution mass spectrometry. *J. Chromatogr. B.* 778,99–111.
7. V. Drevenkar, B. Stengl, Z. Fröbe. (1994). Microanalysis of dialkylphosphorus metabolites of organophosphorus pesticides in human blood by capillary gas chromatography and by phosphorus-selective and ion trap detection, *Analytica Chimica Ac*ta, 290 (3), 277–286
8. C.E. Kientz, (1998). Chromatography and mass spectrometry of chemical warfare agents, toxins and related compounds: state of the art and future prospects, *J. Chromatogr. A*. 814,1–23.
9. E.W.J. Hooijschuur, C.E. Kientz, U.A.T. Brinkman. (2001). Application of microcolumn liquid chromatography and capillary electrophoresis with flame photometric detection for the screening of degradation products of chemical warfare agents in water and soil, *J. Chromatogr. A*. 928,187–199.
10. G.L. Hook, G. Kimm, D. Koch, P.B. Savage, B. Ding, P.A. Smith. (2003) Detection of VX contamination in soil through solid-phase microextraction sampling and gas chromatography/mass spectrometry of the VX degradation product bis(diisopropylaminoethyl)disulfide, *J. Chromatogr. A.* 992,1–9.
11. M. Utriainen, E. Kärpänoja, H. Paakkanen. (2003). Combining miniaturized ion mobility spectrometer and metal oxide gas sensor for the fast detection of toxic chemical vapors, *Sens. Actuators B*. 93,17–24.
12. W.R. Creasy, A.A. Rodriguez, J.R. Stuff, R.W.Warren. (1995). Atomic emission detection for the quantitation of trimethylsilyl derivatives of chemical-warfare-agent related compounds in environmental samples, *J. Chromatogr. A.* 709,333–344.
13. C.S. Kim, R.J. Lad, C.P. Tripp. (2001). Interaction of organophosphorous compounds with TiO2 and WO3 surfaces probed by vibrational spectroscopy, *Sens. Actuators B.* 76,442–448.
14. F. Yan, T. Vo-Dinh. (2007). Surface-enhanced Raman scattering detection of chemical and biological agents using a portable Raman integrated tunable sensor, *Sens. Actuators B.* 121,61–66.
15. M. Pumera. (2006). Analysis of nerve agents using capillary electrophoresis and laboratory-on-a-chip technology, *J. Chromatogr. A*. 1113,5–13.

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