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Voltammetric detection of Monocrotophos from Blood Serum by Enzyme Immobilized Gold Nanoparticles deposited on Graphite Electrode

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Abstract: In this study a working electrode was constructed by immobilization of Acetyl choline esterase, on gold nanoparticles synthesized by electrochemical deposition method on synthetic graphite, using cross linker, gluteraldehyde and 2-Aminoethane thiol for the determination of monocrotophos, an organophosphorus pesticide in blood serum by inhibition of AChE using Cyclic voltammetry. The monocrotophos concentration response was linear in the range from 0.05mgL^{-1} to 3.2mg L$^{-1.}$The detection limit for Monocrotophos was 0.05mg L$^{-1.}$ The results of the present study based on synthetic graphite which being a better conducting electrode offers as a good alternative to other conventional methods as it is economical and with better sensitivity for the detection of analytes like organophosphorus compounds from body fluids for the forensic science related works as the other methods like Gas chromatography/mass spectrometry are costly and time consuming.. The resultant modified graphite electrode surface was characterized using Scanning electron microscopy, EDX and AFM. Immobilization of AChE on AuNPs/Graphite composite is a new approach. The electrodes based on graphite and AuNPs being economical, good conductor offers as a better option for the sensitive electrochemical detection of organophosphorus compounds from body fluids in forensic science scenario in cases of murder, suicide, accidental poisoning, and chemical warfare.

Key words: Acetylcholine esterase, Gold nanoparticles, Synthetic graphite, Immobilization, Organophosphorus pesticide, Forensic Science.

1. Introduction:

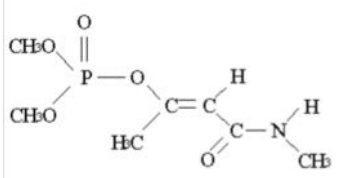


Fig.1. Structure of monocrotophos

Monocrotophos (Empirical Formula;$C_7H_{14}NO_5P$) is an organophosphorus pesticide (Fig.1).It is acutely and chronically toxic to humans. It is a highly hazardous compound as per the EC (No) 1272/2008 classification, and has been responsible for deaths resulting from intentional exposures as in cases of murder,

chemical warfare, mass genocide, suicidal attempt and accidental exposure. Monocrotophos is highly toxic through oral administration, inhalation and absorption through skin. It is a neurotoxin and its toxicity is associated with its capacity to irreversibly inhibit the activity of Acetyl choline esterase in the central and peripheral nervous system,resulting in the accumulation of the neurotransmitter,acetylthiocholine in the nerve junctions which interfere with causes respiratory and cardiac paralysis resulting to death.[1]

Acetyl choline esterase hydrolyses the acetylthiocholine in nerve junctions to choline and acetic acid.[2] Choline formation normally being used for the resynthesis of AChE.[3]In the presence of monocrotophos, the serine nucleophilic hydroxyl group present in the enzyme active site covalently bounds with the phosphorus moiety of pesticide, thus inactivate the enzyme[4] a rapid accurate dermination of organophosphorus pesticides are of crucial importance especially in field analysis and applications.Various conventional analytical techniques such as Gas chromatography coupled with mass spectrometry (GC/MS), Liquid chromatography coupled with mass spectrometry are time consuming, expensive and have to be analyzed by highly skilled technicians and involve extensive time consumption and complicated pretreatment procedure and not suitable for rapid analysis under field conditions, especially in emergency conditions and autopsy examinations of dead bodies.

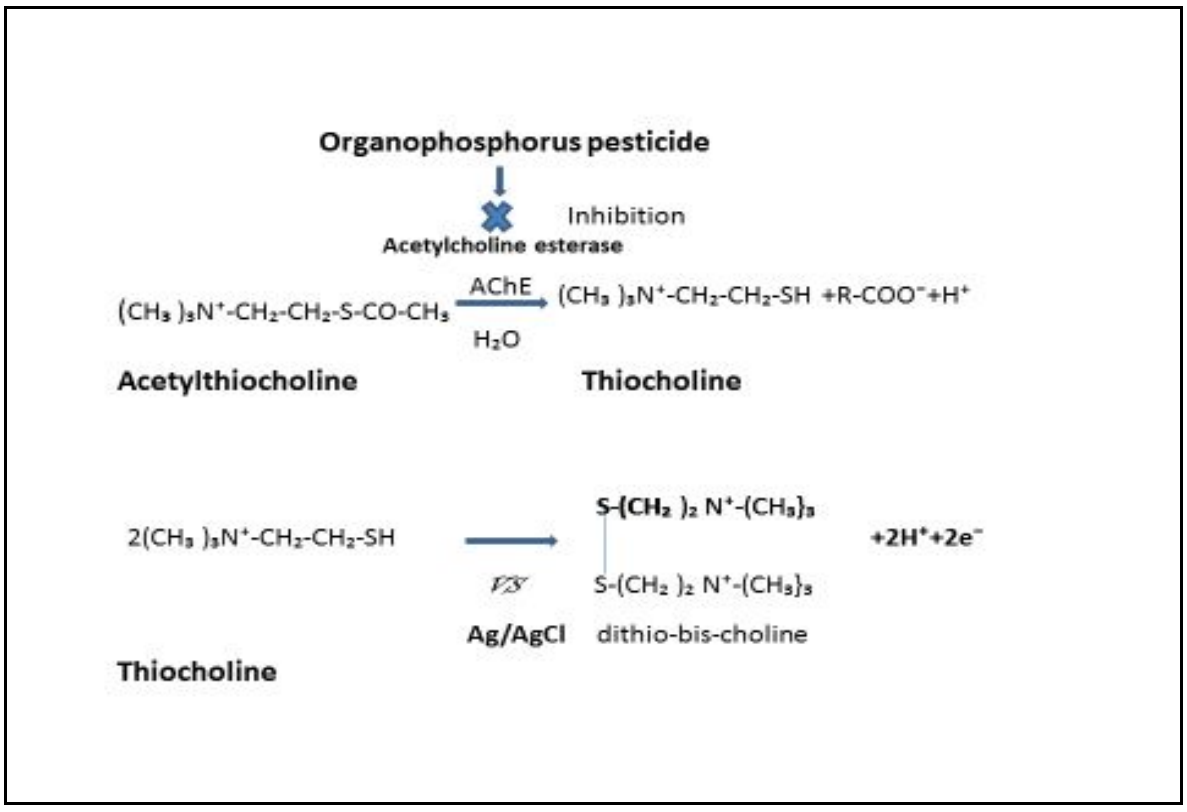


Fig.2 Fundamental concept for the electrochemical analysis using AChE modified Au/GE

Inhibition of Acetyl choline esterase by pesticides like monocrotophos (Fig.2) is the fundamental principle of detection methods of organophosphorus pesticides in immune assays and electrochemical methods [5-7].Recent research trend shows experimenting with different matrices with different designs in analytical electrochemistry to fabricate sensors for various analytes of interest by focusing on characteristics like specificity, sensitivity, reliability, ease of designing and low cost. Immobilizations of biomolecules involving nanomaterials have been found to be a significant phase which directly influence the performance of the sensor. The properties of the nanomaterials is another important factor which strongly influences the performance of the sensor device[8]

Gold nanoparticles have proven to be the best alternative for a stable immobilization of biomolecule by maintaining its bioactivity. Facilitation of electron transfer between redox enzymes/protein and electrode materials is one of the special property of gold nanoparticle which exclude the necessity of any electron transfer mediators. Large surface area, large surface to volume ratio, ability to reduce enzyme/protein-nanoparticle distance and its unique ability to function as a bridge of conduction of electron between enzymatic/protein

groups and electrode surfaces are the major reason for electron transfer between redox enzyme/protein and electrode surfaces.[9-10]

Among different studies already reported, one method involves the attachment of tyrosine by cross linking on Glassy carbon electrode electrodeposited with gold nanoparticle[11].A sensor was prepared by the covalent attachment of glucose oxidase on gold nanoparticle modified gold electrode[12].Another example is the construction of a xanthine oxidase based sensor which make use of carbon paste electrode modified with electrodeposited gold nanoparticle on to which the enzyme was attached through crosslinking through the gluteraldehyde with Bovine serum albumin[13]. Electrodeposition of gold nanoparticles onto a planar gold electrode was also used to attach Acetylcholine esterase [14]

Thiols function as modifying chemicals on gold nanoparticles which helps to immobilize the enzyme.[15]

Composite electrode matrices made after introducing nanoparticles form a better option for the fabrication of enzyme based sensors with better performance. A sensor based on graphite-Teflon composite matrix in which gold nanoparticles and enzyme, tyrosinase was developed [16] 2-amino ethane thiol was used as covalent attachment cross-linker for preparing multilayer films of glucose oxidase/gold nanoparticles on gold electrode by layer by layer technique.[17]

Immobilization of micro peroxidase was done by covalent bonding with gold nanoparticle attached on MWCNT forming a monohybrid film. The development of gold nanoparticle based sensors through immobilization of enzymes on it demonstrate the potential and performance by maintaining the biological activity of enzymes. Main focus in the fabrication and optimization of sensors was the immobilization of enzyme. Enzyme immobilization include methods like covalent attachment through gluteraldehyde and subsequent entrapping by different substrates. [18-19]

The often cited advantages of carbon electrodes include low cost, wide potential window, relative inert electrochemistry and electro catalytic activity for a variety of redox reactions. In the oxidations and reductions of organic and biological molecules in both aqueous and non-aqueous media, properties of carbon electrodes are often found to be superior to those of noble metals. The diversity of carbon as an electrode material stems largely from its structural polymorphism, chemical stability, rich surface chemistry, and strong carbon-carbon bonds present both internally and often between the carbon material and a surface modifier[20-23].

The choice of electrode material and surface preparation method are usually dictated by suitability of the electrode for observing an electrochemical parameter, such as heterogeneous electron transfer rate, surface coverage or redox potential.

Graphitic carbon materials commonly used as adsorbents have a high microscopic surface area and many oxygen containing functional groups. The high polarizability of graphite leads to relatively strong induced dipoles and the permanent dipoles associated with functional groups support dipole-dipole interaction with adsorbents. The ability of carbon to form strong covalent bonds with a variety of materials has been exploited extensively for surface modification.

The propensity of carbon to adsorb molecules from solution and the presence of surface oxides permit electro catalytic reactions on carbon electrodes that are weaker or absent on metal electrodes. A practically important issue related to surface reactivity is the available potential range of carbon electrode materials over which background reactions contribute negligibly to the observed current. The kinetics of surface oxidation and hydrogen evolution are significantly slower on carbon than most commonly used metal electrodes, and the resulting wide potential window is one reason for the widespread use of carbon materials for electrodes.

In this paper we describe a method for creating a sensing platform to achieve a simple reliable immobilization of AChE on gold nanoparticle electrochemically deposited on synthetic graphite and the use of the fabricated electrode for the detection of organophosphorus from spiked blood serum.

To the best of our knowledge AChE immobilization has not been attempted on AuNPs electrochemically deposited on synthetic graphite, till now for the electrochemical detection of organophosphorus pesticides or other analytes from body fluids. In the present study Acetylcholine esterase enzyme was immobilized on gold nanoparticles electrochemically synthesized on the surface of synthetic graphite .Their characterization was done using scanning electron microscopy, energy dispersive X-ray spectrometry and AFM. AChE immobilized AuNP deposited graphite electrode was used for the detection of

organophosphorus pesticide from spiked blood serumby cyclic voltammetry for the forensic science applications.

2 Material and Methods:

2.1 Chemicals and solutions

All reagents including Acetylcholine esterase, Acetylthiocholine chloride,gluteraldehyde, 2-aminoethanethiol ,$HAuCl_4.2H_2O$, $H_2SO_4$and Monocrotophoswere purchased from Sigma Aldrich which were of analytical grade and used without further purification.Graphite electrodes of 5.5cmx 3mm were purchased from local market in Delhi. The purity of the electrode was checked using EDX and was found to be 99.39%.

2.2 Equipments used:

Electrochemical measurements were carried out by using AutolabPotentiostat /Galvanostat, Model, AUT83945; PGSTAT302N .The conventional three electrode system was used with Ag/AgCl as reference electrode, platinum wire as auxiliary electrode and Graphite as working electrode. Silver paste was used to attach a copper wire to graphite electrode.The surface morphology of Bare Graphite electrode, gold deposited surface of graphite electrode and enzyme immobilized AuNP deposited graphite electrode was studied using Scanning Electron Microscopy (SEM-CARL ZEISS EVO40) and Atomic Force Microscopy(Solver-Pro).Elemental analysis of the bare Graphite electrode and Gold electrodeposited graphite electrode was performed using EDX(Bruker AXS Microanalysis ,GmbH Berlin, Germany) attached to SEM.

2.3 Fabrication of Au Nanoparticle modified Graphite electrode

A graphite electrode of 5.5cmx 3mm was cleaned and was sonicated in a mixture of H_2SO_4 and H_2O_2 in a 3:1proportion. The graphite electrode was then polished with alumina slurry. The electrode was then rinsed using double distilled water and was sonicated in absolute ethanol followed by double distilled water for 5 minutes. Cleaned graphite electrode was scanned in 0.5M H_2SO_4 between -1V and +1 V with a scan rate of 0.1V/s till getting a steady-state curve. The electrode was rinsed with distilled water and dried in air.

2.4 Electrodeposition of Au nanoparticles on Graphite electrode surface using Cyclic Voltammetry.

The cleaned graphite electrode was treated for electrodeposition by subjecting the same in solution of 1.0 mM $HAuCl_4$ in 0.5M H_2SO_4 for a potential range of -0.2V - +1.0V at a scan rate of $0.1Vs^{-1}$ with 5 cycles.[24-26] The electrodeposition was carried out in an electrochemical system by Cyclic voltammetry using AutolabPotentiostat /Galvanostat ,Model , AUT83945;PGSTAT302N .The conventional three electrode system was used with Ag/AgCl as reference electrode, platinum wire as auxiliary electrode and Graphite as working electrode. Galvanic reactions at graphite electrode is represented below (Fig.3) Silver paste was used to attach a copper wire to graphite electrode. After the CV, the electrode was washed thoroughly with double distilled water.

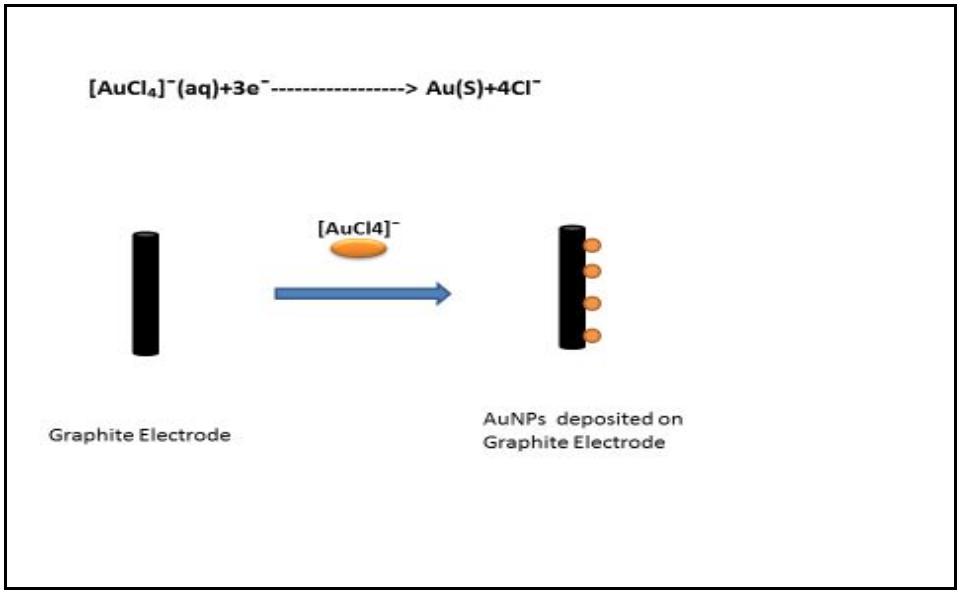
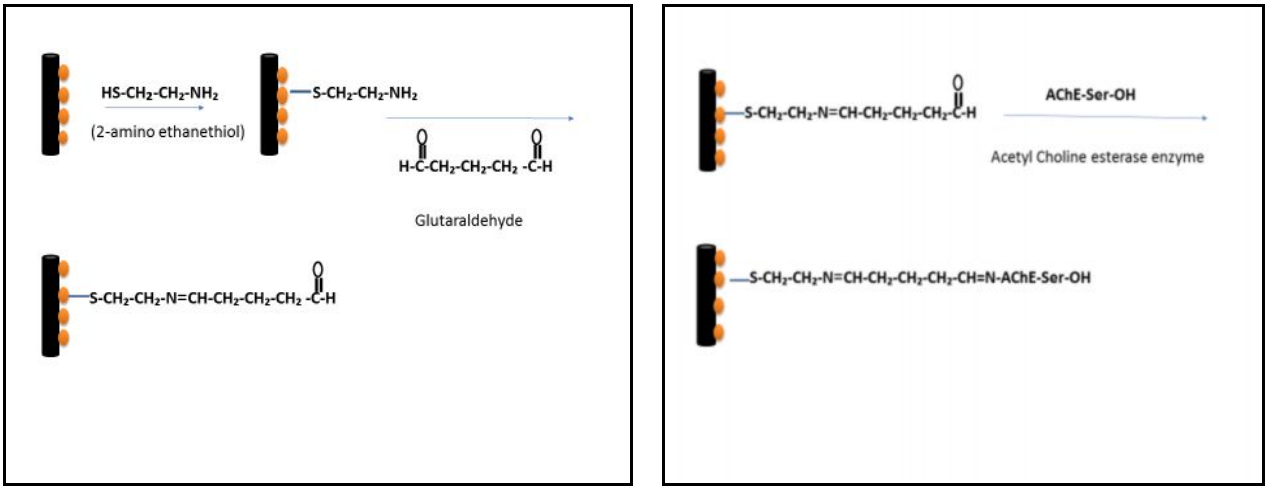


Fig.3. Galvanic reactions during electrodeposition of AuNPson Graphite Electrode

2.5 Preparation of AChE modified Au/graphite working electrode.

The AChE immobilization on AuNP was done by the method mentioned in surface conjugation chemistry. It involved three steps(Fig 4. a-b);

1. The electrode was immersed in $5x10^{-3}$M solution of 2-aminoehtanethiol in 0.01M Phosphate buffer for 12 hours to result in the formation of self assembled monolayers (SAM).Afterwards, the electrode was washed with double distilled water to eliminate excess 2-aminoehtanethiol from electrode surface.
2. As a second step the cross linker gluteraldehyde was linked to the amino groups of 2-aminoethanethiol by immersing the electrode in 25%(V/V) gluteraldehyde solution for 12 hours
3. Thirdly, 25µl of AChE solution was dropped on the surface of activated AuNP/Graphite electrode for immobilizing AChE by cross linking its Amino groups with gluteraldehyde activated electrode surface. The electrode was kept at 4°C for 24 hrs.



a **b**

Fig 4.a,b Preparation of AChE modified AuNPs/Graphite electrode

3. Results and Discussions

3.1 Cyclic Voltammetry of electrodeposition of Au on Graphite electrode surface

The CV showed Oxidation peaks at +0.08V(a) and +0.67V(b) and reduction peaks at -0.104V(c)and -0.539V (d) confirming the electrodeposition of Au on Graphite electrode surface(Fig.5).The oxidative peaks are due to the oxidation of deposited gold to Au(III) (a) and evolution of oxygen(b) respectively. The reductive peaks are due to the hydrogen evolution(c) and reduction of protons (d) respectively.

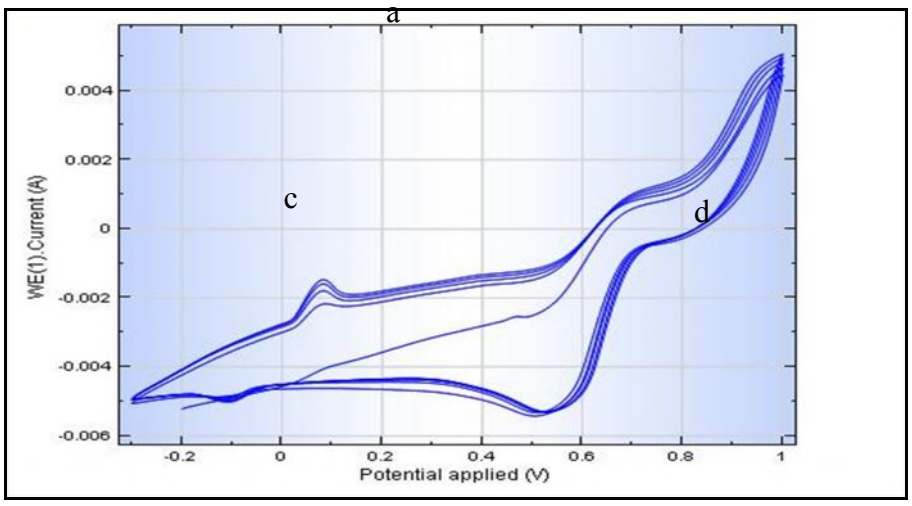
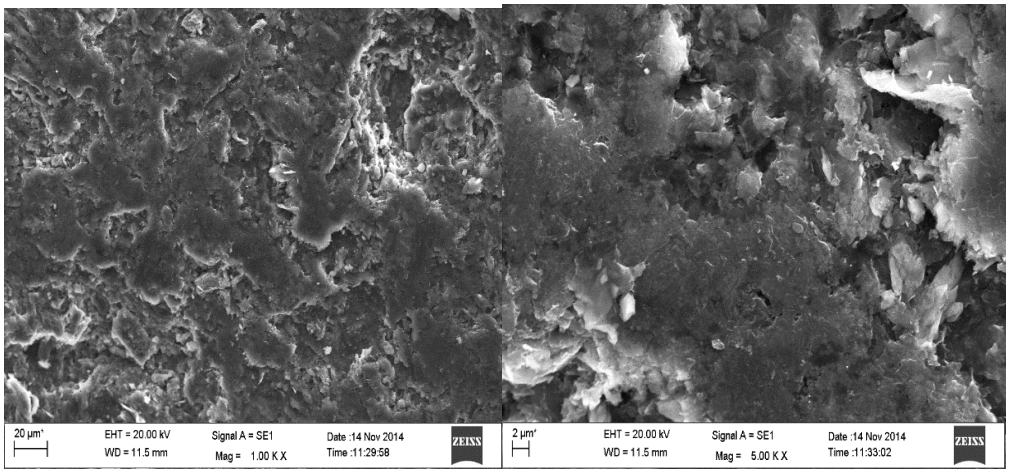


Fig.5. Cyclic voltagramme of 1.0 mM $HAuCl_4$ in 0.5M H_2SO_4 at Graphite Electrode in a potential range of -0.2V - +1.0V at a scan rate of 0.1Vs-1 with 5 cycles

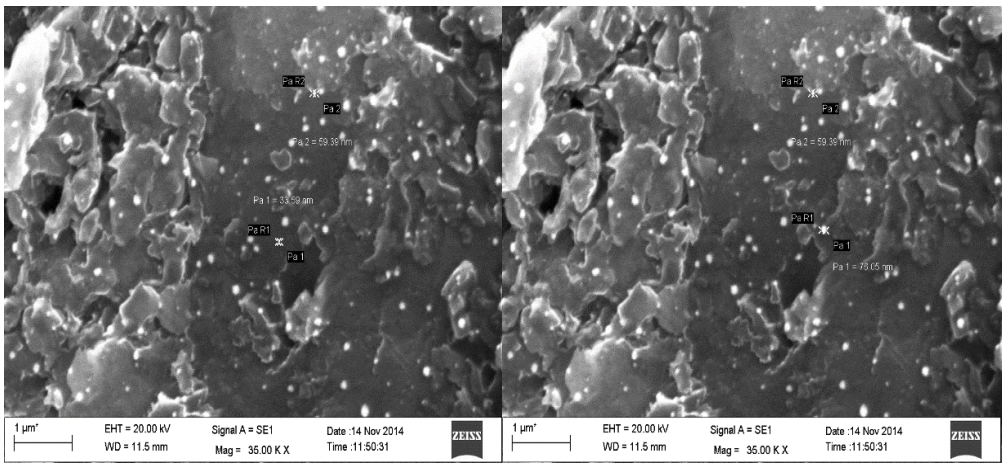
3.2 Morphological Characterization of deposited Gold on the surface of graphite electrode using SEM EDX and AFM

The surface of Bare Graphite electrode and gold deposited surface of GE was studied using Scanning Electron Microscopy and are shown in Figure 6(a,b)&7(a,b). Images of bare Graphite electrode showed an irregular layered surface morphology. But the spherical structures confirmed the presence of AuNP electrodeposited on the surface of Graphite electrode. The sizes of AuNP were ranging from 10nm-100nm



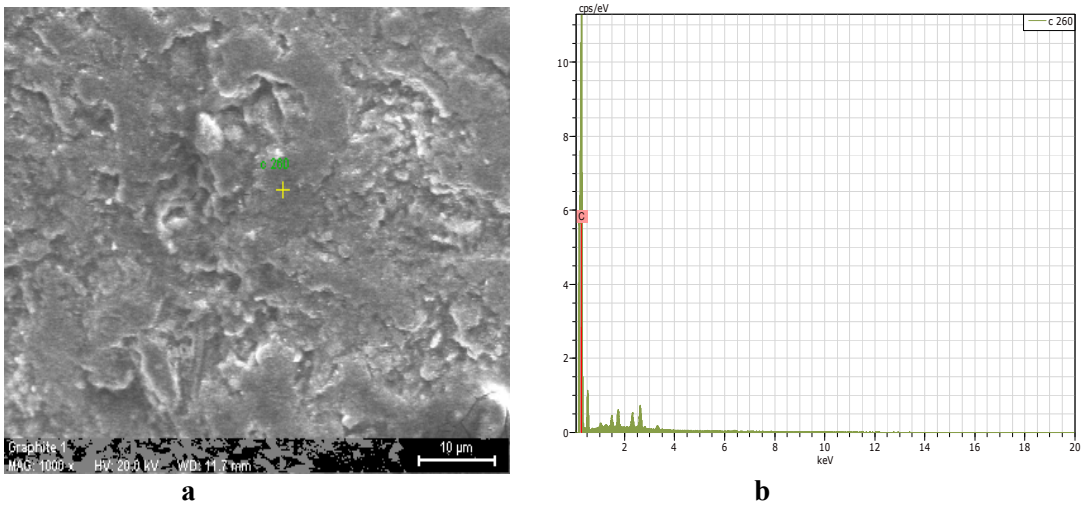
a **b**

Fig.6 (a,b) SEM images of bare synthetic graphite electrode surface



a **b**

Fig 7. SEM images of gold nano particles electrochemically synthesized on the surface of synthetic graphite electrode surface from solution of 1.0 mM $HAuCl_4$ in 0.5M H_2SO_4 using cyclic voltammetry.



a **b**

Fig .8 SEM image (a) and EDX (b) of bare surface of the synthetic graphite electrode surface area

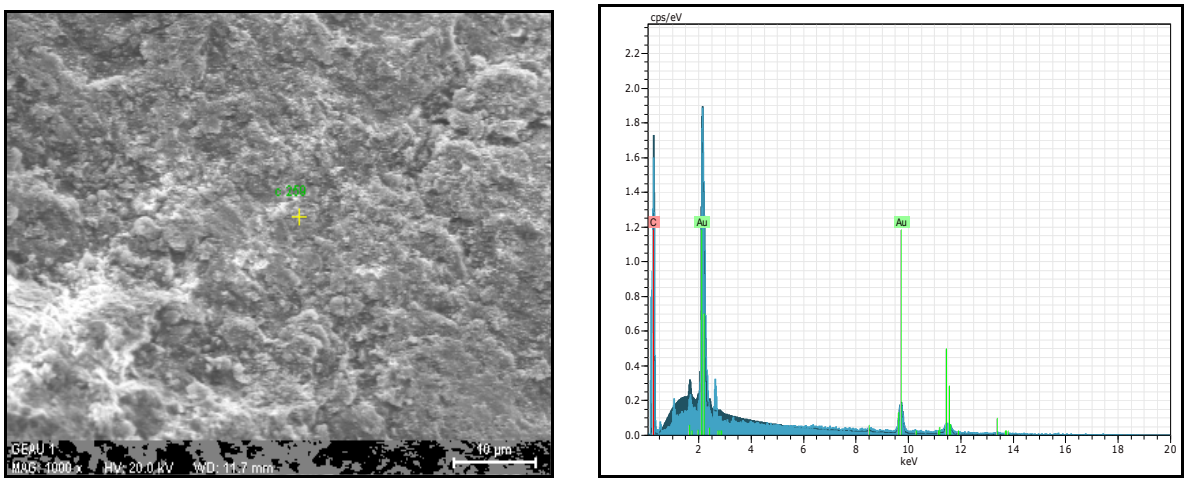
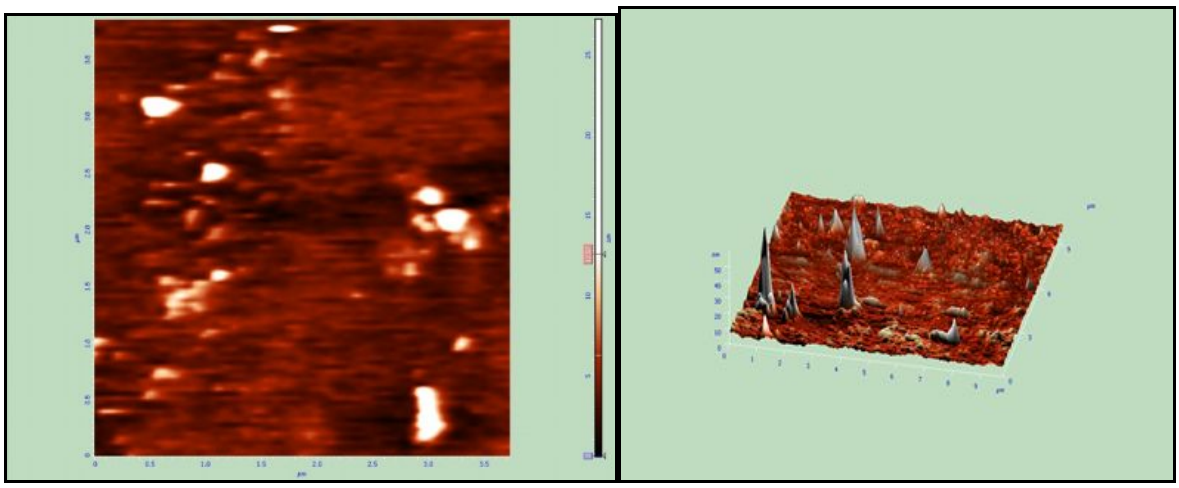


Fig.9.SEM image (a) and EDX (b) of the gold nanoparticles electrochemically deposited on the surface of synthetic graphite electrode surface

Elemental analysis of the bare Graphite electrode and Gold electrodeposited graphite electrode was performed using EDX(Bruker AXS Microanalysis ,GmbH Berlin, Germany) attached to SEM.The EDX results showed that the graphite giving strong peak of carbon presence(Fig.8.) AuNP electrodeposited graphite showed a prominent presence of Au and no impurities were observed on the surface. (Fig.9.)

Surface morphology study of the modified electrode was done using AFM.AFM in tapping mode was used to characterize the AuNPs/Graphite electrode and AChE/AuNPs/Graphite electrode surface.AFM image(Fig 10(a,b) shows rough surface with average roughness height of 1.2nm.The rough surface shows pointed protrusions in the form of humps with larger height profile to a great extent with more surface area due to the AuNP electrodeposition. The height measurement showed AuNPs showing with average height sizes 12.57nm.



a **b**

Fig 10. AFM images (a, b) of surface of bare graphite and AuNPs/Graphite electrode surface

3.3 Characterization of Enzyme immobilized electrode using SEM and AFM

To observe the morphologies and microstructures of AChE immobilized AuNP/ Graphite electrode, Scanning Electron Microscopy (SEM-CARL ZEISS EVO40) was used. The images showed globular aggregations of enzyme immobilized on AuNP/Graphite electrode (Fig.11)

AFM image of surface details of graphite surface with electrodeposited AuNPs with AChE immobilization is shown in Fig.12.a, b.

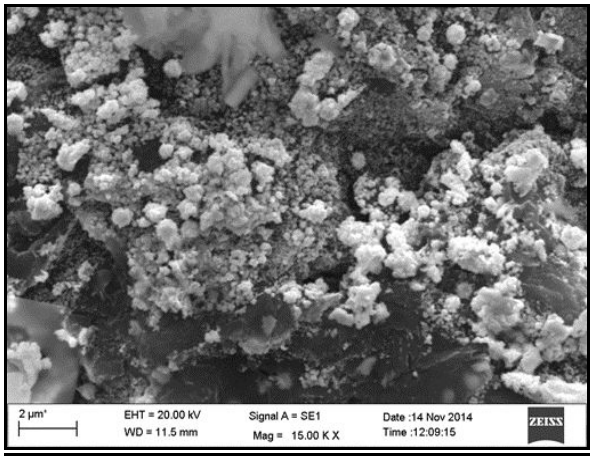
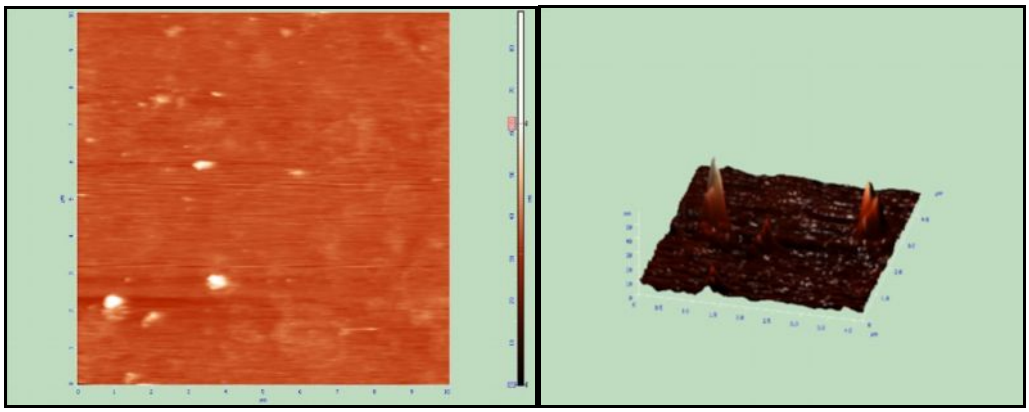


Fig.11. SEM Image of AChE immobilized AuNP/Graphite electrode



a **b**

Fig 12. AFM image(a,b) shows an average size height of 17.80nm.The image shows a fairly smooth surface with Clear Island like structures due to immobilization of AChE

4. Cyclic voltammetry study

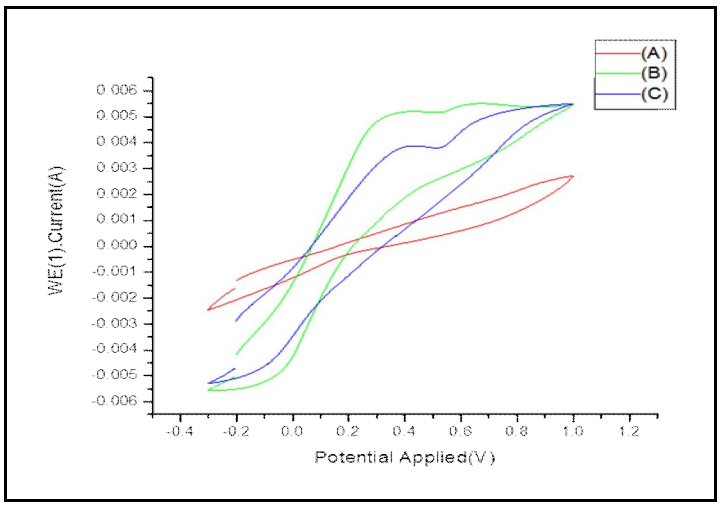


Fig 13. CV of (A) bare graphite electrode, (B) AuNPs/Graphite electrode and (C)AChE/AuNPs/Graphite electrode in pH 7.5 in a potential range of -0.2V - +0.1V in 10 ml of 0.1M phosphate buffer,pH 7.5 in blood serum with substrate 100µl of 0.6M Acetylthiocholine, in 0.5mM $[Fe(CN)_6]^{-3/-4}$ in 0.1 M KCl .Scan rate :0.1VS^{-1}

Acetyl choline esterase (AChE) was covalently immobilized on AuNPs/Graphite electrode in the fabrication of modified electrode. During the stepwise modification, performance of working graphite electrode

was evaluated by Cyclic voltammetry in 0.1M phosphate buffer(pH 7.5).Fig.13shows the CV of bare Graphite electrode, AuNPs/Graphite electrode and AChE/AuNPs/Graphite electrode in the presence of 100µl of 0.6mMAcetylthiocholine chloride in to the cell containing phosphate buffer(pH 7.5) in blood serum at a scan rate of $0.1Vs^{-1}$ .After the introduction of 100µl of 0.6mMAcetylthiocholine chloride in to the reaction cell, no peak was observed for bare graphite electrode(Curve A)in phosphate buffer. An oxidation current peak of 0.003875A at +0.42984mV (Curve C) for the electrode AuNPs/Graphite electrode and the AChE/AuNPs/Graphite electrode produced an oxidation current peak of 0.0051837A at + 0.398102V (Curve B).

Oxidation peak (Curve B) was due to the oxidation of thiocholine, which is a hydrolysis product of Acetylthiocholine chloride due to the action of Acetylthiocholine esterase. The graph shows that there is an increase in the peak and the peak potential shifted negatively in comparison to the electrode without AChE. The increase in the peak current was due to the presence of AuNPs on the surface of graphite electrode, which possess a highly large surface area on the surface of graphite electrode, and have an ability to high electric conduction. This advantage facilitate such electrodes to increase the rate of reactions involving catalyzing enzymes and at lower potential, it could increase the electron transfer rate.

4.1 Optimization of the working electrode

Effect of working potential

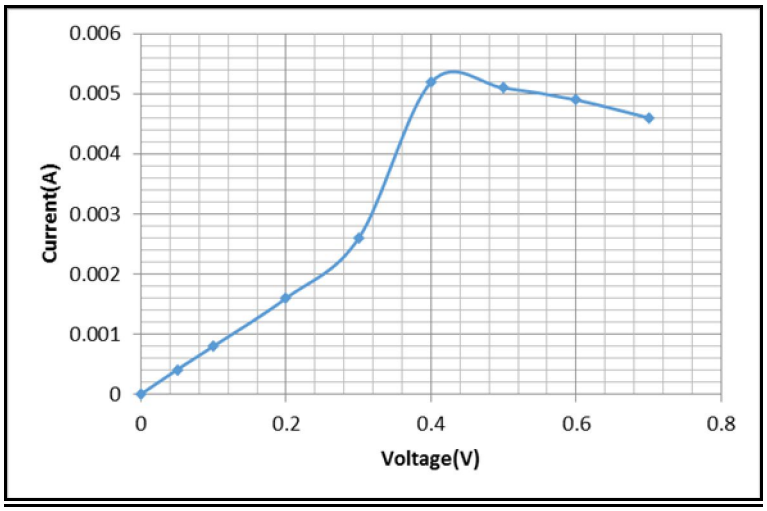


Fig.14.Efect of voltage on the current response of AChE/AuNPs/Graphite electrode. Range of potential used was 0.V to +0.7V.

Fig.14and Table 1 given below shows the results of potential applied on modified electrode. The working potential was stepped from 0.0V to +0.7 V. With the working potential increase from 0.0V to +0.4V the steady-state current response increased and then it reached a plateau from +0.4V to +0.7V.So,the working potential was selected as +0.4V for the modified electrode for the detection of Monocrotophos pesticide

Table.1 showing current readings against different voltages

Sl.No	Voltage(V)	Current(A)
1	0	0
2	0.05	0.00041
3	0.1	0.0008
4	0.2	0.0016
5	0.3	0.0026
6	0.4	0.0052
7	0.5	0.0051
8	0.6	0.0049
9	0.7	0.0046

Effect of pH

The response of the modified graphite electrode was studied using a pH range of 5.0-10.0.For this 0.1M sodium succinate(pH 5.0 and 5.5),0.1M sodium phosphate(pH 6.0,6.5,7.0 and 7.5)and borate buffer(pH 8.0,8.5,9.0 and 10.0) was used. The current response of AChE/AuNPs/Graphite electrode changed between pH 5.0 and 7.0.Maximum current response was shown at pH 7.5.At lower pH the current response was very minimal due to AChE losing its activity.pH, 7.5 was considered for the experiments for the detection of Monocrotophos .The Fig.15&Table.2 show the result of current responses against different pH

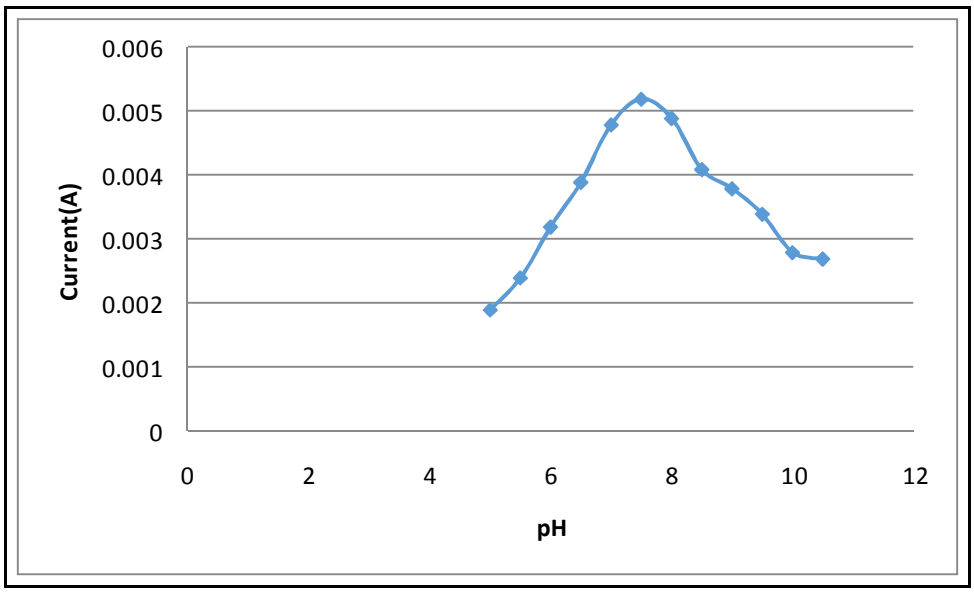


Fig: 15.Effect of pH on the current response of AChE/AuNPs/Graphite electrode. The range of pH used was pH 3.5-10.5

Table: 2 showing current reading against different pH

Sl.No	pH	Current(A)
1	5	0.0019
2	5.5	0.0024
3	6	0.0032
4	6.5	0.0039
5	7	0.0048
6	7.5	0.0052
7	8	0.0049
8	9	0.0038
9	9.5	0.0034
10	10	0.0028

Effect of temperature

Study was conducted on the modified graphite electrode about temperature related effects on its current response .The range of temperature considered for the study was 20ºC - 60ºC. A significant change in the current response was observed with a peak at 35 ºC .Therefore 35ºC was considered as the optimal temperature for conducting the experiments for the detection of organophosphorus pesticide.The Fig.16&Table.3 show the result of current responses against different temperature

Table.3 showing current reading against different temperature

Sl.No	Temperature(ºC)	Current (A)
1	20	0.0029
2	25	0.0033
3	30	0.0039
4	35	0.0051
5	40	0.005
6	45	0.0047
7	50	0.004
8	55	0.0035
9	60	0.0033
10	65	0.003
11	70	0.0028

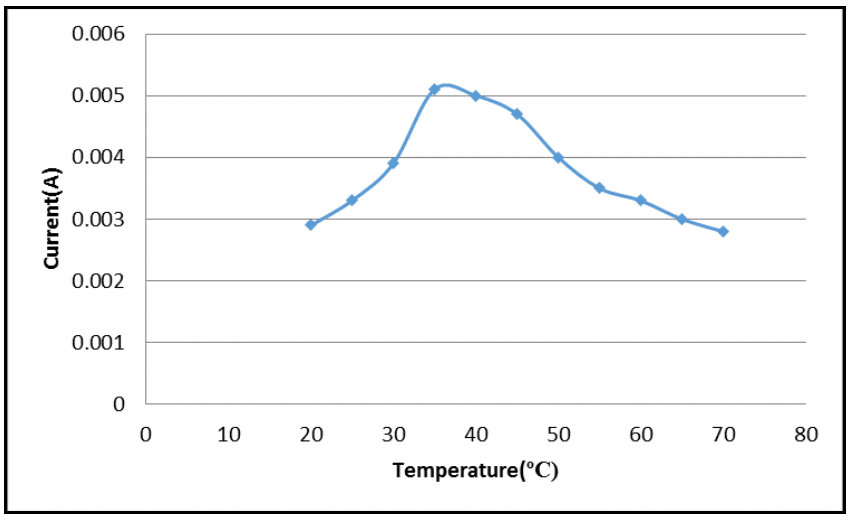


Fig: 16. Effect of temperature on the current response of AChE/AuNPs/Graphite electrode. The range of temperature used was varying from 20ºC - 60ºC

4.1 Monocrotophos determination

The detection of monocrotophos was done through the measurement of inhibition of immobilized AChE activity. This was achieved by immersing he AChE/Au/Graphite electrode in different concentrations of standard inhibitor solution i.e. monocrotophos for 5 minutes. Thisresulted in the formation of enzyme Inhibitor Complexes. After this incubation the modified electrode was then transferred to the electrochemical cell having Acetylthiocholine chloride, phosphate buffer and blood serum to record the reduction in the current after inhibition.

For this study different standard concentrations of monochrotophos i.e.;3.2mg/L, 1.6mg/L, 0.8mg/L, 0.4mg/L, 0.2mg/L, 0.1mg/L and 0.05mg/L were used after spiking in blood serum. With increasing concentrations of monocrotophos, the resulting current from the enzymatic electrode decreased as demonstrated by the cyclic voltammetry (Fig.17&Table.4) enzymatic inhibition was proportional to the concentration of monocrotophos.A calibration curve was plotted between current response against different concentrations of pesticide.

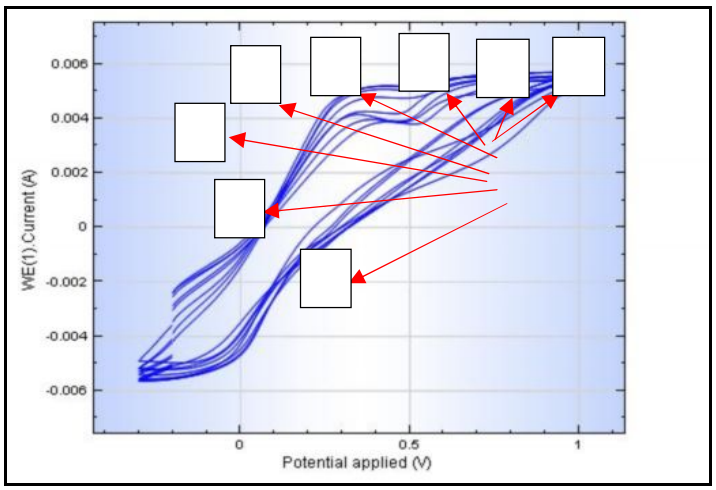


Fig.17. Cyclic voltagramme of AChE immobilized AuNP/ Graphite Electrode in a potential range of -0.2V - +0.1V in 10 ml of 0.1M phosphate buffer,pH 7.5 in blood serum with substrate 100µl of 0.6M Acetylthiocholine, in 0.5mM $[Fe(CN)_6]^{-3/-4}$ in 0.1 M KCl in the presence of Monochrotophos pesticide in different concentration; a) 3.2mg/L b)1.6mg/L c) 0.8mg/L d)0.4mg/L e)0.2mg/L f) 0.1mg/L g) 0.05mg/L at a scan rate of 0.1VS^{-1}
h=current peak without the inhibitor; monocrotophos

Table.4 Showing current reading against different concentrations of pesticide

Sl.No	Concentration(mg/L)	Current(A)
1	3.2	0.00387451
2	1.6	0.00396118
3	0.8	0.00414642
4	0.4	0.00425537
5	0.2	0.00477997
6	0.1	0.0050708
7	0.05	0.0051825
8	No inhibitor	0.00520142

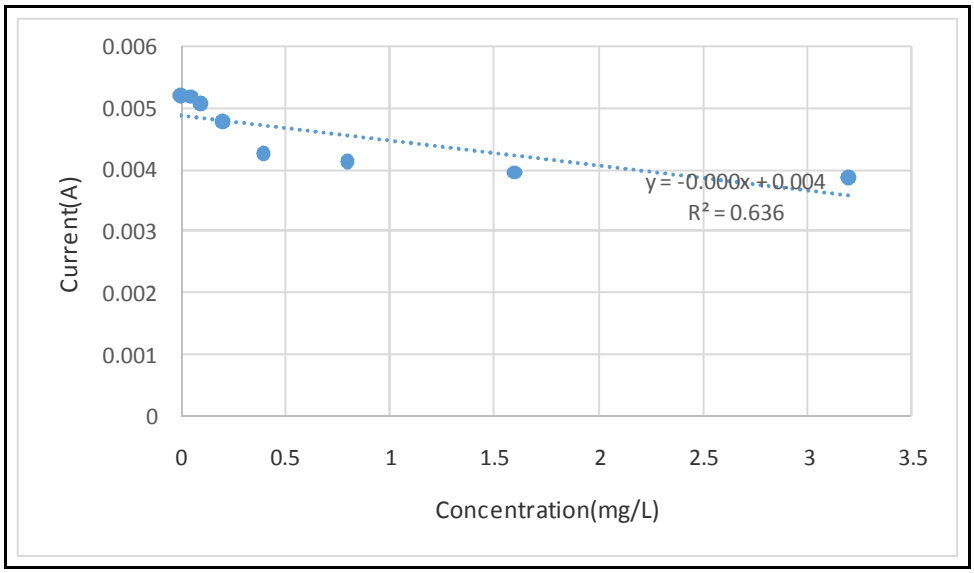


Fig.18. Calibration curve plotted between Monocrotophos concentration and current

5. Conclusions

The determination of monocrotophos in spiked blood serum using the proposed method is better in terms of detection and sensitivity as compared to the Gaschromatography/mass spectrometry(GC/MS) studies already reported for the forensic science related works[27-28].In the present investigation, Acetylcholine esterase was immobilized on AuNP deposited synthetic graphite electrode.The monocrotophos concentration response was linear in the range from 0.05mgL^{-1} to 3.2mg L^{-1} .The detection limit for Monocrotophos was 0.05mg L^{-1} ,which is better than that reported in literature .The literature reports that the presence of organophosphorus pesticides in blood serum is in a range of 0.72-2.90mgL^{-1} in forensic cases involving acute fatalities due to the ingestion of organophosphorus pesticides.[27]The AChEAuNPs/graphite based electrodes offers better application for the electrochemical studies of detection of organophosphorus pesticides present in quantity which is normally encountered in fatal toxicity cases. The present method can also detect lower limits of the pesticides which may also be encountered by forensic scientists. The results of the present study based on synthetic graphite which being a better conducting electrode offers as a good alternative to other conventional methods as it is economical and with better sensitivity for the detection of analytes like organophosphorus compounds from body fluids as the other methods are costly and time consuming. This method shall be useful for confirming the presence of organophosphorus pesticides with its quantity in blood in clinical and forensic cases like murder, suicide, accidental poisoning, and chemical warfare.

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