Biosensor for organophosphorus pesticides detection by the fabrication of ZnO nanoparticles

A

Thesis submitted

in partial fulfillment of the requirement of degree of

Master of Science

in

Chemistry

Under the supervision of

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Submitted By

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Acknowledgement

I am greatly indebted to my supervisor Prof. Dr. Susheel Mittal, Deputy Director of Thapar University for his guidance, encouragements, valuable comments and endless supports. I am tremendously thankful to him for giving me such an interesting topic. I am feeling proud of being a member of his laboratory.

I wish to thank my senior Ms. Manisha Pabbi, Research Scholar for always being helpful and support throughout writing the thesis, for her advices in experiments, critical views throughout my study and endless patience and valuable friendships whenever I needed.

I would like to thank to all my friends and all chemistry department for their suggestions and criticism.

I would also to thank my lab mates and colleagues in Biosensor laboratory. One by one for their technical and moral supports and valuable comments.

Finally, to my beloved family; I love you mom, dad, bro thank you for everything.

It is my duty to express my gratitude to Thapar University for the support that I have received as student during my post-graduation education.

Date: 15/7/15

Place: Patiala

Amanpreet Kaur

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Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled **"Biosensor for organophosphorus pesticides detection by the fabrication of ZnO nanoparticles"**, in partial fulfillment of the requirements for the award of the degree of Masters in Chemistry, School of Chemistry and Biochemistry, Thapar university, Patiala, is my own work during the period of January 2015 to July 2015, under the supervision of Dr. Susheel Mittal, Deputy Director, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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Certificate

This is to certify that the thesis entitled **"Biosensor for organophosphorus pesticides detection by the fabrication of ZnO nanoparticles"**, being submitted by Ms. Amanpreet kaur in partial fulfillment of the requirements for the award of degree of Master of Science in the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Susheel Mittal and that no part of this thesis has been submitted for the award of any other degree.

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Abstract

Flower shaped ZnO nanoparticles (NPs)were synthesized by sol gel process using $Zn(NO_3)_2$ as precursor and were used in the fabrication of ISFET biosensor for detection oforganophosphorus pesticides(OPs).In this work, whole cell algae *Chlorella* sp. having surface bound alkaline phosphatase (AP) was exploited for study.Three experimental sets were done which include (i) direct interaction of enzyme and pesticie without immobilizing algae (ii) immobilizing algae on surface of ISFET Ta_2O_5electrode (iii) modifying electrode surface by ZnO NPs incorporated –algae. The basis of detection states that AP dephosphorylates substrate 2-Phospho-L-ascorbic acid trisodium (PAA) to ascorbic acid (AA), which was detected by ISFET transducer. OPs inhibits the activity of enzyme AP, which gives current change on the change of the pH of the solution. The extent of inhibition gives the concentration of OP present.OPs studied were chloropyrifos, triazophos and malathion. Among these, chlorpyrifos showed highest AP enzyme inhibition upto 10^{-9} M and malathion was the least. It was found that among three methods studied ZnO modified electrode showed better results.

Introduction

In modern phase of technology, improvements are constant and with these improvements, some further demand for a higher level of technology. Apparently, everybodynowadays is waiting for nanotechnology to provide a new revolution. Thus, nanoscale science and engineering have found great potential in the development of nano-biosensors with more rapid response and higher sensitivity than of planar sensor configurations.A biosensor is an analytical device, used for the bioanalyte detection that combines a physicochemical detector.Among various categories of nanoparticles (NPs), ZnO nanostructures have attracted much attentionin the field of biosensors. It possesses high specific surface area, non-toxic nature, biocompatibility with biomolecule, optical transparency, high stability and ease of fabrication that make it an excellent candidate for bio sensing properties.

ZnO is ann-type, II–VI semiconductorwith band gap of 3.37 eV and a large excitonic bindingenergy of 60 meV at room temperature.ZnO crystallizes in three forms: hexagonal wurtzite, cubic zinc blende, and the rarely observed cubic rock salt[1].However, hexagonal wurtzite structure is most stableat ambient temperature and pressure. ZnO formshexagonal wurtzite structure with Zn^{2+} ion surrounded by tetrahedral O^{2-}ions and vice versa.This tetrahedral co-ordination gives rise to a polar symmetry, which is responsible for its number of thephysical and chemical properties.The difference in electrical propertiessuch as conductivity is because of the presence of oxygen vacancies,shallow zinc, interstitial space, hydrogen impurity and other donortype point defects [2]. Moreover, the ZnO near-surface region can behighly conductive due to H donors in this region and a large densityof near-surface electrons.

Fig 1 shows the systematic diagram for ZnO incorporated biosensor [2].Biosensor comprises two fundamental unitsconnected in series that are “biologicalrecognition or bio-receptor” and “transducer.”Bio-receptor or biomolecule provide specificity and sensitivity to biosensor by interacting with analyte to produce recognition signal and transducer converts recognition signal into measureable signal. Incorporation of ZnO in the matrix of bio-receptor would tend to increase its permeability, sensitivity andprovide

large number of adsorption sites for biomolecule. Depending upon the type of bio-receptor and transducer, biosensors are of various types which are explained below.

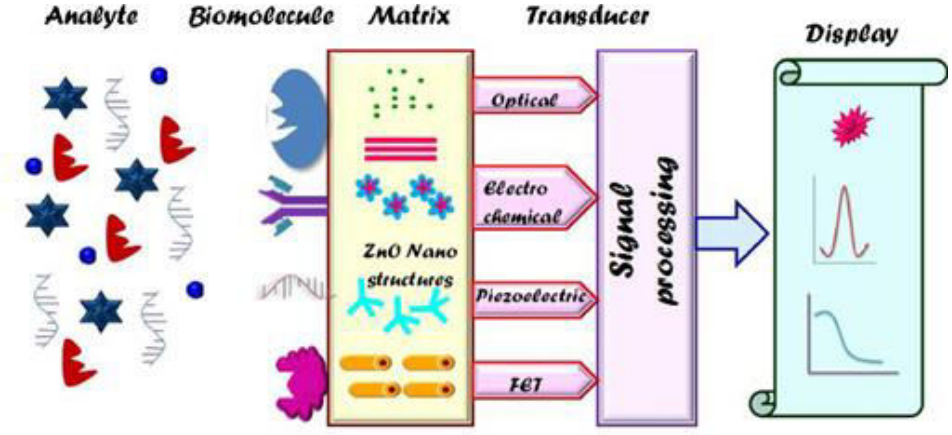

Fig.1 Systematic diagram of biosensor

CLASSIFICATION OF BIOSENSORS

Biosensors can be classifiedbased on:

a) Bio-receptor or biomolecule

b) Transducer

1. Classification of biosensors on the basis of bio-receptor

Biomolecule undergoes a binding process by taking up the desired target analyte and produces signal, which is fed to transducer. Biosensors can be categorized into following types depending on the kind ofbioreceptor used:

(a) Enzyme based biosensors: There are biosensors in which enzyme is used as bioreceptor to produce metabolic changes during conversion of a substrate to product. The majority of enzyme based biosensors detect a particular analyte by converting it into detectable product by an immobilized enzyme. The product thus formed should have either electro activity (e.g. redox active) or optical property (e.g. fluorescence, absorbance) which can be detected by a suitable transducer.

(b) Nucleic acid based biosensors: Nucleic acidbiosensors involve DNA or RNA as bio recognition element which are based on highly specific hybridization of complementary strands of DNA or RNA molecules.

(c) Immunoassay or antibodies based biosensors: It involves recognition between an antigen and its corresponding antibody. This recognition is very specific and binding depicts its concentration.

(d) Cell organelles based biosensors: Various cell organelles like mitochondria, plasmid etc. and their various actions and activities are used as recognition element for various analytical determinations.

(e) Whole cell based biosensors: Various whole cells like bacteria, fungi, algae, etc. are used as bio-receptor for various estimations and measurements in analytical samples.

Although, purified enzymes offer great deal about sensitivity and selectivity and whole cells lack selectivity in most of analyte determinations, yet they are better alternate to rest of biological recognition elements as they provide following advantages:

i. Can be handled easily.

ii. Can be easily cultured, cells proliferate with ease in both anaerobic and aerobic conditions.

iii. Cost effective.

iv. Enzyme system used for the study will work best within the system because it will get its optimum working environment of pH and temperature in the cell.

v. Can be miniaturized with ease allowing potential rapid multi target analysis.

The whole cell used in our work is algae *Chlorella* sp. having surface bound alkaline phosphatase.

2. Classification of biosensors on the basis of transducer

Transducer converts interaction of the analyte with bio-receptor i.e. recognition event into measurable signalsuch as an electrical signal. It can take many forms depending upon the parameter being measured-electrochemical, optical, mass and thermal changes.

(a) Electrochemical transducers based biosensor

Electrochemical biosensor senses any electrical change produced due to consumption or release of electrons during chemical reaction. It can be of various types such as potentiometry, voltammetry, amperometry and conductometry.

Potentiometricbiosensor

Potentiometric biosensors measure the potential change due to the gathering of charge i.e. in the form of electrons on the working electrode with respect to a reference electrode while current is not flowing. The transducer employed in the potentiometric technique is often ion-selective membrane and field effect transducer (FET)as these have better sensitivity and selectivity than traditional potentiometer.The sensitivity and selectivity of potentiometric biosensor are exceptional due to the species selective working electrode used in the system. Mainly, there are two types of FET-based biosensors as per their structure ISFET (ion selective field effect transistor) and MOSFET (metal-oxide-semiconductor field-effect transistor).In MOSFET the gate terminal is electrically isolated from the source and drain terminals but in ISFET the metal gate is replaced by an ion-selective membrane, electrolyte and a reference electrode. In the present study, we arefocusing on ion selective field effect transducers(ISFET).

The ISFET usually mentioned to as a pH sensor. ISFET monitors interface potential on the gate insulator due to ions concentrations (H+ or OH−) in a solution. A field-effect transistor (FET) consists of three terminals source, drain, and gate as shown in Fig 2. The voltage among the source and drain of the FET controls the current flow in the gate voltage.

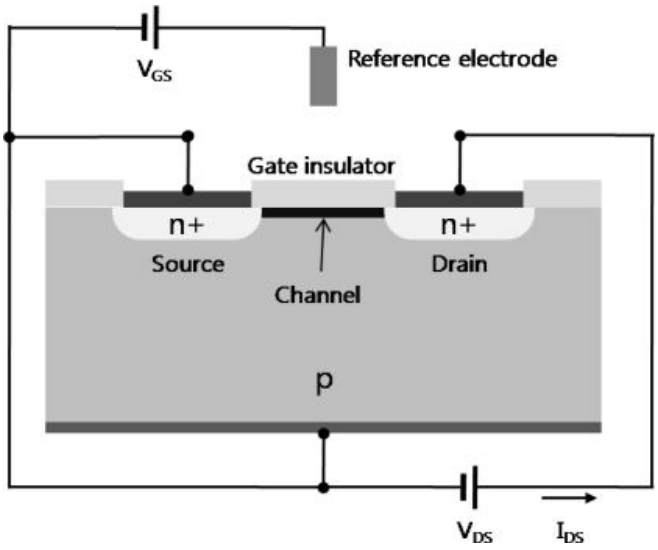

Fig. 2 Overview of ISFET

In the case of an ISFET biosensor, the amount of the current movement will be not only monitored by the charges of biomolecules interacting on the gate dielectric but it is also sensitive to pH, various kinds of ions, products of enzyme reactions, etc. Wecan visualize bimolecular interactions in a label-free method through a directchange inconductance which is measured by ISFET.Enzyme based ISFET biosensors are known as ENFET generally fabricated by immobilizing an enzyme onto the gate insulator of an ISFET. When enzymatic reaction between enzyme and substrate occurs than products is produced or reactant is consumed, which results in concentration change this change can be observed with the help of ISFET. Therefore, an analogous alteration of the ISFET signal can be associated with the original analyte concentration. These are useful in the detection of the analytes glucose, urea, penicillin, ethanol, lactose, sucrose, maltose, ascorbic acid, lactate, acetylcholine, organophosphorus pesticides, formaldehyde, creatinine, etc. reported by Soldatkin et al [3], Pijanowskaet al [4].

Conductometric biosensor

It involves the conductivity change in the solution due to the consumption or manufacturing of ionic species, for example, by the metabolic activity of the microorganisms. The measurement of conductance is extremely fast and sensitive. It is too worthy to observing that such biosensors are suitable for miniaturization meanwhile it

requires no reference electrode in the system. However, all charge carriers could result in the conversion of conductivity, consequently the selectivity of conductometric biosensor is comparatively less[5].

Amperometric biosensor

It functions at a given potential between the working electrode and the reference electrode and the current signal is recorded associated with the concentration of target compounds. In the amperometric detection, the current signal is produced due to the oxidation or reduction of an electro active metabolic product or intermediate on the surface of a working electrode [6].

Voltammetric biosensor

It is a multipurpose technique in electrochemical detection. Current as well as potential both parameters are recorded. The location of peak current is associated to the definite chemical and the peak current density is proportional to the concentration of the parallel species. Furthermore, voltammetry is capable to identify numerous compounds, which have dissimilar peak potentials, in a single electrochemical experiment, therefore offering the instantaneous detection of multiple analytes.

(b) Optical biosensor

This type of detection is generally on the basis of fluorescent, luminescent, colorimetric, or other optical signal by the interaction of microbes with the analytes and associates the detected optical signal with the concentration of target compounds. Optical detecting methods are particularly striking in high quantity screening meanwhile they permit biosensors to display multiple analytes concurrently[7].

(c) Mass- sensitive biosensor

Piezoelectric crystals act as mass sensors i.e. these transducers have been used where the biorecognition reaction causes change in mass. The utility of the piezoelectric crystal as a mass sensor arises from the linear relationship between the change in mass at the crystal surface and the change in its oscillating frequency. The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass[8].

ZnO based ISFET biosensor for pesticide detection

In our present study, we fabricated ZnO incorporated whole cell ISFET biosensor for the detection of organophosphorus pesticides (OPs). OPs like chlorpyrifos, triazophos, malathionare extensively used in agriculture for crop protection. However, due to their low solubility and degradation, they accumulate in environment causing serious health issues and contamination of soil, water and food. Therefore, it becomes obvious need to determine pesticides very rapidly and at trace level. Various methods are available for their determination but biosensors are better alternate to conventional methods as these are more sensitive, low cost and less laborious than spectrophotometric methods.The whole cell exploited for the construction of biosensor is algae *Chlorella sp.*having surface boundalkaline phosphatase (AP) and ISFET as transducer to quantitate the binding taking place between enzyme and pesticide.ZnO-based ISFETbiosensor showed high pH sensitivity and high stability comparable toother NPs modified ISFET because near-surface region of ZnO are highly conductive due to H donors in this region and a large densityof electrons.

Literature Review

The selectivity and chemical sensitivity of the ISFET are entirely controlled by the properties of the electrolyte/insulator boundary. For instance, numerous different kinds of oxide coverings of inorganic constituents such as SiO_2, Ta_2O_5, Al_2O_3, Si_3N_4 etc. can be used for obtaining a pH response. Protonation/deprotonation of the gate material is influenced by pH gate area [9, 10].Furthermost ion sensors are potentiometric sensors, which means that the electrical potential difference, $\Delta\varphi$, at a solid/liquid interface as function of the ion concentration to be determined according the Nernst equation:

$\Delta\phi = RT/F \ln a_1/a_2$

Here R is the gas constant, T the absolute temperature (K) and F the Faraday constant. Ion concentrations ci are noted in terms of activities, $a = f_i.c_i$, with f_i being the activity coefficient. In diluted electrolytes $f_i = 1$. This equation shows that, providing that at one side of the interface the activity "a" of the ion of interest is kept constant, the electrode potential is a direct logarithmic function of the ion activity on the other side. Thus, for instance, a metal electrode in its own solution, for instance copper in a copper sulphate solution and i is the molar concentration of the free uncomplexed ionic species.

ISFET based biosensors

Gate is altered by different kind of nanomaterials [4], biological species [11] for the construction of different kind of biosensors such as chip based ISFET biosensors [12], DNA basedISFET biosensors [13, 14], Enzyme based ISFET biosensors ENFET [15] reported by Soldatkin et al [3], Pijanowska et al[4], etc.Immune based ISFET biosensorspresented by Schleck et alin 1978 [16], nanoparticles based ISFET biosensors [17], Cell-based Based ISFET biosensor [18, 19]. These various kinds of biosensors are used to detect different compounds which are discussed below.

ISFET biosensor for glucose sensing

Glucose sensors are very prominent for fabrication of measurement arrangements. These sensors have many applications in the field of environment monitoring, medical

diagnostics, food products and many more [20]. ISFET glucose sensors measure the glucose concentration by detecting the pH variation due to the hydrogen ions that are generated by the dissociation of gluconic acid. In the starting these sensors was showing low sensitivity due to low dissociation constant [21]. With the advancement of technology, these sensors are modified by various methods for high sensitivity and better results such as electrolysis of hydrogen peroxide [22]. In this method glucose oxidation as well as the dissociation of gluconic acid was proposed [23]. In this process, two extra hydrogen ions are created by the electrolysis of the hydrogen peroxide:

$$\beta\text{ - D - glucose} + H_2O + O_2 \longrightarrow \text{Gluconic acid} + H_2O_2 \quad 1$$

$$\text{Gluconic acid} \longleftrightarrow \text{Gluconate} + H^+ \quad 2$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^- \quad 3$$

The sensitivity would beenhanced by the addition of two hydrogen ions. It would also be expected that the additional oxygen from reaction (24) would increase the reaction (25) which is responsible for broader dynamic range. In addition to that, there are another advanced techniques to increase sensitivity for instance semiconductor nanowires, grown from bottom-up methodologyhave been established as good aspirants for glucose sensors [26].

ISFET biosensors for biological sensing

It is one of the most stimulating methods in electrical bio sensing technology [27]. It is highlysensitive for the monitoring of innumerable biomolecules such as DNA, proteins, enzymes, and cells [28].In addition to that because of high accuracy and sensitivity it also detects biological events such as nucleic acid hybridizations, protein-protein interactions, antigen-antibody binding, and enzyme-substrate reactions [29, 30]. There properties of biological sensing make it useful for various applications in the detection of vivo, vitro, medicinal field and many more.

ISFET biosensors for urea sensing

The development of ISFET sensors for the determination of urea is a significant in research because such analytical systems are small sized, fast and easy to use [31, 32].

The immobilization of urease enzyme is responsible to sense the urea [3]. Urease is an enzyme which catalysis the hydrolysis of urea according to the reaction:

$(NH_2)_2CO+2H_2O+H+urease \rightarrow 2NH_4^+ + HCO_3^-$

Recognition of pH changes due to the enzymatic reaction was measured around the gate surface of a pH-FET [33]. These urease based biosensors can be used urea detection in biomedical or food samples etc.

ISFET biosensor for penicillin sensing

There are also biosensors for the determination of penicillin [36]. The penicillin biosensor consists of pH sensitive field structure with immobilized enzyme [57]. Penicillinase is an enzyme which catalysis the hydrolysis of penicillin which results in the change in the concentration of H^+ ions [32]. This change of ions also cause change in drain current which would be noticed by the ISFET. It has lots of application in analysis.

ISFET biosensors for sensing heavy metals

ISFET sensor is highly sensitive to heavy metals as compared to traditional methods such as atomic absorption spectroscopy.Stardub et al [34,37] reported enzymatic sensors based ISFET for detection of heavy metal ions. There different sensors are developed for the sensing of heavy metals by the immobilization of enzymes such as urease, cholinesterases[3, 16, 25]. It was shown that a urease sensor is more sensitive to ions than cholinesterases (ChEs) sensor reported by Stardub [38]. The sensitivity of the urease sensor to heavy metal ions metals was found as follows

$$Hg^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+}.$$

ISFET biosensor for sensing pesticide

The majority of biosensors for pesticides are based on the inhibition of acetylcholinesterase or butyrylcholinesterase and some another enzymes integrated with electrochemical transducers such as potentiometric and amperometric and optical transducers etc. While being sensitive, these biosensors undergo some limitations like poor selectivity, multi-step indirect determination, and irreversible inhibition by many compounds however ISFET is able to overcome these problems. There are many

successful ISFET based sensors to sense pesticide.Schoning et al reported an EIS-based ISFET sensor for the direct determination of organophosphorus pesticides by immobilizing enzyme organophosphate hydrolase (OPH) [29]. Flounder et al, 1999 reported a pH-sensitive field effect transistors (FET) modified with OPH and used for direct detection of organophosphate compounds [40]. Hai et al. reported a sensor by immobilizing enzyme acetylcholine esterase for pesticide detection [41].Gamal A. E. Mostafa has prepared and discussed about enzymatic determination of pesticides by the inhibition of the activity of selected enzymes such as cholinesterase, acid phosphatase, ascorbate oxidase, acetolactate synthase and aldehyde dehydrogenase [42].

ISFET biosensors for sensing of miscellaneous compounds

ISFET biosensors due to their specialties are used to sense a plenty of compounds such as arginine [67], toxic substances [43, 44, 45] with an accurate way as compared to traditional methods [35], environment monitoring [46], pH determinations at varying temperatures [47],hypochlorite,glycoalkaloids [48] and many more.

Nanoparticles based ISFET biosensors for sensing

Advancement in technology have improved the characterization techniques and new synthesis methods in recent years. This is responsible for enhancing biosensor performance. Nanoparticle fabrication is one out of these techniques responsible for better performance of biosensor. There are various types of nanoparticle incorporated ISFET sensors such as ZnO aluminum ISFET (AZO) sensor prepared bydoping ZnO nanostructuresreported by Wang et al [49]. A new polycrystalline ZnO-based ion-sensitive field effect transistors (ISFETs) and FET-type biosensors are reported byYanoet al[50]. The ZnO-based ISFET showed high pH sensitivity and high stability comparable to commercially available silicon-based biosensors. Lee et al reported intrinsic-ZnO sensing membrane and various ZnO layers in the ZnO-based FETs. These layers were deposited by using the vapor cooling condensation system to sense ammonia [51]. ZnO compound has miscellaneous nano structures includes particles, wires, rods, needles, belts, tubes, fibers, tetrapod-like, flower-like and hedgehog-like morphologies [52, 53]. Zhang et al reported that nanostructure matrices provide a solid support to immobilization to sense molecules [54]. Schlecket al reported method of immobilization, physical,

chemical and surface properties of the preferred support material in immobilization to monitor the molecules, and the complete biosensor performance[66].Schoning et al reportedbiosensors based on urease and BuChE were investigated and compared upon modifying the ISFET electrodes using zeolite Beta nanoparticles with varying Si/Al ratios. Biosensor responses obtained were the highest for N-BEA-100 and N-BEA-50 samples for BuChE and urease, respectively. The results suggested that the Si/Al ratio strongly influenced the biosensor [55].Reyes et al reported zinc oxide thin film transistor for immune sensing with high selectivity and sensitivity [56].Ibupoto et alreported ZnO based ISFET sensor to detect penicillin by the enzyme penicillinase[57].Rahman et al [58] reported nanostructured based field effect transistorsfor the determination of glucose by electrochemical oxidation.These nanostructures have been widely discovered to develop biosensors with high sensitivity, fast response times, and stability. There are developments of different nanostructured metal-oxide such as ZnO, Cu (I)/ (II) oxides, MnO_2, TiO_2, CeO_2, SiO_2, ZrO_2, and other metal-oxides based glucose biosensors [59]. ZnO matrices are superior to their equivalents nanoparticles such as TiO_2 because these are accurately suitable to the adsorption and bio electrochemistry of proteins [65]. Nano composites of ZnO including metal oxide semiconductor-metal hybrids and inorganic-organic hybrids which is also used as matrices for immobilization of enzyme and other materials [60]. The fabrication of nanostructures can improve the sensing properties of components [61]. Renault et al [62] reported that these biosensors are used to detect pesticides reported by the inhibition of enzymeacetylcholinesterase or butyrylcholinesterase [63, 64]. Hahm et alreported these are used to detectDNA, biology, medicine and protein detection[68] and other biological materials and diseases detection reported by Pramanik et al [69].

Gap in the Studies

It is evident from the literature review that numerous ISFET based biosensors have been developed for the determination of various analytes such as glucose, urea, penicillin, heavy metals etc. In addition, nanoparticles (NPs) were used to modify ISFET electrode to enhance the sensitivity and detection limit. Among various categories of NPs, predominantly ZnO NPs were used for modification, as these are more compatible with biomolecule. However, for pesticide determination very few reports using cholinesterase enzyme are reported in literature without any improvisation with NPs.

Research Problem

In our present work, we are using *Chlorella sp.*for the determination of OPs such as chlorpyrifos, triazophos and malathion. Whole cells offer many advantages over enzymatic bioreceptor which include low cost, easy to handle, optimum stability of enzyme which is not possible in case of purified enzymes. Although whole cells have low selectivity but they recognize a wide range of analytes.

The activity of alkaline phosphatase (AP) enzyme bound on the surface of algae is inhibited in the presence of inhibitors (OPs); the degree of inhibition provides the concentration of pesticide present. The study was carried out in free as well as immobilized algae. ZnO was incorporated in the immobilized algal cells to increase the permeability of membrane and also to increase the detection limit. Thus, ZnO incorporated whole cell ISFET biosensor was fabricated for the determination of OPs.

Experimental

Material

2-Phospho-L-ascorbic acid trisodium, $MgCl_2$ were purchased from Sigma-Aldrich (India) and used without further purification. NaOH and $Zn(NO_3)_2$was purchased from S D Fine-Chem Limited (India). Bovine Albumin serum (BSA) and gluteraldehyde were purchased from LobaChemie (India).

Algae Culture

Algae *Chlorella* sp. was identified, cultured and sub-cultured in BG -11 [93] media every three weeks. Algae was harvested by centrifugation and starved by suspending in phosphate free BG-11 media to induce maximum alkaline phosphatase activity.

Instruments

All experiments were performed on ISFET pH sensor model no. 3330, Agilent U8001A single output DC power supply (0-30 V, 3A) and Agilent U1233A True RMS Multimeter.

Cell Immobilization

Algae *Chlorella* sp. having surface bound AP was immobilized on the surface of ISFET electrode by following method:

1. 75 mg of BSA was added to the starved algal solution (1mL).
2. By drop method,20µL of above solution was deposited on electrode and placed in gluteraldehyde vapour for two hours.
3. After drying at room temperature electrode was washed with distilled water before experiment.

For ZnO incorporated algal immobilization, different amount of ZnO powder (e.g.3, 5, 7 mg) were added to stock solution (1 mL of algae and 75 mg of BSA) and same steps were followed as above.

Synthesis of ZnO

ZnO nanoparticles were prepared from $Zn(NO_3)_2$salt precursor and NaOH. 100 mL solution of 0.2 M metal salt precursor was prepared in water by dissolving 5.94 g of $Zn(NO_3)_2$.The solution of $Zn(NO_3)_2$was put on stirring for 15 minutes at 60^0C. Meanwhile, NaOHsolution of 0.5 Mconcentration was prepared by dissolving2 gof NaOH in 100 mL water andheatedupto 60^0C. After heating,NaOHsolution was added to $Zn(NO_3)_2$solution at continuous stirring within one minute. The reaction mixture was allowed to stirred at 60^0C for 1 hour.Then reactor was allowed to cool at room temperature without stirring. White colored precipitates of ZnO began to form in mother liquid. The solution was left for aging overnight and washed several times with water to remove impurities. Prior to each washing, 15 minutes of ultra-sonication was done for proper washing.After washing precipitates were dried in oven at 80 ^{0}C [70].

Pesticide testing

Solutions of organophosphate pesticides were prepared from the concentration range 10^{-1}M to 10^{-9}M in ethanol and water mixture in ratio of 6.4. Organophosphate pesticides selected for thestudy includedchlorpyrifos, triazophos and malathion.

ISFET study

Experiments for the detection of pesticides were done with both free as well as with immobilized algae.In case of free algae (i.e. without immobilization), experiment was done by adding *Chlorella* sp.(2%)in a solution containing $MgCl_2$(10^{-3} M)which is an enzyme activator and2-Phospho-L-ascorbic acid trisodium salt was added as a substrate with constant stirring. Ascorbic acid was generated as a product and current was measured by using ISFET transducer as pH sensor. Same procedure was followed in case of immobilized algae i.e., with and without ZnO. The only difference in the procedure

was that enzymenot added in the solution instead immobilized on the surface of ISFET electrode.

Results and Discussion

XRD analysis of ZnO

The XRD pattern of the powder material was studied with the diffraction angle 15° - 80° as shown in Fig 3.The diffraction peaks for 2θ = 31.71°, 34.40°, 36.42°, 47.58°,56.84°, 63.12°, 66.52°, 68.12°, 69.18°, 72.58°, and 77.08° wereobserved, which are matching with the ZnO hexagonal phase of JCPDF No. 36-1451. The crystallite size of ZnO,determined using Scherrer formula is found to be~ 30 nm.

$$D= 0.89\lambda/\beta \cos\theta$$

Where, D is crystalline size, λ is wavelength of X-ray (0.15406 nm), β is peak width at half-maximum (FWHM) and θ is Bragg diffraction angle.

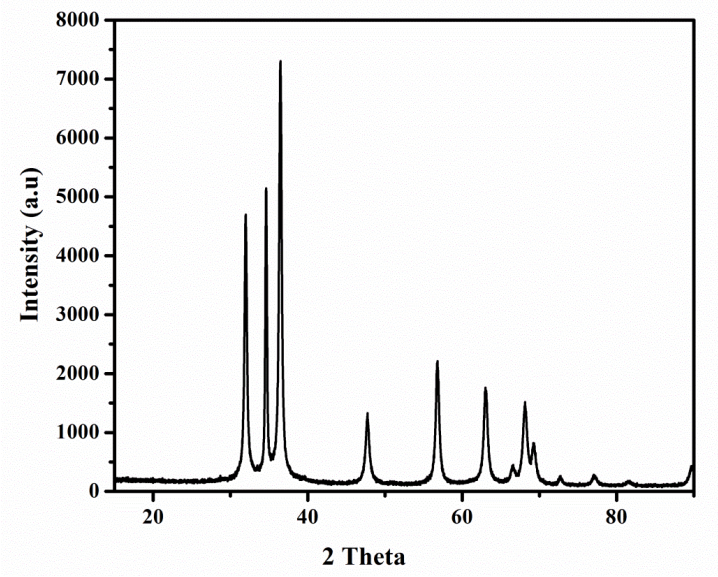

Fig. 3 XRD pattern of ZnO NPs

SEM analysis of ZnO

The morphology and the grain size of ZnO NPs were elucidated from SEM images as shown in Fig. 4. It is apparent from SEM image that nano ZnO particles have arranged over one another in a flower shape manner. The estimated grain size is approximately 500 nm.

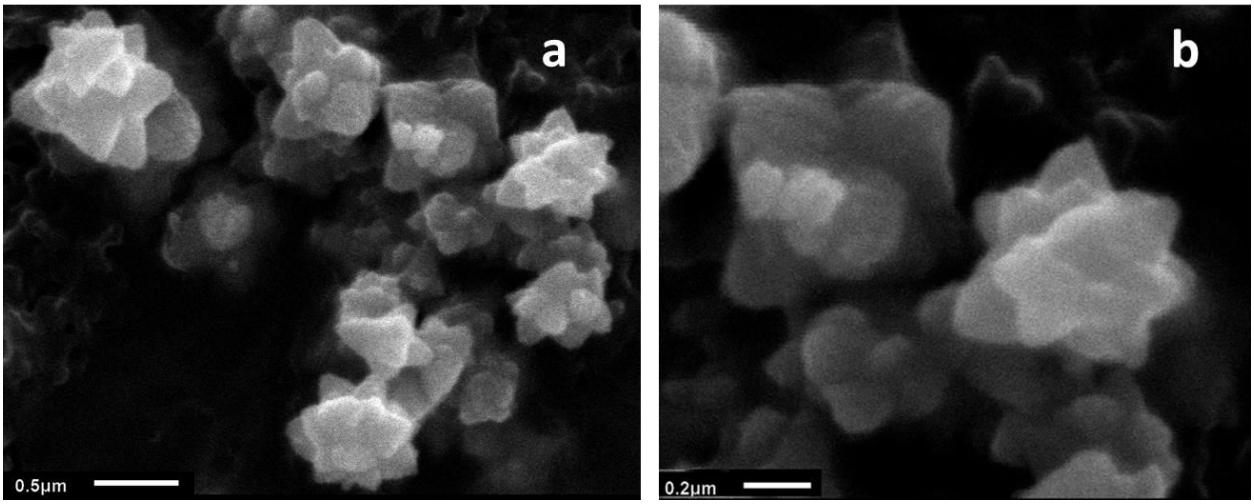

Fig. 5 SEM image of ZnO NPs

FT-IR spectra of ZnO NPs

FT-IR spectra of ZnO NPs are shown in Fig. 5. The absorption peaks at 470 cm^{-1} corresponding to Zn–O vibrations, suggest the presence of ZnO NPs. Absorption peak observedat 3433 cm^{-1} is due to O–H stretching vibrations. In addition, the small intensity peaks at 1636, 1482 and 1429 cm^{-1} were assigned to COO^- ions, which were difficult to remove completely even after intense washing.

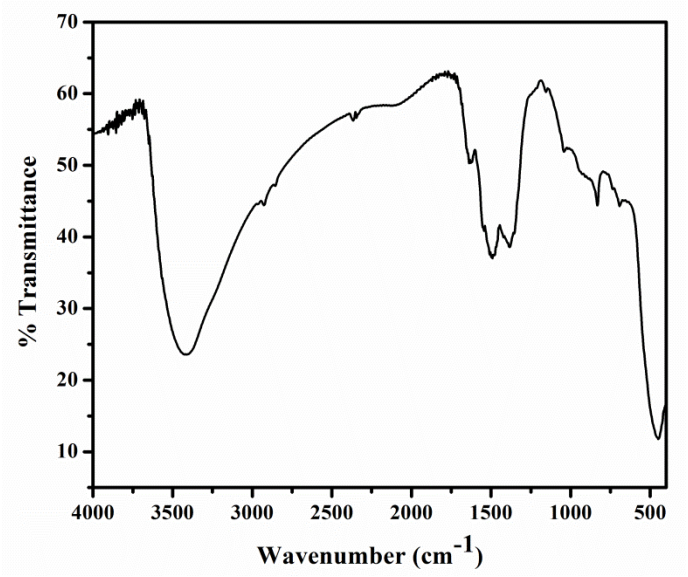

Fig. 5 FT-IR spectra of ZnO NPs

EDS analysis ofZnO NPs

Electron dispersion spectrometric (EDS) analysis of ZnO NPs is shown in Fig. 6. Element analysis shows the presence of Zn and O in the weight percentage of 73.79 and26.21 respectively.

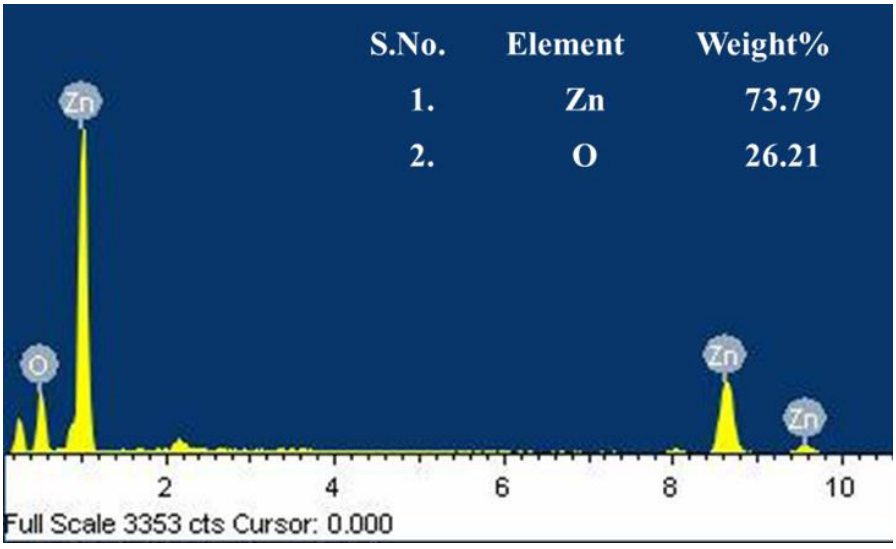

Fig. 6 SEM-EDSof ZnO NPs

ISFET based whole cell biosensor for detection of pesticides

Detection principle

Alkaline phosphatase (AP) enzyme present on the cell membrane of algae *Chlorella* sp. is used as a tool for the detection of pesticides.AP is a metalloenzyme containing Mg^{2+} and Zn^{2+} ions and serine residues at its active sites. AP dephosphorylates2-phospho-L-ascorbic acid (PAA)to generate product ascorbic acid which further releases H^+ ions. ISFET transducer detects these H^+ ions and produce current equivalent to their concentration. Presence of pesticide would hinder the formation of ascorbic acid (AA) thereby decreasing the magnitude of current. This decrease indirectly corresponds to the concentration of pesticide present.

Organophosphorus pesticides (OPs) under study are chlorpyrifos, triazophos and malathion. These OPs inhibit enzyme to different extent depending upon the type of interaction they are forming. In our study, inhibition study was carried out in three different ways

(i) By direct interaction of enzyme and substrate without immobilizing thealgae.

(ii) By immobilizing algae on gate surface of ISFET Ta_2O_3electrode.

(iii) By immobilizing ZnO NPs incorporated-algae on surface of ISFET Ta_2O_3electrode.

Optimization of parameters

For the finest functioning of biosensor it is required that the working parameters such as enzyme concentration and substrate concentration should be optimized. It is well-known that the current generated isdue to the presence of product ascorbic acid (AA) that isproduced by the enzyme alkaline phosphatase (AP) from substrate 2-phospho-L-ascorbic acid (PAA). Therefore, the magnitude of current generated depends upon the amount of the product AA formed, which further depends upon the amount of algal cells,substrate and incubation time.These parameters were measured using bare ISFET as a sensor of H^+ ions are described as follows:

(A) Standardization of substrate

To understand the effect of different substrate concentrations on enzyme, experiments were conducted by taking different substrate concentrations in the range of 0-0.5 mL for both, with and without immobilization. From the results, as shown in given Fig. 7, it can

be seen that minimum concentration of 0.2 mL of substrate is required to get stable current magnitude. Thus, all the experiments were done at this concentration.

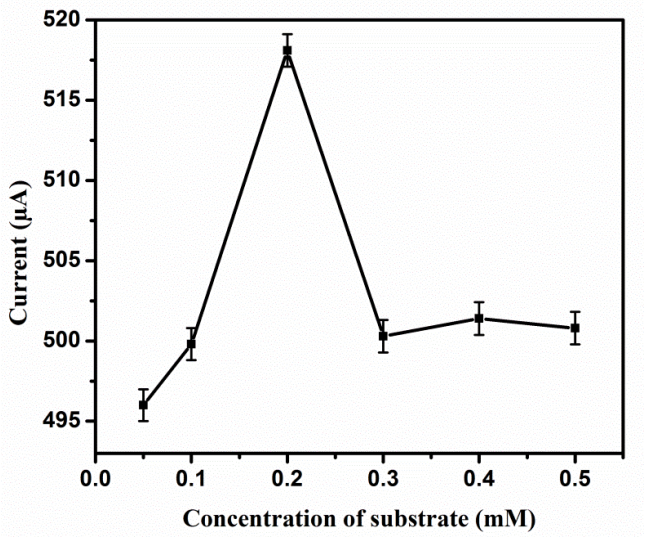

Fig. 7 Plots of current as a function of substrate concentration for ascorbic acid phosphatase system using bare ISFET as H^+ - ion sensor

(B) Standardization of Algal cells

Experiments were conducted foroptimization of algal cells concentration to obtain maximum current during experiment.

In case of without immobilization

Different amounts of the algal cells were taken from 0.1 mL to 0.5 mL and current for each amount of thealgal cells were measured. Results are given in Fig.8 and it is seen that the current increases with increase in amount of algal cellsup to 0.2 mL. Beyond this concentration, the current decreases. Hence, 0.2 mL can be taken as optimized concentration of algal cells. Further increase beyond 0.2 mL leads to overcrowding of algal cells, resulting in decrease of current.

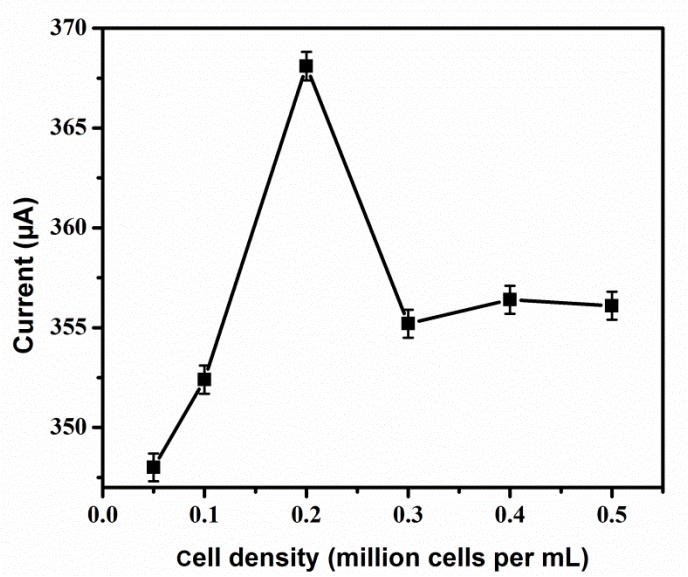

Fig. 8 Plots of current as a function of cell density for ascorbic acid phosphatase system (without immobilization)

In case of without immobilization

In this case,algal cellswere immobilized on the surface of electrode using BSA-gulteraldehyde system. For the immobilization, we have to optimize the quantity of algal cells which produces maximum current. From the Fig. 9it is evident that 20 µL of algal cells produces maximum current. Thus, for further experiments this was optimized concentration.

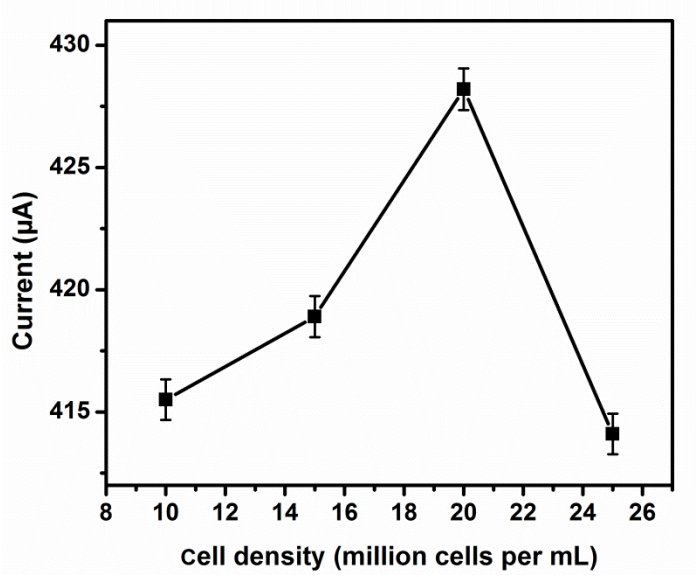

Fig. 9 Plots of current as a function of cell density for ascorbic acid phosphatase system (with immobilization)

(C) Standardization of incubation time

The incubation time is the time given for the interaction of enzyme with substrate to produce signal. This factor is crucial factor.

In case of without immobilization

Enzyme activity measurement experiments was carried out at different durations of incubation of the mixture solution. Samples were incubated from 1 to 7 hours to know the most stable incubation stable time. Results are shown in Fig. 10. It can be seen that after 3 hours of incubation, there was no further change in current. All subsequent experiments of enzyme without immobilization were run with 3 hours incubation period.

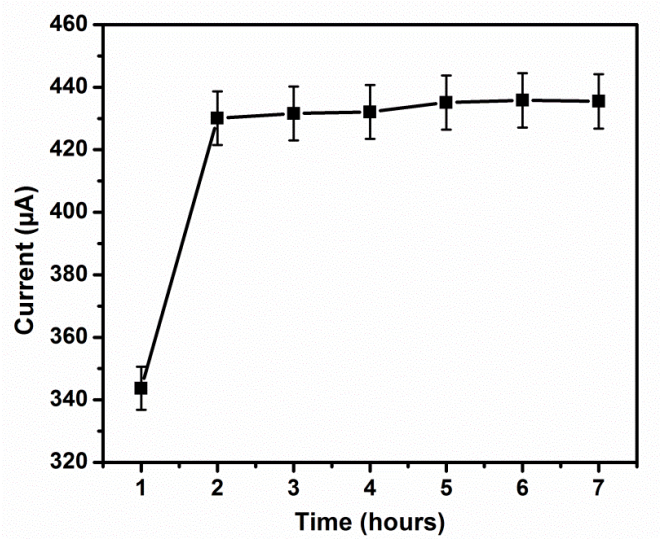

Fig. 10 Plots of current as a function of time for ascorbic acid phosphatase system (without immobilization)

In case of immobilization

Samples were incubated from 1 to 7 minutes to know the most stable incubation time. By optimizing the time, we are able to know how much time is required by the ions to pass through the membrane. Results are shown in Fig.11. It can be seen that after 3 minutes of incubation there was no further change in current magnitude. Thus, all subsequent experiments were run with 3 minute of incubation period.

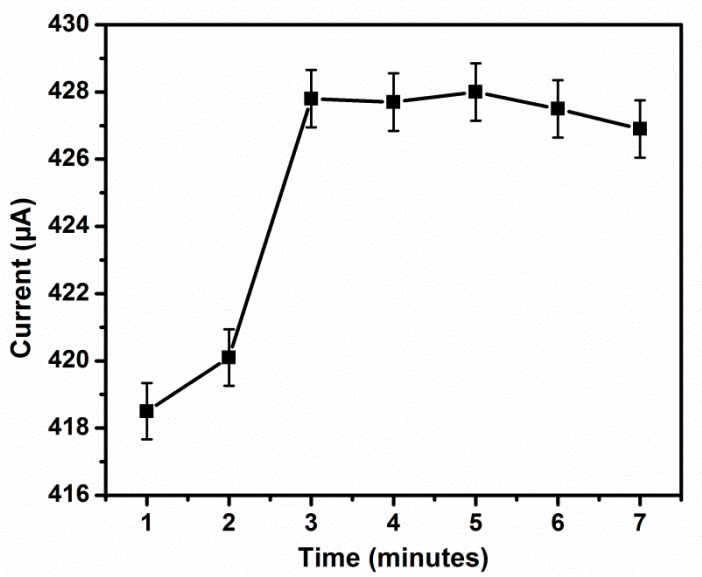

Fig. 11 Plots of current as a function of time for ascorbic acid phosphatase system (with immobilization)

Inhibition Study of Pesticides

OPs act as enzyme inhibitors and different pesticides inhibit enzyme activity to different extent. The extent of inhibition depends upon the complexation that occursbetween enzyme and the inhibitor, more the complexation, more will be the inhibition. Pesticides alter the active site structure of enzyme, thus shutting its normal functioning.

In our study, inhibition study was carried out in three different ways (i) By direct interaction of enzyme and substrate without immobilizing the algae (ii) By immobilizing algae on the surface of ISFET (Ta_2O_3electrode) (iii) By immobilizing ZnO NPs incorporated -algae on surface of ISFET Ta_2O_3electrode.It has been observed that in all three cases the inhibition trend of pesticides were same i.e.Chlorpyrifos shows highest inhibition then triazophos and least inhibition is shown by malathion.

(i) By direct interaction of enzyme and substrate without immobilizing algae

In this method,algal cells having surface bound AP enzyme, substrate, $MgCl_2$, pesticide, water all were put together on stirring then current was measured through the transducer ISFET. Chlorpyrifos shows highest inhibition rate upto concentration 10^{-9}M while triazophos upto 10^{-5}M. Least inhibition is shown by malathion up to only 10^{-3} M.Calibration curves were obtained for different concentration ranges for the different

OPsas shown in Fig. 12. Percentage inhibition is plotted against pesticide concentration. Beyond a linear trend of inhibition the results were irregular and irreproducible.

Percentage inhibition can be calculated as **% inhibition = I_o-I/I_o×100**

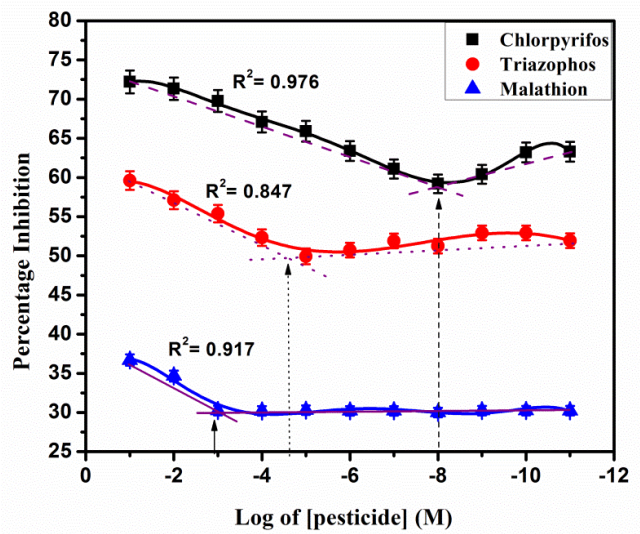

Fig. 12 Calibration curves for chlorpyrifos, triazophos and malathion (without immobilization)

(ii) By immobilizing algae on surface of ISFET Ta_2O_3electrode

In nextsystem,algal cellswere immobilized on the surface of Ta_2O_3electrode with the help of bovine serum albumin and gluteraldehyde. Immobilization has decreased the response time i.e. signal can be obtained in minutes rather than in hours. However, the major disadvantage was the membrane permeability. Because of this, the current magnitude was less as compared to the system where there was direct interaction between the product formedand surface of electrode. The trend of inhibition of enzyme activity remained same i.e. chlorpyrifos> triazophos >malathion.Nevertheless,due to low permeability, chlorpyrifos can only showed a low detection limit of 10^{-8} M, which is less as compared to the former. To overcome this problem, ZnO NPs were incorporated into the system.

(iii) By immobilizing ZnO NPs incorporated -algae on surface of ISFET Ta_2O_3electrode

In third method,optimized amounts of ZnO NPs were immobilized along with the algaeas shown in Fig. 13 about 5 mg wasadded in the algal cells for immobilization. Incorporation of ZnO greatly enhanced membrane permeability, hence current magnitude has increased to many folds as compared to ZnO unmodified immobilized electrode and

also low detection limit for chlorpyrifos up to 10^{-9} M was obtained.This effect can be credited to highly conductive surface of ZnO and H donors in this region.

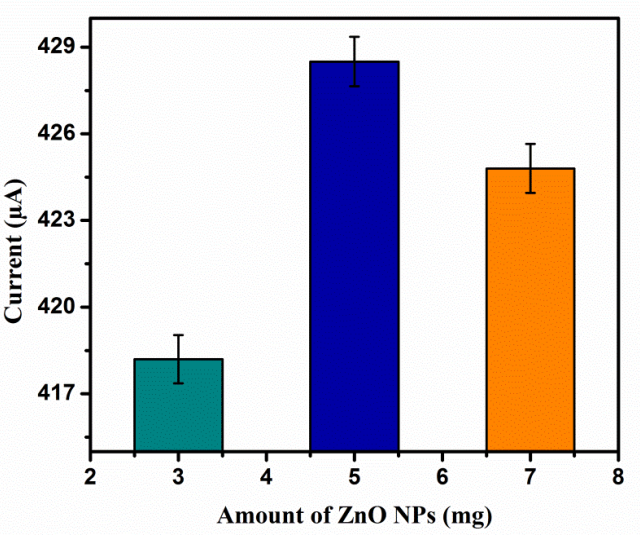

Fig. 13 Current vs amount of ZnO NPs for immobilization

Taking chlorpyrifos (CPF) as a model compound,Fig. 14 showedcalibration curves obtained for three set of experiments, asdiscussed above.Itdemonstrates the comparison between percentage inhibition of enzyme activity obtained in presence of enzyme inhibitor in case of without algal immobilization, with algal immobilization and ZnO incorporated algal immobilization.From graph, it can be seen that percentage inhibition is higher in case of ZnO incorporated algal immobilization for detection of CPF.

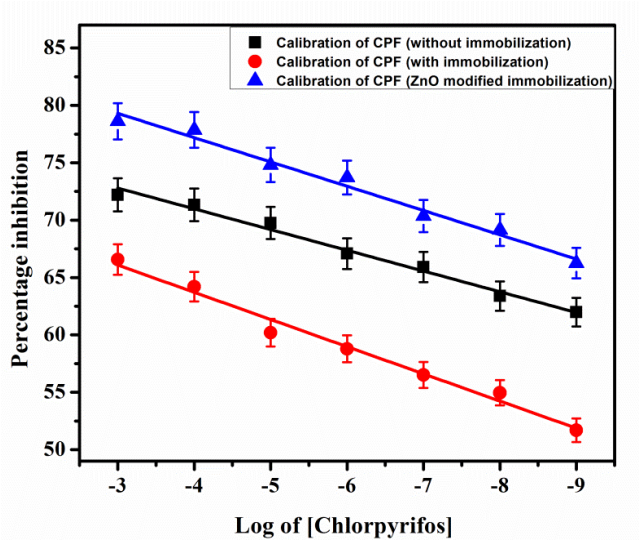

Fig. 14 Comparison calibration curves for Chlorpyrifos (CPF)

Similar trend were seen in case of triazophos and malathion as shown in Fig. 15 and Fig. 16 respectively.

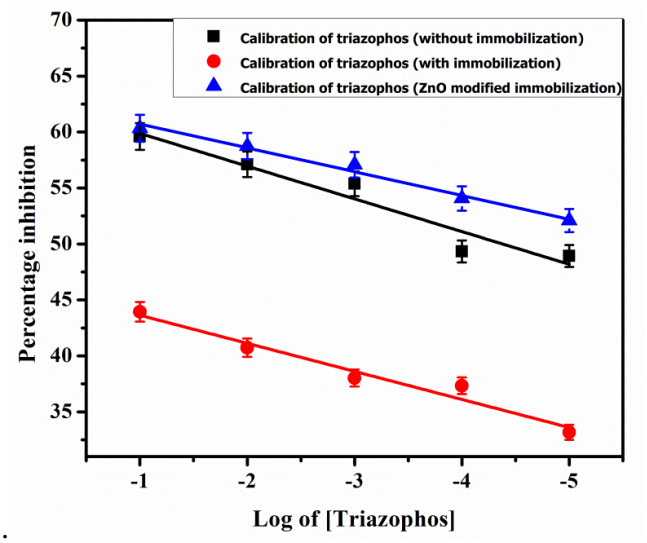

Fig. 15 Comparison calibration curves for Triazophos

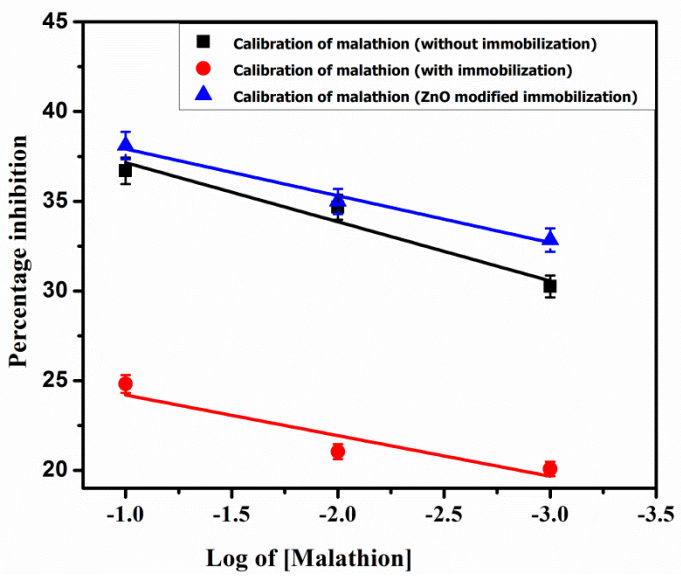

Fig. 16 Comparison calibration curves for Malathion

Interference Study

From the study, it can be noticed that chlorpyrifos showed maximum inhibition up to10^{-9} M in case of ZnO modified electrode and 10^{-8} M in case of unmodified electrode. So

taking it as primary analyte, interference study was carried out in presence of triazophos and malathion. The concentration range studied for chlorpyrifos was 10^{-9} - 10^{-1} M and that of interfering species was 10^{-4} M. It was found that the current increased for triazophos while malathion decreased the magnitude of currentappreciably. Overall there was no change in inhibition trend.

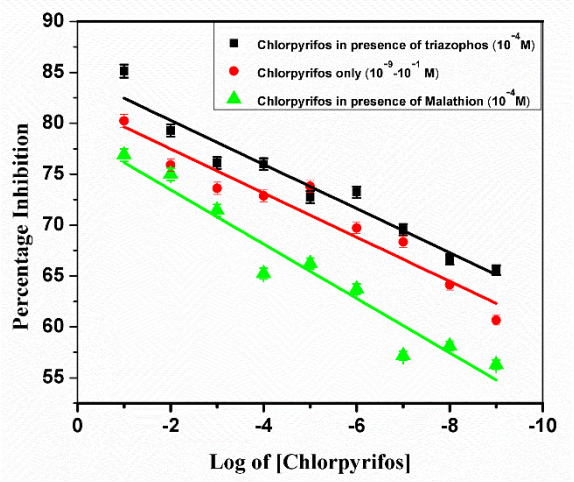

Fig. 16 Interference study for chlorpyrifos

Conclusion

Flower shaped ZnO NPswere synthesized from precursor $Zn(NO_3)_2$ and they were used in the fabrication of ISFET biosensor for detection of OPs.In our study, three experimental sets were done which include (i) By direct interaction of enzyme and substrate without immobilizing algae (ii) By immobilizing algae on surface of ISFET Ta_2O_5electrode (iii) By modifying electrode surface by ZnO NPs incorporated –algae. OPs studied were chloropyrifos, triazophos and malathion. Among these, chlorpyrifos showed highest AP enzyme inhibition and for malathion was the least. The basis of detection of OPs was inhibition of AP (enzyme present on the membrane of *Chlorella* sp.) and the interaction was detected by ISFET transducer. ISFET transducer is a pH based sensor which gives current change on the change of the pH of the solution. It was found that among three methods studied ZnO modified electrode showed better results.

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