Voltammetric algal biosensor for Hg (II) ion improvised with ZnO nanoparticles

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Partial fulfilment of the requirements for the degree of

Master of Science in Chemistry

Submitted by

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Certificate

This is to certify that the thesis entitled **"Voltammetric algal biosensor for Hg (II) ion improvised with ZnO nanoparticles"**, being submitted by **Ms. Bhumika Bhardwaj** in partial fulfilment of the requirements for the award of degree of Master of Science in the School of Chemistry and Biochemistry, Thapar Institute of Engineering & Technology, 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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Candidate's declaration

I hereby declare that the work being presented in the thesis entitled **"Voltammetric algal biosensor for Hg (II) ion improvised with ZnO nanoparticles"**, in partial fulfilment of the requirements for the award of the degree of Masters in Chemistry, School of Chemistry and Biochemistry, Thapar Institute of Engineering & Technology, Patiala, is my own work during the period of January 2018 to June 2018, under the supervision of Dr. Susheel Mittal, Senior Professor, Thapar Institute of Engineering & Technology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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

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Abstract

Chlorella sp. algae whole cells were immobilised on the surface of glassy carbon electrode and platinum electrode for detection of mercury ions. To improve the sensitivity and detection limit, immobilized electrodes were modified with flower shaped ZnO nanoparticles. ZnO nanoparticles formation was confirmed by various techniques such as FTIR, SEM, photo- luminance (PL), EDS and DRS. Various parameters such as algal concentration, response time, substrate concentration, pH of buffer were optimised for better results. Chronoamperometry, cyclic voltammetry (CV), and differential pulse voltammetry (DPV) techniques were performed for the detection of mercury ions at different concentration.

The studies were investigated using algal/GC electrode, ZnO modified algal GC electrode, algal/platinum electrode and ZnO modified algal platinum electrode. It was found that incorporation of ZnO nanoparticles have increased the overall performance of biosensor in terms of detection limit, current response and sensitivity. Results were more effective in ZnO/algal/GC biosensor with the sensitivity 5.42µA/M. ZnO modified algal GC electrode could detect Hg(II) ions through upto 10^{-13} M by chronoamperometry and 10^{-9} M by DPV. Further, ZnO modified algal platinum electrode could detect Hg(II) ions in concentration range of 10^{-11} M -10^{-3} M. Interference study was carried out by interfering ions such as Ag^{+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} with Hg(II) ions. It was found that the proposed biosensor could detect Hg(II) ions without any inference with above mentioned ions.

INTRODUCTION

The term 'heavy metal' is defined as the metallic element that has a high density or has relative high atomic weight. Some heavy metals like cobalt (coenzyme), copper (co-factor in enzymes), iron (haemoglobin, myoglobin) and zinc (in enzymes) have essential physiological roles in human body at trace concentration levels. However, a few heavy metals such as mercury (Hg^{2+}) are the most toxic and have adverse effect on environment and human health even at low concentration. Mercury contamination can be caused by natural sources including volcanic and oceanic emissions, forest fires or by human activities such as combustion of fuels, discharge from gold mining, pulp, paper and hydroelectric industries. Hg^{2+} is non-biodegradable in nature, thus persist in environment for longer period. It enters our food chain and accumulate in human beings causing various diseases. It is likely to invade and potentially damage digestive system, central nervous and endocrine system of the human body.[1]

Most frequently used traditional methods for Hg^{2+} determination are atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS) and fluorimetric detection.[2] These methods are highly selective, sensitive but suffer some drawbacks in term of complicated instruments and professional handling, which limit their utility in mercury monitoring. Therefore, a convenient and sensitive approach is required. Biosensors provide a better alternative to above techniques in respect of easy handling, continuous online analysis and are cost effective.

Biosensors

Biosensor is a device that uses biological signals from analyte and convert it into electrical signals. Biosensors play very significant role in detecting analyte, in the field of diagnostics, detecting the traces of harmful substance in saliva, urine etc. Working of biosensors are based on the two components – (i) on the bioreceptor which sense the analyte (ii) on the transducer which is responsible for the conversion of biological signals to electrical signals.[3] Transducer can be capacitance or voltage controlled oscillator.

Components of Biosensors

Main parts of biosensor are as follows:

- **Analyte:** Analyte is a material which is to be detected by biosensor. For example, Hg^{2+} ion is an analyte to be detected by biosensor.
- **Bioreceptor:** Bioreceptor is biological molecule which can be enzyme, protein, DNA or aptamer that interacts with analyte and produce signals.
- **Transducer**: It converts the signal generated by product of binding process of bioreceptor with desired analyte, analyses it and changes to electrochemical or physical signal, which is further processed and displayed. The strength of signals usually depend upon the concentration of analyte and bioreceptor interactions. There are various types of transducers like electrochemical (voltammetry amperometry, potentiometry), thermal, and polarimetric.
- **Electronics:** It is a component of biosensor, which operates the signal coming from transducer. Mainly, the signal is amplified by an amplifier and transform analog signals into digital signals.
- **Display:** Display generally represents the results of biosensor in more convenient way. The result is in the form of binary digit or in a graphical representation.

The working of a biosensor with its components is shown in Fig 1. Biological elements like enzymes and proteins are immobilized on transducer through covalent and non-covalent bonding. Analyte interacts with biological element, producing a chemical signals that is converted into electrical signal by transducer. Further, electrical signals are amplified and results are displayed on computer.

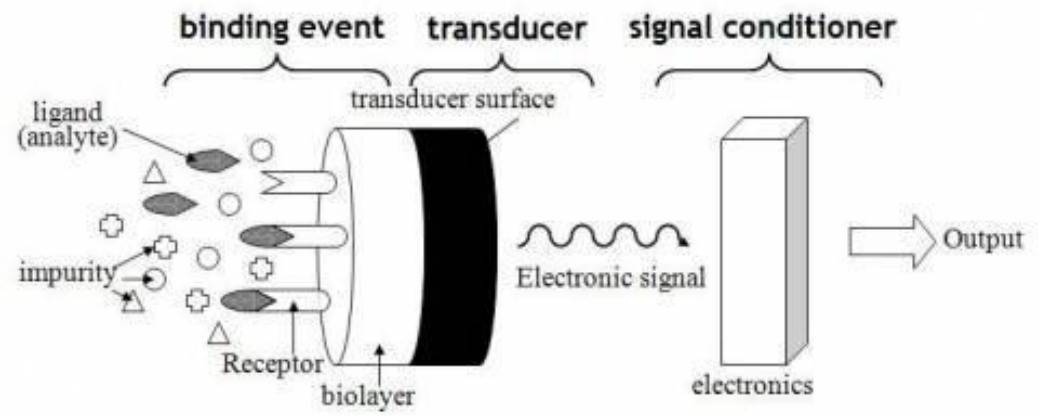

Fig. 1 Representation of a biosensor
(research.in.melab: biosensor [Medical Electronics Laboratory])

Types of biosensor

Biosensors can be categorized on the basis of bioreceptors and transducers, which are described as follows:

A) On the basis of receptor

a) Bio-catalytic biosensor

b) Bio complexing biosensor

c) Receptor based or antagonist based biosensor

a) Bio-catalytic biosensor

Bio-catalytic biosensor is based upon the use of catalysts as a bioreceptor. Catalysts are substances that alter or speed up the reaction. These catalysts may include enzymes, protein etc. This type of biosensors may follow either of the two mechanisms, (i) first mechanism involves the catalytic transformation of a species, (ii) Second mechanism involves the sensing of analyte by inhibiting the enzyme activity. These type of biosensors are concerned with enzyme kinetics and their stability under typical conditions. These are further of three types: (i) Enzyme based, (ii) Whole cells based, (iii) Tissue based.

b) Bio-complexing biosensor

This type of biosensor includes the binding of analyte with macromolecules that has been separated from biological environment. An equilibrium is achieved, which is detected by detector. After this equilibrium, there is no net consumption of macromolecules. The best known example of an affinity biosensor is the enzyme-linked immunosorbent assay (ELISA). The affinity biosensors are designed in such a way that dissociation of target analytes is minimized. So, these sensors do not provide kinetics information of the analyte.

c) Receptor based or antagonist based

Antagonist are substances that interfere or inhibit the activity of analyte or other molecule. Ion channel membrane receptors have been used in conductometric or optical biosensors. A method that is based on immobilization and stabilization of a bioreceptor for the detection of analyte.[4] There is a polymer film, which contains bioreceptor in it and is capable of binding with analyte. Specificity of bioreceptor for the analyte is the important parameter for the detection of analyte. For example, aspartate as a target antagonist may be determined in the presence of various interfering antagonist by

detecting sodium ions.

B) Based on transducer

Electrochemical transducers can be of various types for sensor applications such as potentiometry, voltammetry, amperometry and conductometry.

1. **Amperometric:** This type of biosensor converts electrochemically non- active species into product that can be oxidized or reduced at a working electrode. It measures current by the movement of electrons at a constant voltage. Concentration of substrate is directly proportional upon the amount of current produced at the electrode. This type of sensor is similar to potentiometric biosensor in terms of response time, dynamic range and sensitivities. Common example of amperometric biosensor is Clark oxygen electrode. It is a first glucose biosensor invented. This type of biosensor is based on the reduction of O_2 and determines the glucose by glucose oxidase enzyme.[5]

2. **Potentiometric:** These biosensors use ion selective electrodes, which convert biological signals into electrical signals. This type of transducer based biosensor is used for the detection of organic as well as inorganic materials. This type of sensor measures activity of the analyte with respect to internal standard. Signal obtained is in the form of voltage and is independent of the size of sensor. Common examples are carbon dioxide and ammonia selective electrodes. In this type of biosensor, potential difference is directly proportional to amount of the analyte used. The main advantage of this sensor is its simplicity and adaptability.[6]

3. **Conductometric:** This type of biosensor uses ionic species and measure the electrical conductivity that are measured in terms of ohmmeter. These biosensors do not require reference electrode. These biosensors prevent faradaic processes on electrodes.[7] The major drawback of this type of biosensor is that enzymes are sensitive to ionic concentration such as H^+ and NH_4^+. Common example of conductometric biosensor is urea based biosensor. In addition, this type of biosensor is used to determine the urea concentration in blood serum, glucose in human body. Other common examples include ion-selective field effect transistors (ISFET). This type of sensor is used to monitor pH during open-heart surgery.[8]

Optical Transducer based biosensor

Optical technique that could potentially be used in biosensors is UV- visible spectroscopy, fluorescence, luminescence (bioluminescence, chemiluminescence) and optical fibres. Here there is no requirement of reference electrode.[9] This type of biosensor

produce signal which is directly proportional to the concentration of the analyte. For example, optical biosensor is used for checking the amount of glucose in human body. Fibre optics are used to detect dissolved oxygen, carbon dioxide and pH of a substance. The main application of this type of biosensor is to detect the oxygen concentration by identifying the effect of oxygen in dye Piezoelectric Transducers based biosensor.[10]

Piezoelectric transducer based biosensor

The word 'piezo' means pressure. This type of biosensor converts biological signals into electrical signals by applying pressure. Enzymes, antibodies, and antigens are used as bioreceptors in this type of biosensor. This type of biosensor is used in automobiles, home security alarms and in object detectors. These are used in various forms like actuators, cantilever, and ultrasonic piezoelectric biosensor.[11]

CHARACTERISCTICS OF BIOSENSORS

An ideal biosensor depends upon the following characteristics which result in better performance. These characteristics not only increase the performance level but also enhance its reproducibility. These characteristics are mentioned below:

Accuracy: Accuracy is one of the most important factor of an ideal biosensor. It can be defined as the capability of a bioreceptor to analyse an analyte containing other components of the substrate.

Reliability: It is one of the main principles of scientific method. It is the ability of an entire experiment to be duplicated either by the researcher or by the person working independently. In other words, reliability is replication of the experiment.

Constancy: It is the ability of biosensor to remain unchanged over a time or a condition. Conditions include temperature and humidity that effect the response of transducer. Also binding between analyte and bioreceptor affects the response of biosensor. If the binding between analyte and bioreceptor is strong then there would be strong covalent and ionic interactions which ultimately result in greater stability of the biosensor.

Susceptibility: It is the ability of responsiveness to internal and external changes occurring in biosensor. Biosensor must have a capability to detect the analyte in trace amount of concentrations.

Compatibility: Biosensor must be able to carry out from one environment to other.

Linearity: Linearity depicts the correctness of measured data by a straight line. A range

of analyte concentration is measured which changes the response of biosensor linearly with the concentration.

Modification of electroanalytical biosensors by incorporating nanostructures

The efficiency and sensitivity of a biosensor can be increased by integrating nanostructures into its assembly. These are immobilized on the surface of electrode and act as an enhancer for sensing the analyte. Various types of nanomaterials are available such as Au, Cu Ag nanoparticles and metal oxide nanoparticles like CdS, Fe_2O_3, CuO. ZnO based metal oxide nanoparticles are better for fabrication of biosensors, as these are non-toxic, easy to synthesize, have high surface area and high electron bandgap of 3.31 eV. Modifying biosensor with ZnO nanoparticles would increase the sensitivity and have less response time, which is beneficial for performance of the biosensor. Apart from this, ZnO has high isoelectric point, which increases the ability of immobilization of enzyme which has low isoelectric strength through electrostatic interactions. Moreover, large surface area would provide more surface active sites for analytes to bind and have high rate of electron transfer from bulk to electrode surface .[12] And also, the shape of ZnO nanoparticles have great impact on the preface of a biosensors. In the present study, flower shaped ZnO based electroanalytical algal biosensor has been developed for the detection of mercury ions in aqueous solution. Flower shaped ZnO has three- dimensional structure thus, have high electron transfer rate than one and two-dimensional nanoparticles.[13] This is due to the fact that surface morphology of particles also affect the surface defects and porous nature of ZnO nanoparticles. Three-dimensional nanoparticles have better electron transfer mechanism, greater efficiency in catalytic activity and have better adsorption ability for the analytes than the one and two-dimensional ZnO.

In the present study, flower shaped ZnO incorporated *Chlorella* sp. algae based biosensor for the determination of mercury ions has been fabricated. The comparison study of two electrodes i.e. glassy carbon (GC) and platinum (Pt) has been discussed.

LITERATURE SURVEY

There were various attempts and efforts which have led to the progress and development in the research on biosensors in last five years. These electroanalytical techniques for the detection heavy metal ions in various analytes have increased the demand of biosensor in scientific community.

Whole cell based biosensor

P-benzoquinone-mediated biosensor was incorporated with Psychrobacter sp. by Wang *et. al.*, (2013) for the detection of heavy metal ions. EC_{50} values of Cu^{+2}, Cd^{+2}, Zn^{+2}, Cr^{+6}, Hg^{+2} and Pb^{+2} to Psychrobacter sp. were determined at incubation time 30min and were in the range of 2.6mg/L, 47.3mg/L, 10.9mg/L, 14.0mg/L, 0.8mg/L and 110.1mg/L, respectively.[14] Fibre optic biosensor was developed for the detection of heavy metal toxicity based on the marine bacterium *Aliivibrio fischeri* (*A. fischeri*) by Futra *et. al.*, (2014). They detected Cu(II), Cd(II), Pb(II), Zn(II), Cr(VI), Co(II), Ni(II), Ag(I) and Fe(II) ions in marine water.[15] A transgenic zebrafish biosensor for zinc and cadmium was developed by Pawar *et. al.*, (2015) for the detections of Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2}. Cadmium was considered to be the most potent inducer with 4.6-fold induction followed by zinc (2.3-fold).[16] Vopálenská *et. al.*, (2015) has designed a biosensor for the detection of copper ions. This biosensor was immobilised with *Saccharomyces cerevisiae* in alginate beads. They studied variation of concentration of copper ions on the basis of beads colour. The colour of beads appeared white if copper ions were present below the detection limit beads appeared red or pink if concentration of copper ions increased.[17] Similarly, Homaei (2017) has designed a biosensor which was based on Penaeus merguiensis alkaline phosphatase (PM ALP) immobilized on gold nanorods (GNRs) for heavy metal sensing. He has studied the kinetic behaviour and calculated the values of K_m and V_{max}.[18] A sensitive voltammetric method for detection of trace heavy metal ions were applied by Dali *et. al.*, (2018). Glassy carbon electrode was used which was immobilised by mixture of (SWCNTs/Biomass). The fungus was used as an inactive biomass. The detection limits were found to be 10^{-8} M and 10^{-7} M for lead and cadmium respectively.[19]

DNA based biosensor

In this survey, Au@Ag core-shell nanoparticles were prepared and further functionalized by DNA Enzyme to fabricate a biosensor. Linear range of current was found to be in the range of 0.002-20 μg L^{-1} with limit of detection of 0.006 μg L^{-1}. Cui *et. al.,* (2016) has developed biosensor on gold interdigitated electrodes (GIE) for the detection of heavy metal ion i.e., lead.[20] The sensor was fabricated by immobilizing GR-5 DNAzymes onto the GIE surface. Detection limit of 6.61 nM was observed which results into a greater sensitivity. Xie *et. al.,* (2016) has developed a biosensor for the sensitive detection of mercury (II) ions. They deposited the silver nanoparticles (AgNPs) on terminal deoxynucleotidyl transferase (TdT) extended ssDNA for signal output and cycling amplification. Biosensor showed a better response with a detection limit of 3 pM.[21] A biosensor for Hg^{2+} detection was fabricated that was based on dye sensitization effect induced by Y-shaped DNA transformation by Zhang *et. al.,* (2017). The current has increased linearly by the increase in the concentration of mercury levels in the range of 5pM - 500 pM with the detection limit of 1.5 pM.[22] Hu *et. al.,*(2018) has fabricated the biosensor using $[Ru(bpy)_3]^{2+}$/β-cyclodextrin-Pd nanoparticles (β-CY-PdNPs)/Gelatin (Gel) complex and ferrocene-labeled DNA probe (Fer-DNA) for the detection of Hg^{+2}. They have studied that in the absence of mercury there F-DNA based biosensor has regained its hairpin structure and results in quenching of $[Ru (bpy) 3]^{2+}$ signal. Current response was better in the range 0.003 ~ 600 ng/mL and detection limit was found to be 0.0015 ng/mL. Also DNA based biosensor was constructed for the detection of mercury ions using gold nanoparticles.[23] Fluorescence study was carried out and it was observed that number of fluorescent particles were decreased in the presence of Pb^{+2}.[24]

Enzymatic based biosensor

Biosensor was fabricated by using tassel multiwalled carbon nanotube (MT-MWCNT) by Moyo *et. al.,* (2014). Horseradish peroxidase (HRP) enzyme was immobilized on maize tassel multiwalled carbon nanotube (MT-MWCNT).[25] The inhibition rates were dependent on their concentrations in the range of 0.092–0.55 mg L^{-1}, 0.068–2 mg L^{-1} for Pb^{2+} and Cu^{2+} respectively. Similarly, the same enzyme was immobilised on polymerised neutral red (NR) for the detection of Cr (III) and Cr (VI) by Attar *et. al.,* (2014) .They studied the kinetic behaviour of HRP enzyme which

showed the interaction of chromium (+3) with I_{50} = 3.8 µM.[26] Ayenimo *et. al*., (2015) has introduced a method which was based on the inhibition of an ultrathin polypyrrole-glucose oxidase (PPy-GOx) on a biosensor for analysing the response of Cu^{2+}, Hg^{2+}, Cd^{2+} and Pb^{2+} ions.[27]Similarly, the same enzyme was immobilised on polymerised neutral red (NR) for the detection of Cr (III) and Cr (VI) by Attar *et. al.*, (2014) .They studied the kinetic behaviour of HRP enzyme which showed the interaction of chromium (+3) with I_{50} = 3.8 µM.[26] Ayenimo *et. al*., (2015) has introduced a method which was based on the inhibition of an ultrathin polypyrrole-glucose oxidase (PPy-GOx) on a biosensor for analysing the response of Cu^{2+}, Hg^{2+}, Cd^{2+} and Pb^{2+} ions. [27] A Glucose based biosensor was constructed by Rust *et. al.*, (2015). They have immobilised glucose oxidase nitrogen-doped carbon nanotubes (N-CNTs) for the detection of Ag^{+}, Cu^{+2} and Co^{+2} ions. They have immobilised glucose oxidase nitrogen-doped carbon nanotubes (N-CNTs) for the detection of Ag^{+}, Cu^{+2} and Co^{+2} ions. The Ag^{+} biosensor showed a sensitivity of 2.00 x 10^{8} ± 0.06 M^{-1}.[28] Do and Lin (2016) have fabricated biosensors by the immobilization of urease onto nano-structured polyaniline (PANi)-Nafion /Au/Al_2O_3. The sensitivity of lead ion was obtained to be 743.5 µA ppm for the linear dynamic range of 0.1-1.0 ppm Pb^{2+} .[29] Also a biosensor based on GOD immobilised on liposome reactor and chitosan (CS) was constructed for the detection of mercury by Yu *et. al*., (2017). Linear range was found to be from 0.5 to 5.00 µmol/L and detection limit was 0.076 µmol/L.Choline oxidase (ChOx) was immobilized on a glassy carbon electrode (GCE) and was modified with multiwalled carbon nanotubes (MWCNT) through cross-linking with glutaraldehyde.[30] The ChOx/ MWCNT/GCE gave a linear response from 0.1 to 1.0 nM Pb^{2+} and a detection limit of 0.04 nM. An amperometric urease inhibition-based biosensor was fabricated to detect Pb^{2+} and Hg^{2+} ions in water matrix by Gumpu *et. al.*, (2017).[31] A hydrogen peroxide enzyme based inhibition biosensor was constructed for the indirect determination of toxic mercury ions that has been reported by Elsebai *et. al.*, (2018). The limit of detection was calculated as 1.8x10^{-11} M. Also lead ions were detected by the inhibition of choline oxidase enzyme. The modified Pt/CeO2/urease electrode was developed by immobilizing CeO_2 nanoparticles. The developed sensor showed a detection limit of 0.019 ± 0.001 µM with a sensitivity of 89.2 × 10−3 µA $µM^{-1}$ for Pb^{2+} ions. A detection limit of 0.018 ± 0.003 with a sensitivity of 94.1 × 10−3 µA $µM^{-1}$ was achieved in detecting Hg^{2+} ions. . This

modified biosensor showed a fast response time (<1 s) with a linear range of 0.5–2.2 μM and 0.02–0.8 μM for Pb^{2+} and Hg^{2+} ions respectively.[32]

Nucleic acid biosensors

Biosensor based on functional nucleic acid is developed for the determination of Hg^{2+} and Pb^{2+} by Han *et. al.*, (2016). The biosensor show detection limit of 22 pM for Hg^{2+} and 20 nM for Pb^{2+}.[33] Similarly Bala *et. al.,* (2016) has developed gold disk electrodes immobilised with single-stranded PNA (peptide nucleic acid) for the determination of Hg^{2+}. Current difference increases within the concentration range 0.05 to 10 $\mu molL^{-1}$, while for higher concentrations, the saturation of PNA layer with Hg^{2+} is observed. Detection limit was found to be of 3.33 $nmolL^{-1}$.[34]

Aptamer based biosensor

Aptamer based biosensor was constructed for Hg^{+2} which was based on "T–Hg^{+2} –T" coordination by aptamers and "gate-controlled" amplification by switch of the channel probe by Li *et. al***.,** (2015).[35] The oxidative current was found to be decreased. Detection limit was observed at 5.0×10^{-15} mol/L. Wang *et. al.*, (2016) have developed an aptasensor based on graphene field effect transistor (FET) for detecting lead in children blood having detection limit less than 37.5 ng/L .[36]

Biopolymer based biosensor

Heavy metal ions were detected by biopolymer based biosensor constructed by Wong (2015). Here, β-carotene in palm kernel oil was used as the biological reporter and was embedded with by polyurethane polymer. The presence of copper (Cu), lead (Pb), zinc (Zn), and Al was detected through this technique. The results showed that the optical density has increased with the presence of heavy metals and Al within 0.1 mg/L – 10.0 mg/L.[37] An epoxy based graphite electrode was deposited by mercury film for the detection of lead ions by Cankurtaran *et. al.*, (2016). They studied the detection limit of lead for ten minutes. This was found to be as 0.98 μg/L.[38]

Protein based biosensor

The protein based biosensor was fabricated for the detection of silver ions. Mercury drop electrode (HMDE) was constructed and was immobilised with the heavy metal binding protein metallothionein (MT) for silver (I) ions detection. The detection limit

for silver (I) ions was estimated to be 500 nM. Biosensor was fabricated by using native proteins (albumin) that was bound with Cu^{+2} and got denatured. Wang *et. al.* (2014) demonstrated the K_D value of native and denatured albumin gold surface as 5.87 $\pm 0.2\times10^{-5}$M and 5.47$\pm$ 0.1×10^{-4} M respectively. This biosensor was used to detect Cu^{+2} with concentration below 0.1 mg/L in water, deionised water.[39] Hg^{2+} ion in water samples were detected by Prasad *et. al.,* (2016) using a biosensor modified with glutamine (Gln) and histidine (His) functionalized silver nanoparticles (Gln-His-Ag NPs). They observed of detection limit of 25.48 μM. The calibration graph was constructed between absorption ratios (A500/A402) and observed the concentration range in the range of 1.0 – 500 μM with the detection limit of 0.90 μM.[40] A fluorescence based biosensor was designed by immobilising a protein named of C-phycocyanin(C-PC) by Bhayani *et. al.,* (2016) for the sensing of mercury ions in polluted water. He has studied the fluorescence quenching ability of C-phycocyanin(C-PC) with different heavy metal ions.

Nanoparticles based biosensor

Wang *et. al.,* (2014) have developed a graphene and gold nanoparticles based biosensor for the detection of $Cu^{2+.}$ It was found that stripping peak current increased with increase in the concentration of Cu^{2+}. The limit of detection was found to be 0.028 nM (S/N = 3).[42] A biosensor was fabricated for the detection of heavy metal ions by Xue *et. al.,* (2016). They have used hemin @ reduced graphene oxide (hemin@rGO) immobilised with flower-like MnO_2 along with hollow AuPd (hAuPd-fMnO_2-hemin@rGO) and integrated with DNAzymes. This type of biosensor showed a response time between 0.1pM–200nM and detection limit was found to be 0.034pM.[43] ZnO nanofibers (ZnO NFs) glassy carbon electrode was fabricated by Liu *et. al.,* (2016) to detect current response for Cd (II) ions. They observed better current response from 4.8×10^{-9} to 1.3×10^{-6} mol L^{-1} concentration range. The detection of limit was found to be 1.8×10^{-9} $molL^{-1}$ (S/N > 3) .[44]

MATERIALS AND METHODS

Chemicals

Sodium hydroxide and zinc chloride were purchased from SD Fine-Chem Limited (India), p-nitrophenol (substrate), magnesium chloride (enzyme activator), Tris-HCl buffer (pH 8.5) were of analytical grade and purchased from Sigma-Aldrich, India. Bovine albumin serum and glutaraldehyde were obtained from Loba Chemie (India).

Instruments used

All experiments were done on potentiostat (Auto lab/PGSTAT12/Eco Chemie/Netherlands). Glassy carbon (GC) and platinum (Pt) electrodes were used as a working electrode, Ag/AgCl electrode (Metrohm make) was used a reference electrode, and platinum electrode was used as a counter electrode for the detection of Hg (II). Powder X-ray diffraction (XRD) patterns for ZnO nanoparticles were analysed using a XPERT-PRO diffractometer (Cu K α, ƛ = 0.15406 nm, 45 kV, 40 mA, step size = 0.013°). The morphology of ZnO nanoparticles were studied by using SEM (JEOL JSM-6510LV) and TEM (FEI TECNAI G^2 F-2D, 2 °A, 200 kV) instruments. Stretching and functional groups present in ZnO nanoparticles were analysed by Fourier transform infrared (FT-IR) instrument using Agilent Resolutions Pro (Cary 660) by preparing KBr pellets in the volume ratio of 1:100 (sample: KBr). For the elemental detection of ZnO nanoparticles, EDS (INCA x-act Oxford) instrument was used. Photoluminescence measurements (PL) of ZnO were done on a Perkin Elmer Spectrofluorometer (LS-45, Xe lamp).

Algal culture

Algae *Chlorella sp.* was identified, cultured and sub-cultured in BG-11 media every three weeks. Algae was harvested by centrifugation and starved by suspending in phosphate free BG-11 media for 15 days to induce maximum alkaline phosphatase activity.

ZnO nanoparticles preparation

ZnO nanoparticles were prepared from $ZnCl_2$, which act as a salt precursor. 5.5 g of $ZnCl_2$ was dissolved in 100mL of distilled water in a volumetric flask. This solution was kept under constant magnetic stirring until $ZnCl_2$ is totally dissolved in distilled water. Temperature of the beaker containing $ZnCl_2$ solution was raised to 90º C on heating mantle. Then, 2.0 g of NaOH was dissolved in 100mL of distilled water in a separate beaker. From the prepared NaOH solution, 16mL of NaOH was added to the beaker with constant stirring touching the walls of beaker. The solution turned into a milky colloid. Reaction was allowed to proceed for 2 hours after complete addition of NaOH solution. White colored precipitates of ZnO began to form. Washing was done and supertant was removed by centrifugation method. This technique was done to remove the impurities. Further the residues left behind were soniceted for 10-15 minutes for proper washing. Centrifugation and sonication steps were repeated 8-10 times to remove the impurities. After washing, precipitates were dried in oven at 80ºC. Dried powder of ZnO were used for characterisation and fabrication of biosensor.[45]

Preparation of modified biosensor

Glassy carbon (GC) and platinum (Pt) electrodes were polished with 1 mm of alumina slurry and then soniceted with ethanol and de-ionized water for 30 seconds, respectively. This step was done to remove suspended impurities on the electrode surface. Then, 3mg of ZnO nanoparticles were put in 1000µL of ethanol. Firstly, 10µL of ZnO nanoparticles solution was deposited on the glassy carbon electrode which acted as a working electrode. Electrode was kept for drying for half an hour.

Then, 1 mL of the starved algae was mixed with 75 mg of BSA. Further, 10µL of BSA-algae was immobilised on the ZnO immobilized electrode with the help of micropipette. The electrode was kept for drying in the atmosphere for 2 hours. Further, gluteraldehyde was deposited over the electrode and was dried for 2 hours. During this time, glutaraldehyde cross linked with algae through covalent bonding. This electrode was further used for the measurements. The same procedure was followed for immobilization of the algae on platinum electrode.

Thus, four electrodes were prepared, (i) algal immobilized GC electrode, (ii) ZnO incorporated algal immobilized GC electrode, (iii) algal immobilized Pt electrode, (iv) ZnO incorporated algal immobilized Pt electrode.

RESULT AND DISCUSSION

Structural, optical and morphological characterization of ZnO nanoparticles

Powder XRD analysis of ZnO nanoparticles

The crystalline structure of ZnO NPs was characterized using X-ray diffraction using CuKα radiation (λ = 1.54016 Å) over the range of 2θ = 10–80 degrees. The XRD analysis was employed to determine the phase structure and purity of the synthesized nanoparticles. A definite line broadening of the XRD peaks indicates that the prepared material consist of particles in nanoscale range. X−ray diffraction (XRD) patterns of ZnO NPs revealed that ZnO has hexagonal wurtzite structure. [12,46]

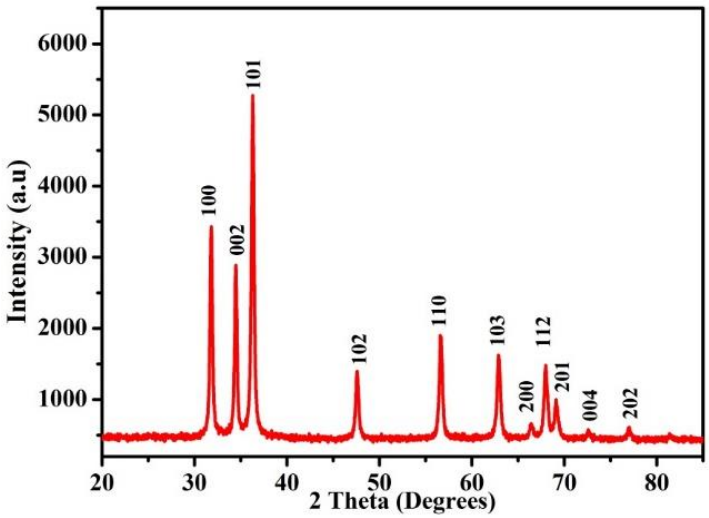

Fig. 2: Powder XRD analysis of ZnO nanoparticles

Peaks at 2θ value of 31.85°, 34.57°, 36.31°, 47.61°, 56.74°, 62.93, 66.30, 67.93, 69.24, 72.61, and 76.84 arising from the diffraction planes of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), (202) planes respectively, shown in Fig 2. The (101) diffraction peak in the XRD of ZnO NPs is more sharp as compared to (100) and (002) peaks, which implies that the preferred growth of NPs is in the (101)

crystallographic plane. The synthesized ZnO nanoparticle diameter was calculated using Debye-Scherrer formula as follows:

$$d = \frac{0.89\lambda}{\beta cos\theta}$$

where 0.89 is Scherrer's constant, λ is the wavelength of X-rays, θ is the Bragg diffraction angle, and β is the full width at half-maximum (FWHM) of the diffraction peak corresponding to plane (101). The average particle size of the sample was found to be approximately 40 nm which is derived from the FWHM of more intense peak corresponding to 101 plane located at 36.31° using Scherrer's formula. [46]

Optical characterization of ZnO nanoparticles

DRS and Photoluminescence (PL) spectrum

The DRS spectrum was taken in the range of 200-800 nm. Fig 3(a) shows absorbance as a function of wavelength with exciton absorption at about 390 nm. Photoluminescence (PL) spectroscopy is an important technique used to study the optical properties of semiconducting materials. Fig 3(b) showed PL spectra of ZnO nano powder recorded in range of 350 nm to 550 nm. It was observed that spectra have broad excitation peak at 383 nm and 406 nm which correspond to the blue region. These peaks originate either from the recombination of free excitations through exction-exction collision process or due to intrinsic defects such as oxygen and zinc interstitials. The peaks observed at 429 nm and 468 nm caused by radiative combination of a photo-generated hole with an electron. Another important fact says if PL intensity is high then it means rate of recombination of electrons and holes are high. [49]

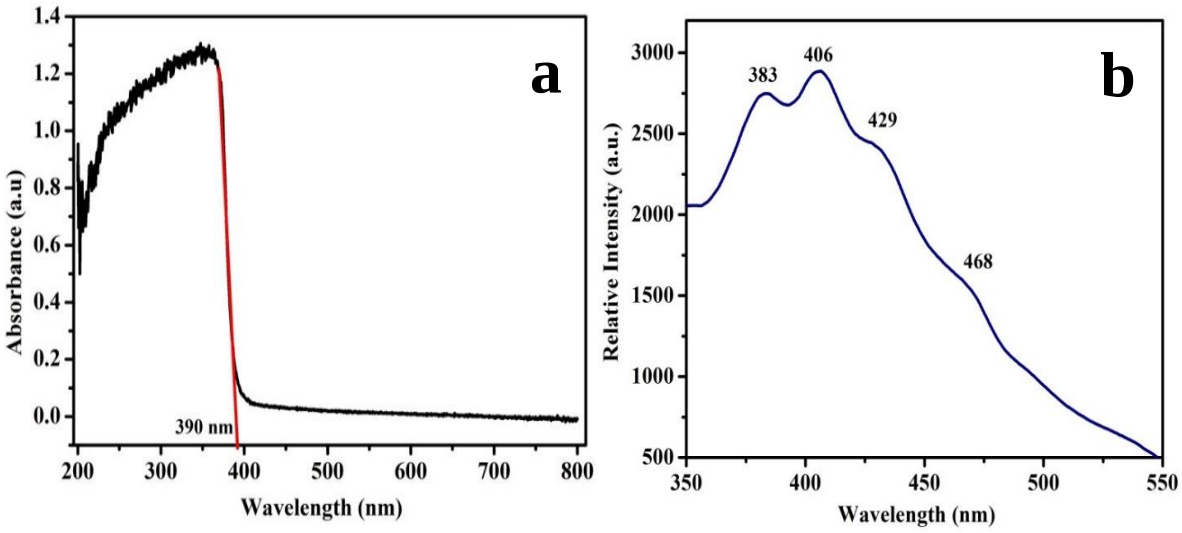

Fig.3: (a) DRS spectra of ZnO nanoparticles recorded in range 200 nm – 800 nm (b) PL spectra of ZnO nanoparticles recorded in range 350 nm – 550 nm

FT-IR spectrum

FT-IR study was done in order to check the purity and nature of the nanoparticles. Metal oxides generally give absorption bands in fingerprint region i.e. below 1000 cm^{-1} arising from inter-atomic vibrations. It was observed from Fig 4 that the peak below 500 cm^{-1} i.e. 466 cm^{-1} corresponds to Zn-O vibration confirms the formation of ZnO.[12,47] Peak observed at 905 cm^{-1} corresponds to bending mode of carbonates. Peaks observed at 1040 cm^{-1} and 1618 cm^{-1} are due to C=C stretching vibrations modes.[50] The peak observed at 3494 cm^{-1} is due to O-H stretching. Very weak peaks observed at 2388 cm^{-1} and 2947 cm^{-1} might be due to atmospheric carbon dioxide.

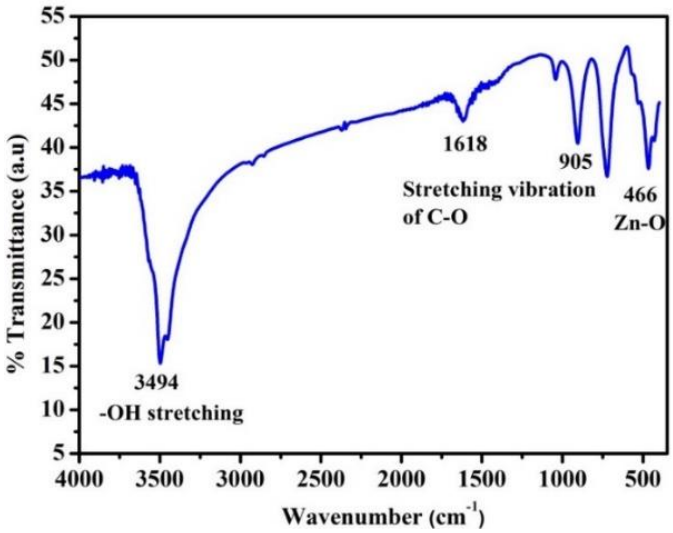

Fig. 4: FT-IR spectra of synthesized ZnO nanoparticles

Scanning electron microscopy (SEM) and EDS analysis of ZnO nanoparticles

SEM is a type of electron microscope that produces images of a sample by examining it with a focused beam of electrons. The electron beam interfaces with electrons in the sample, producing various signals that can be detected and give idea about morphology and composition of sample. SEM analysis is advantageous because the x-rays produced do not lead to loss of volume of the sample, so it becomes viable to repeatedly examine the same sample. From SEM, the size of the particles was found to be approximately ~ 300 nm. Morphology of the synthesized ZnO nanoparticles was like a rose flower. EDS analysis of flower shaped ZnO is shown in Fig 5. Element analysis shows the presence of Zn and O in the weight percentage of 73.7 and 26.3, respectively.

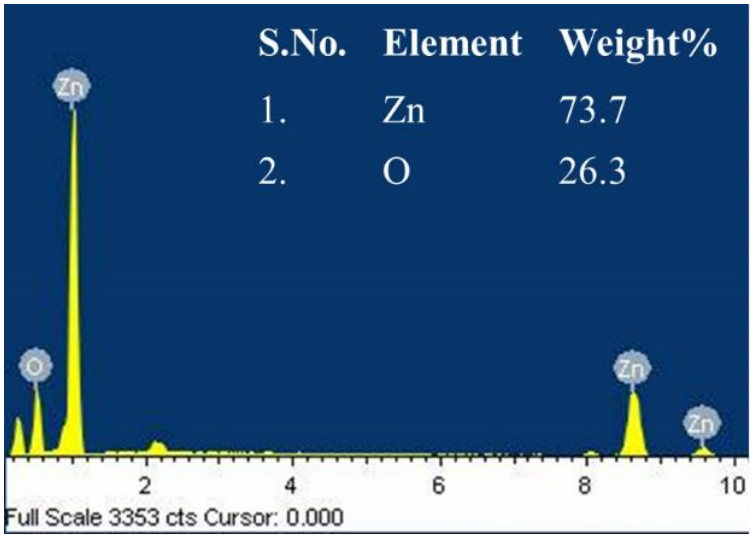

Fig. 5: EDS analysis of synthesized ZnO nanoparticles

Optimization of parameters for optimal functioning of enzyme for biosensor development

The magnitude of current developed at the working electrode surface depends upon the amount of p- nitrophenol (p-NP) produced by surface bound alkaline phosphatase (ALP) from substrate p- nitrophenylphosphate (p-NPP). The amount of p-NP formed is directly proportional to alkaline phosphatase (ALP) enzyme activity immobilized at the electrode surface. Thus, it is essential that all parameters like pH, cell density,

substrate concentration etc., which affect the performance of a biosensor should be optimized.

Optimization of pH and algal cell density

The pH environment for working electrode loaded with ALP enzyme of algal cell entrapped in membrane matrix was studied in pH range of 4 – 10 at pH 9, the current obtained was maximum as ALP showed maximum activity (Fig 6a). Before and after this pH, there was a sharp decrease in output of the current, which can be attributed to denaturation of the enzyme.

The generation of p-NP is an essential step in the development of voltammetric biosensor, which depends upon the enzyme present in the immobilized matrix. Hence, the number of cells per unit surface area of the membrane immobilized on the surface of the electrode has to be optimized. Different concentrations of algal cells ranging from 5µL to 25µL of six million cells per mL were taken. It can be observed from Fig 6(b) that the current increases from 5µL to 10µL and then decreases upto 25 µL. The decrease can be attributed to overloading of algal cells on the electrode surface. Therefore, 10 µL of six million cells per mL was optimized concentration for immobilization.

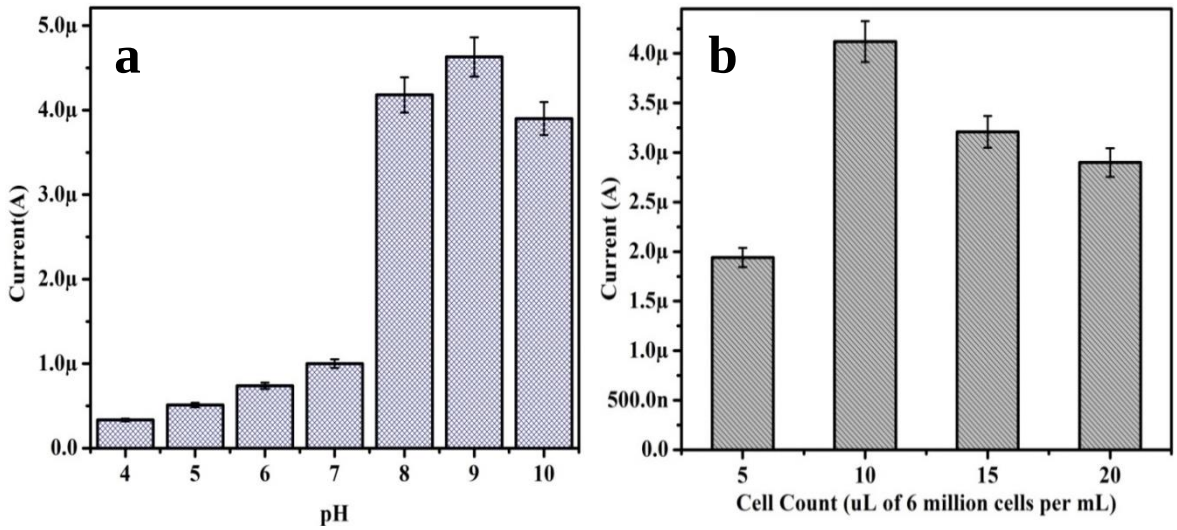

Fig. 6: (a) Amperometric responses of algae modified glassy carbon electrode at different pH conditions (b) Amperometric response of algae modified glassy carbon electrode at different cell densities

Optimization of substrate concentration

Experiments were done by adding different amounts of the p-NPP substrate to the reaction media ranging from 0.1 mL to 0.5 mL of 9mM concentration. The extent of current formed is additionally reliant upon the amount of substrate being acted upon by enzyme to produce the product p-NP. It was observed from Fig 7 that the current increased from 0.1 mL to 0.3 mL but became almost stable after 0.3 mL concentration of the substrate. As the amount of substrate is increased beyond the capacity of the enzyme, a steady state or saturation is achieved. Thus, this optimized concentration of the substrate (0.3 mL of 9mM) was used for further studies on the modified electrode.

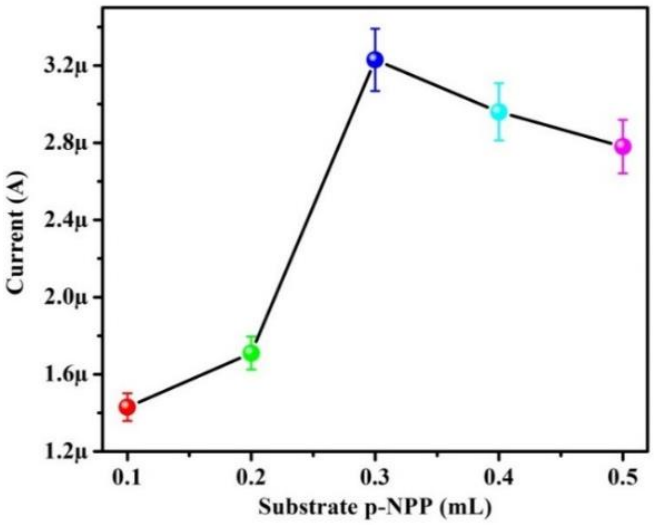

Fig.7: Amperometric responses of algae modified glassy carbon electrode at different substrate concentrations

Optimization of incubation time

Incubation time is the time taken by the enzyme to react with the analyte to produce stable output current. The activity of the enzyme was carried out at different intervals of time. From the Fig 8, it can be seen that value of the output current increased up to 10 minutes as this was the maximum time required by the enzyme to react with substrate to produce p-nitrophenol (p-NP). After this, it becomes almost constant because now enzyme has totally reacted with the substrate. Therefore, the response time for an algal biosensor was 10 minutes.

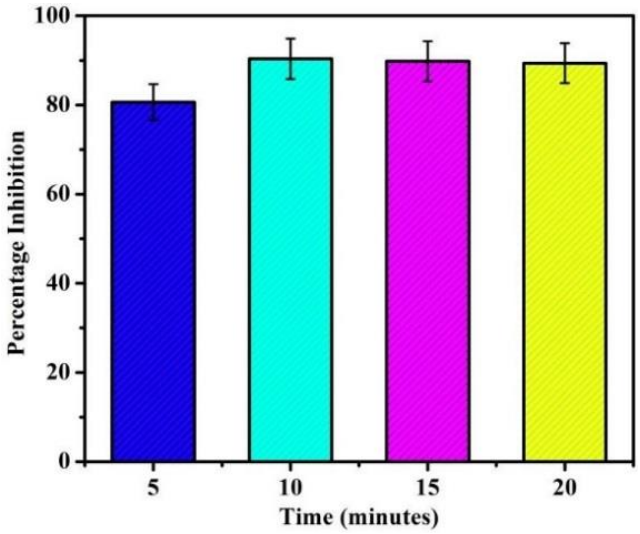

Fig. 8: Amperometric responses of algae modified glassy carbon electrode at different time intervals

Determination of Hg (II) ions using Glassy carbon (GC) and Platinum (Pt) electrodes

Detection Principle: Alkaline phosphatase (ALP) of *Chlorella sp.* algae is a metalloenzyme containing one Mg^{2+} ion and two Zn^{2+} ions near active site of enzyme. It also contains a number of amino acids like aspartate, glutamate, histidine and cysteine. Hg^{2+} ions are non-competitive inhibitors of enzyme. It binds with –SH group present in cysteine residue would acts as soft base and forms a stable Hg^{2+}-enzyme complex.

Hg (II) ions were determined on glassy carbon (GC) and platinum (Pt) electrodes in aqueous medium. Two types of biosensors of each electrode were prepared: (i) algal immobilized GC electrode and ZnO incorporated algal immobilized GC electrode, (ii) algal immobilized Pt electrode and ZnO incorporated algal immobilized Pt electrode.

1. Biosensor prepared using glassy carbon electrode

(a) Characterization of algal/GC biosensor and ZnO-algal/GC biosensor

The chronoamperometric study to characterize immobilized electrodes were carried out by dipping algal/GC and ZnO-algal/GC electrodes separately in Tris–HCl buffer solution (pH ~ 9) containing $MgCl_2$ acting as an enzyme activator. The ALP enzyme

of algae acts upon the substrate p-nitrophenyl phosphate (pNPP) converting it into electroactive p-nitrophenol (pNP). In this study, 100µL of p-NPP were consecutively added to Tris HCl buffer (pH ~ 8.5) upto 1.0 mL and corresponding current was recorded. The current is obtained from the oxidation of product p-NP. A graph of anodic current versus concentration was plotted as shown in Fig 9.

It can be observed from the Fig 9 that ZnO-algae/GC electrode has higher current as compared to AP-algae/GC. This is due to the fact that flower shaped ZnO nanoparticles have higher surface area and large number of electron voids, which facilitate faster electron transfer between the immobilized layer to electrode surface. The parameters like sensitivity, concentration range, co-relation coefficient (R^2) for both electrodes are summarized in Table 1.

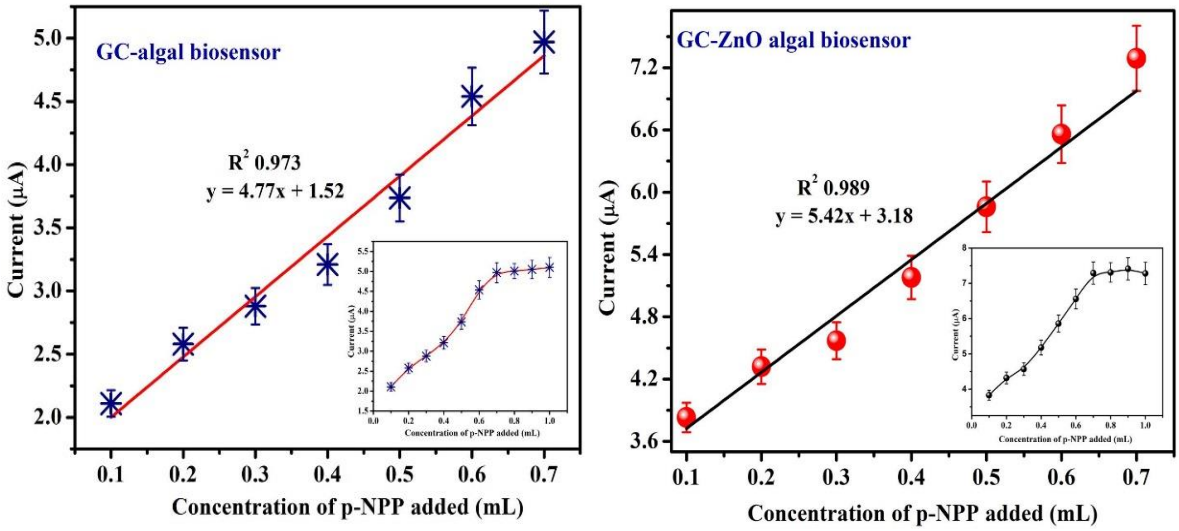

Fig. 9: Calibration curves plotted between current vs concentration of substrate p-NPP for immobilized glassy carbon electrodes

Table 1: Summary of characteristics of immobilized glassy carbon electrode.

Working electrode	**Sensitivity (µA/M)**	**Concentration range (mL)**	**R^2**
Algae/GC	4.77	0.1-0.7	0.973
ZnO/Algae/GC	5.42	0.1-0.7	0.989

From Table 1, it can be seen that the ZnO-algal/GC electrode has high sensitivity of 5.42 µA/M and high co-relation coefficient (R^2) 0.989 than algae/GC electrode. Thus, the mercury detection was carried out by ZnO-algal/GC electrode.

(b) Determination of Hg(II) ions using ZnO-algal/GC electrode

Chronoamperometric study: Chronoamperometric study was done by immersing ZnO-algae/GC electrode, Ag/AgCl reference electrode, and platinum counter electrode in the mixture of 0.1 M Tris–HCl buffer, $MgCl_2$ (10^{-3} M) and substrate p-NPP (0.3 mL of 9 mM) . Measurements were done at the working potential 0.95 V. ALP dephosphorylates substrate to mM) produce product p-NP, an electroactive species. P-NP is oxidized at electrode surface, the current obtained was labelled as control current (I_o). For Hg^{2+} ions determination, the immobilized electrode was dipped in different reaction mixture containing mercury solutions. The Hg^{2+} ions inhibit ALP enzyme, thus producing less amount of p-NP, which in turn produce less current (labelled as I_c). the percentage inhibition was calculated using following equation:

$$\mathbf{I\% = (I_o - I_c)/ I_o *100}$$

Fig 10 shows calibration curve obtained by plotting percentage inhibition vs concentration of Hg^{2+} ions. A linear curve was obtained in the concentration range of 10^{-13}- 10^{-3} M and detection limit of 10^{-13} M for ZnO-algal/GC electrode. The algal/GC electrode could detect mercury ions upto 10^{-10} M.

Voltammetric study: Cyclic voltammetry (CV) measurement for Hg^{2+} ions were performed at scan rate of 50 mV/s in potential range of 0.20 V to 1.20 V. Differential pulse voltammetric (DPV) studies were done in potential range from 0.50 V to 1.5 V with amplitude of 40 mV and scan rate of 50 mV/sec. Fig 11(a) shows cyclic voltammogram of p-NP generated at 10^{-4} M concentration of Hg^{2+} ion. DPV studies are shown in Fig 11(b). p-NP showed an oxidation peak at +1.18 V. With DPV, Hg^{2+} ions can detected upto 10^{-9} M with sensitivity of 5.71µA/M and co-relation coefficient (R^2) of 0.997 as shown in Fig 12.

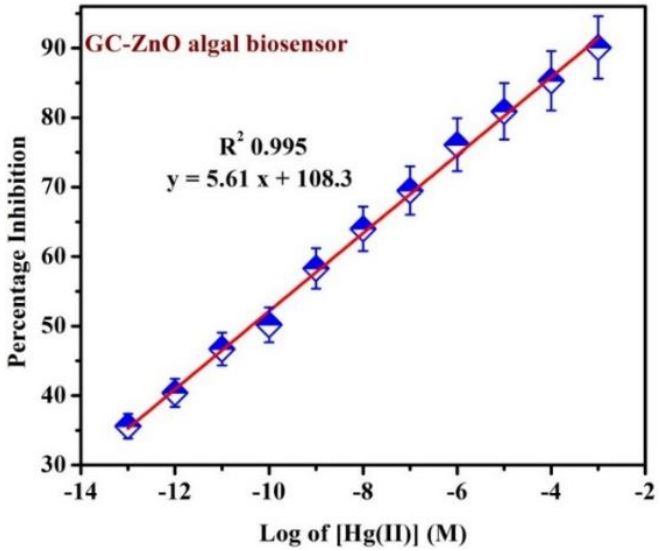

Fig. 10: Chronoamperometric calibration curve for detection of Hg (II) ions on modified glassy carbon electrode

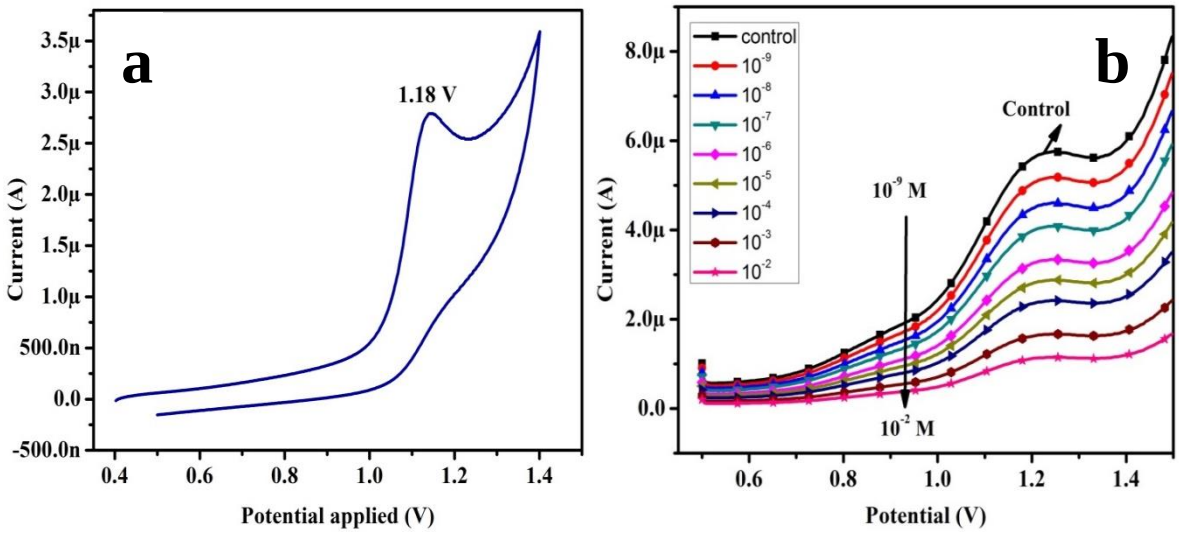

Fig. 11: (a) Cyclic Voltammogram of p-NP in presence of 10^{-4} M Hg (II). (b) Differential pulse voltammogram of p-NP in presence of Hg (II) in concentration range 10^{-9}M -10^{-2} M

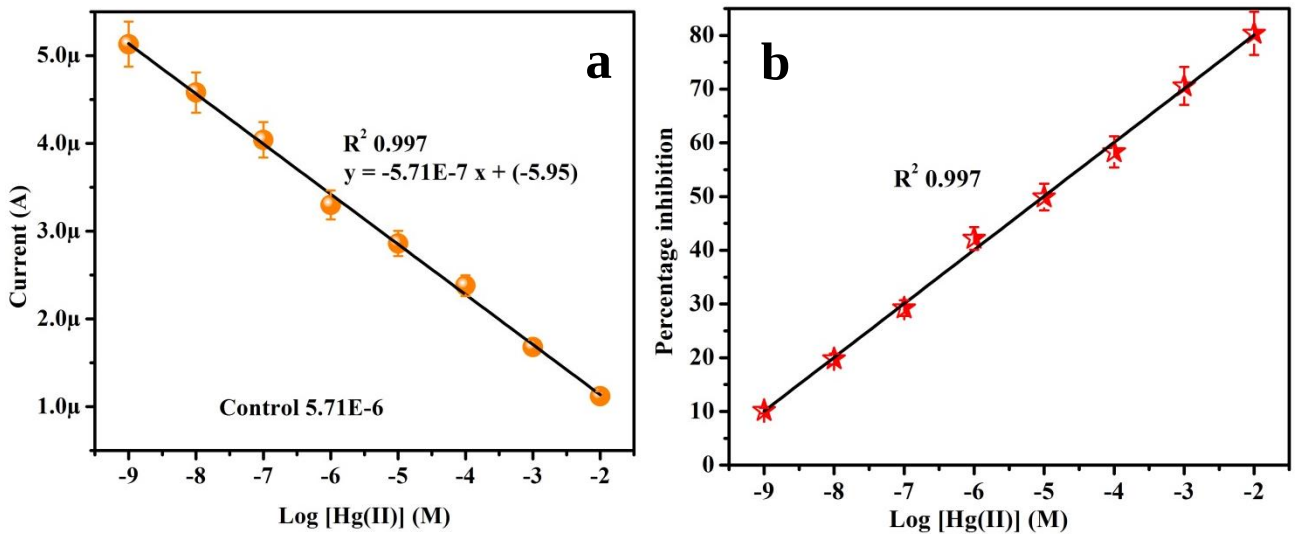

Fig. 12: (a) Calibration curve plotted between Current vs Log of concentration of Hg(II) ions using ZnO/Algae/GC electrode (b) Plots of percentage Inhibition vs Log of concentration of Hg (II) ions

2. Biosensor prepared using Platinum electrode

(a) Characterization of Pt/algal biosensor and ZnO-algal/Pt biosensor

Plots of current versus concentration of substrate for Pt immobilized electrodes are shown in Fig 13. It can be observed from the plot that ZnO-algal/Pt has higher sensitivity and showed a large current as compared to algal/Pt/electrode. The various parameters such as sensitivity, concentration range and R^2 for both Pt immobilized electrodes are summarized in Table 2. It can be observed that algal/Pt and ZnO-algal/Pt both electrodes showed a linear response from 0.1 mL to 0.5 mL concentration of substrate. However, sensitivity for ZnO incorporated Pt electrode was found to be 3.03 µA/M and R^2 was 0.985, which is higher than algal/Pt electrode. Hence, Hg (II) ions were determined using ZnO-algal/Pt electrode.

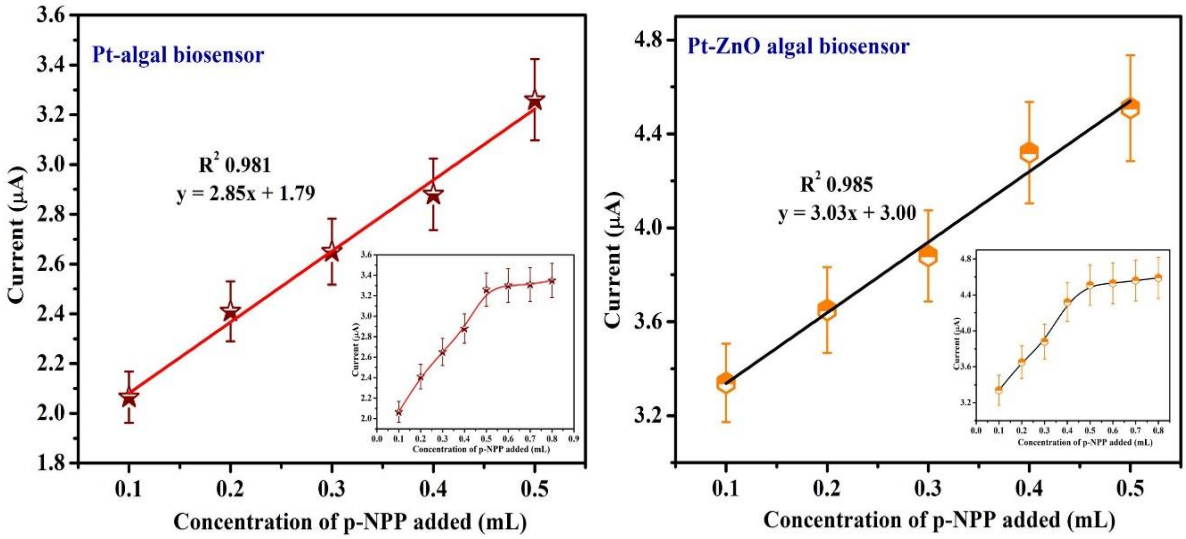

Fig. 13: Calibration curves plotted between current vs concentration of substrate p-NPP for Pt- Algal and ZnO/Pt- Algal electrodes

Table 2: Summary of characteristics of immobilized platinum electrode.

Working electrode	**Sensitivity (µA/M)**	**Concentration range(M)**	**R^2**
Algal/Pt	2.85	0.1 - 0.5	0.981
ZnO-algal/Pt	3.03	0.1 – 0.5	0.985

(b) Determination of Hg(II) ions using ZnO-algal/Pt electrode

Chronoamperometric study was done with ZnO modified algal-Pt electrode for the determination of Hg (II) ions in concentration range from 10^{-12} M to 10^{-3} M. The working electrode was biased at 0.95 V for the study. The principle for the detection is similar to the glassy carbon electrode method. With ZnO modified algal-Pt electrode, Hg (II) ions can be detected upto 10^{-11} M as shown in Fig 14.

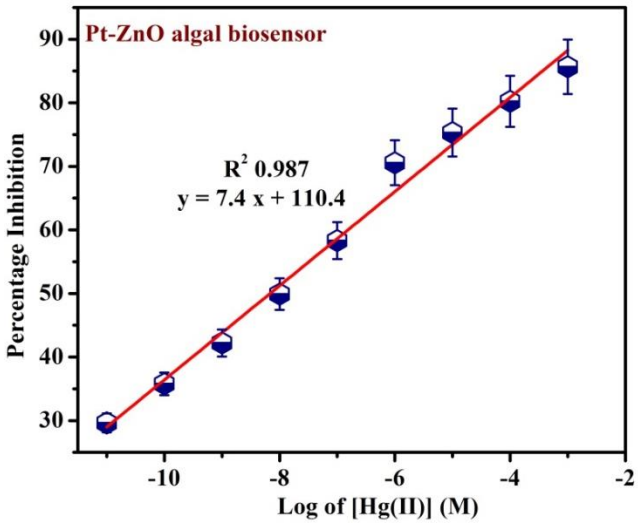

Fig. 14: Chronoamperometric calibration curve for detection of Hg (II) ions on modified platinum electrode

Interference study

Interference study was done by using interfering ions such as Ag^{+}, Cu^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} with Hg^{+2} ion. For this, equal amounts of each interfering ion of concentration 1×10^{-3} M was added to 1×10^{-4} M concentration of Hg^{+2} ion in reaction mixture containing 300µl of the substrate p-NPP, 200µl of $MgCl_2$, and Tris HCl buffer. The change in the current magnitude with respect to Hg^{+2} ion was plotted as shown in Fig 15. From graph it can been seen that no major change is observed in presence of interfering ions. Thus, this developed biosensor is selective to mercury ions.

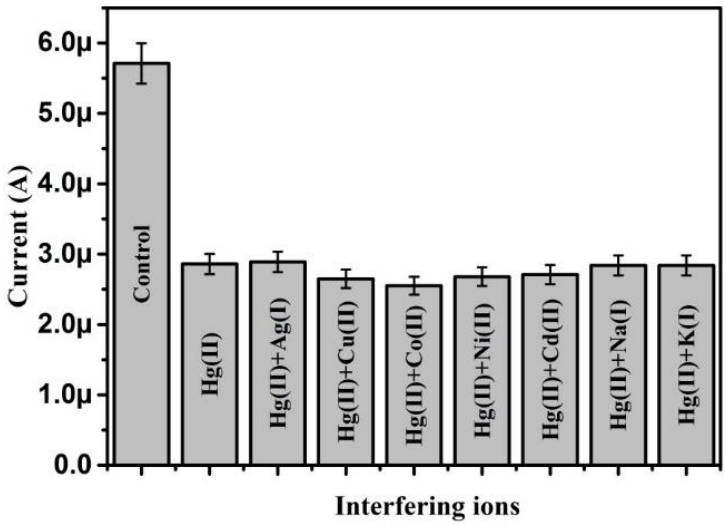

Fig. 15: Performance of ZnO-algal biosensor for Hg (II) in presence of interfering species

CONCLUSION

An electroanalytical algal biosensor by incorporating flower shaped ZnO nanoparticles in the algae culture immobilized on the surface of the working electrode for the detection of Hg (II) ions in aqueous medium. The algae was immobilized on two electrodes- glassy carbon and platinum electrodes using BSA-glutaraldehyde method to study the performance efficiency of the two commonly used electrodes materials. Before the detection of mercury ions, working electrode parameters such as algal concentration, incubation time, substrate concentration, and pH were optimized for better working of the developed biosensor. Four types of electrode were prepared, (i) algal immobilized GC electrode, (ii) ZnO incorporated algal immobilized GC electrode, (iii) algal immobilized Pt electrode, (iv) ZnO incorporated algal immobilized Pt electrode. Firstly, glassy carbon immobilized electrodes were studied. It was found that incorporation of ZnO nanoparticles to the immobilized layer has increased sensitivity of the GC-algal biosensor. ZnO-algal/GC could detect Hg(II) ions through chronoamperometry and differential pulse voltammetry (DPV) upto 10^{-13} M and 10^{-9} M, respectively whereas algal/GC electrode could only detect upto 10^{-10} M by chronoamperometry. Similar trend was observed in Pt modified electrode. In the case of ZnO-algal/Pt electrode, a linear concentration range of 10^{-11} M to 10^{-3} M of Hg(II) was observed as compared to algal/Pt electrode which showed a linear response in range of 10^{-10} - 10^{-3}M. Interference study was done to validate that prepared biosensor for Hg^{2+} detection. It has showed good stability and selectivity for Hg (II) as compared to other interfering ions.

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