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# New Assembly for Biosensing of Ultra-Trace Level Mercury in Continuous Flow System

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A new assembly was designed for the determination of mercury ions in a continuous flow system with *Chlorella* sp. based amperometric biosensor. The biosensor system was used for the measurement of bioavailable mercury ions in an aqueous medium. Calibration curve for Hg (II) measurement was reproducible in the concentration range  $10^{-11}$  M to  $10^{-6}$  M. The biosensor shows an excellent selectivity for mercury ions in presence of silver and other potential interfering ions and shows sensitivity and reproducibility in aqueous stream with a flow rate varying in the range of 0.5 mL/min to 1.5 mL/min. Real sample analysis in continuous flow stream was carried out and validated with chronoamperometric measurements taken in batch mode and with atomic absorption spectrometry. Results are also compared with the existing measurement methods and found better in terms of selectivity and detection limit.

## Introduction

During the last few decades human race has perceived the contamination of fresh water (ground, river and canal water) by heavy metal ions due to various anthropogenic processes like industrial and agricultural waste waters. Hence, environmental security has come up as a fundamental requirement for sustainability of human race<sup>1</sup>. Determination of heavy metal ions in flowing fresh water sources has been of great prominence as heavy metals are highly toxic at high concentrations<sup>2</sup>. Bioaccumulation<sup>3</sup> of heavy metals in flora and fauna changes the non-lethal dose to lethal dose.

In the last two decades biosensors have come up as an alternate technique to various traditional techniques for the determination of heavy metal ions like inductively coupled plasma-mass spectrometry (ICP-MS)<sup>4,5</sup>, atomic absorption spectrometry (AAS)<sup>4</sup>, anodic stripping voltammetry, etc., as preparation of biosensors are cost effective<sup>6</sup>, easy to handle and can be miniaturized for in-situ and online determination of various analytes and above all they give us the bioavailable content of heavy metal ions<sup>7</sup> than total metal ion<sup>8</sup> concentration as given by such traditional methods.

Some of the heavy metals are known to human race from ages<sup>9-12</sup> and play an important role in human body as micro nutrients and cofactors of various enzymes<sup>13</sup>, but metals like cadmium and mercury are having no physiological role in human body<sup>14</sup>. Out of all such metals mercury and its compounds are extremely toxic as these cause various disorders of central nervous system, renal system and liver. Hence, its sensitive and selective determination becomes of great

significance. In recent years, a number of biosensors have come up in the market using the property of mercury to inhibit various enzymatic activities<sup>15-17</sup> and cell fractions<sup>18</sup>.

In the present work, inhibition of a cell wall bound enzyme alkaline phosphatase of *Chlorella* sp., immobilized on glassy carbon electrode with mercury ions in a continuous flow system has been studied. Alkaline phosphatase activity (APA) is responsible for dephosphorylation of p-nitrophenylphosphate to produce p-nitrophenol. Current generated due to oxidation of enzymatically generated p-nitrophenol has been measured amperometrically. Effect of the flow rate and the use of biosensor for in-situ determination of an artificial stream has been studied.

## Experimental

### Algae culture

Algae, *Chlorella* sp. was identified, cultured and recultured in BG-11<sup>19</sup> media every three weeks. Algae was starved and suspended in phosphate free BG-11 media after harvesting, before being used for immobilization on the electrode surface.

### Biosensor preparation

Biosensor was prepared as per the method reported previously by our group<sup>20</sup>. The glutaraldehyde would react with amines of bovine serum albumin and does not affect the microbial proteins<sup>21</sup>.

### Instrument and the electrodes used

All chronoamperometric experiments were performed using

Autolab PGSTAT12 (Eco-Chemie-Netherlands), with platinum electrode, saturated Ag/AgCl electrode and modified glassy carbon (GC) electrode as counter, reference and working electrodes, respectively. All electrodes were of CH instrument (USA) make.

### Chronoamperometry

Chronoamperometric study was performed by immersing algae modified GC electrode in 0.1 M tris-HCl, 1mM MgCl<sub>2</sub> (enzyme activator) solution and p-nitrophenyl phosphate, added as the substrate. Chronoamperometric scans were recorded by biasing the electrode at 0.85V vs Ag/AgCl reference electrode<sup>20,22</sup> and the current generated due to oxidation of enzymatically generated p-nitrophenol was detected as performed in previously reported work<sup>20</sup>.

### Heavy metal testing

Stock solution of mercury nitrate (0.1 M) was prepared by dissolving nitrate salt of mercury (Sigma Aldrich) in double distilled deionized water. Fresh solution of required concentration was made by diluting the stock solution just before starting the experiment. The Millipore purified deionised distilled water was having conductivity of  $5.5 \times 10^{-6}$  S/m or less.

### Assembly for flow system

An indigenously designed assembly (Fig: 1) was used for studying the experiments of mercury ion determination using the biosensor. A continuous but controlled flow of the electrolyte medium was maintained by electrical pump (with an adjustable flow) from the reservoir into the voltammetric cell of radius 0.75 cm and height of 1.5 cm with a dead volume of approximately 1.5 mL through a channel, simulating a stream of waste water sample containing the heavy metal contamination. Filtration system was fitted in the channel to prevent any particulates entering the voltammetric cell. Supporting electrolyte and the heavy metal salt solutions were injected as and when required through the openings in the teflon lid on voltammetric cell kept for the purpose. A stopcock and the pump were used to control the flow of liquid as per requirement.

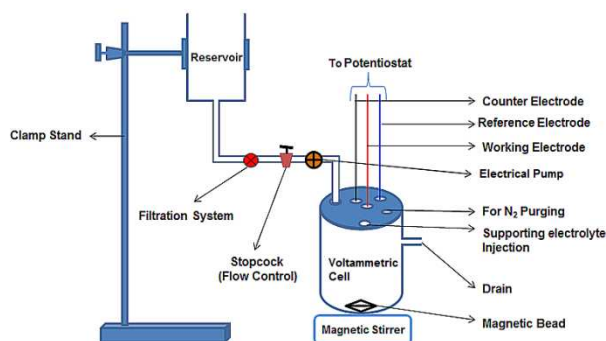


Fig: 1 Diagrammatic representation of assembly used in study of biosensor in a flow system

### Results and discussion

Biosensor was prepared and characterized as previously reported<sup>20</sup>. Alkaline phosphatase from the surface of immobilized algae causes dephosphorylation of electro-inactive phosphate substrate p-nitrophenyl phosphate and changes it to electroactive p-nitrophenol, which on biasing a higher voltage undergoes oxidation releasing two electrons being measured in a chronoamperometric scan (Fig: 2). Mercury causes inactivation of alkaline phosphatase, causing a decrease in the production of electroactive p-nitrophenol, hence, a decrease in current generation.

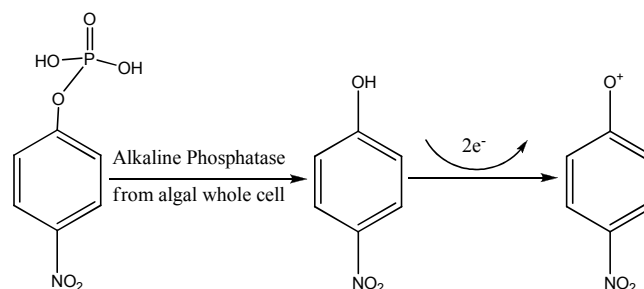


Fig: 2 Detection principle of algal biosensor

### Online determination of mercury

The reported method<sup>20</sup> can determine mercury upto  $10^{-11}$  M in aqueous samples taken in batches. The chronoamperometric technique used for the determination of mercury has been used in a continuous flowing stream of the sample.

An experimental set up was designed for the variable flow rate ranging between 0.5 mL/min to 5.0 mL/min. A continuous stream of aqueous medium consisting of the substrate, supporting electrolyte and enzyme activator was varied from 0.5 mL/min to 5.0 mL/min and chronoamperometric experiments were conducted to optimize response time of the set up for each flow rate (Fig 3).

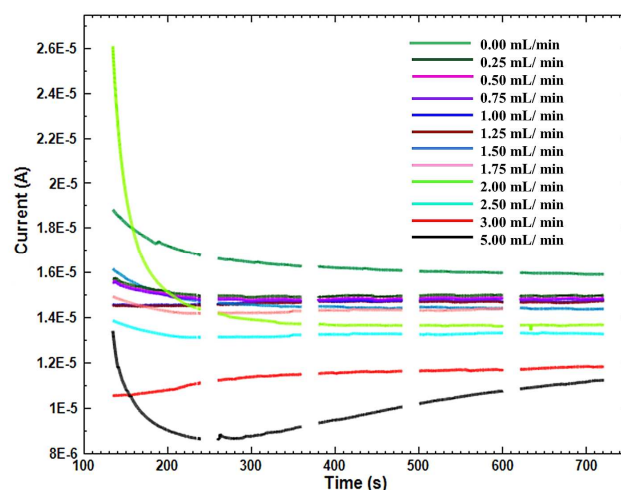


Fig: 3 Overlay of chronoamperometric plots at different flow rates

Overlay of plots showed that the current is optimized after about 5 minutes from start of the experiment and was used for all further experiments. Similar experiments were done for

current measurement as a function of rate of flow to find out the optimum flow at which the current is sustained. It was observed (Fig 4) that after an initial decrease in current, it more or less stabilized upto 1.5 mL/min flow rate and there was a sudden drop in current on further increment inflow rate. Hence, the optimum rate can be taken as 1.5 mL/min.

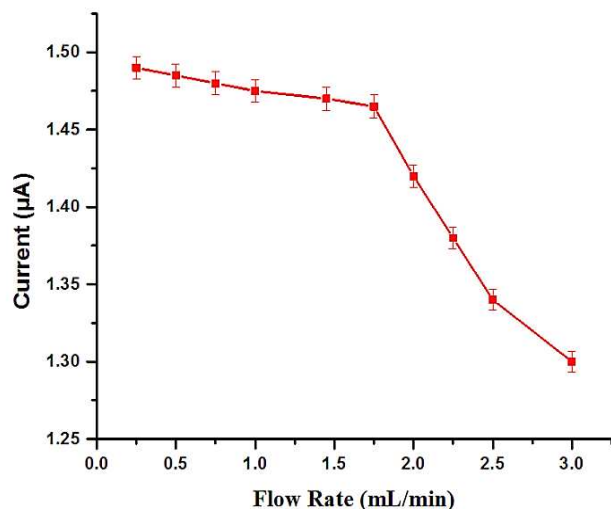


Fig: 4 Plot of variation in current with varying rate of flow

#### Calibration curve for mercury ions in a flow stream

Applying the optimized conditions of response time and flow rate, a calibration curve for  $\text{Hg}^{2+}$  ions was drawn in a concentration range of  $10^{-11}$  M to  $10^{-6}$  M. The experiment was repeated 5 times and a representative response of the electrode system in terms of current plotted against  $-\log [\text{Hg}^{2+}]$  is shown in Fig 5 (RSD = 3%). The proposed biosensor responded linearly in a concentration range of  $10^{-11}$  M to  $10^{-6}$  M, while above and below this concentration, the response of the electrode deviates from linearity. The calibration curve has an  $r^2$  value of 0.99 and is acceptable for  $\text{Hg}^{2+}$  ion determination using a continuous flow system.

The proposed system responds sharply for the detection of mercury, as it is seen from the calibration plot (Fig 5) that even a slightest amount of  $\text{Hg}^{2+}$  ( $10^{-11}$  M) causes the current to decrease by more than 7%. The lower detection limit of the proposed system was calculated using standard  $3\sigma$  method and detection limit comes out to be  $7.1 \times 10^{-11}$  M for the continuous flow analysis. Where ' $\sigma$ ' is standard deviation ( $8.9 \times 10^{-11}$ ), the intercept value of the calibration plot or of blank solution having no mercury ions in it.

In order to check validity of this calibration curve, some real time samples of  $\text{Hg}^{2+}$  ions prepared artificially were determined with the proposed set up of biosensor with continuous flow and compared with analysis using atomic absorption spectroscopy and the batch analysis method reported earlier<sup>20</sup> (Table 1).

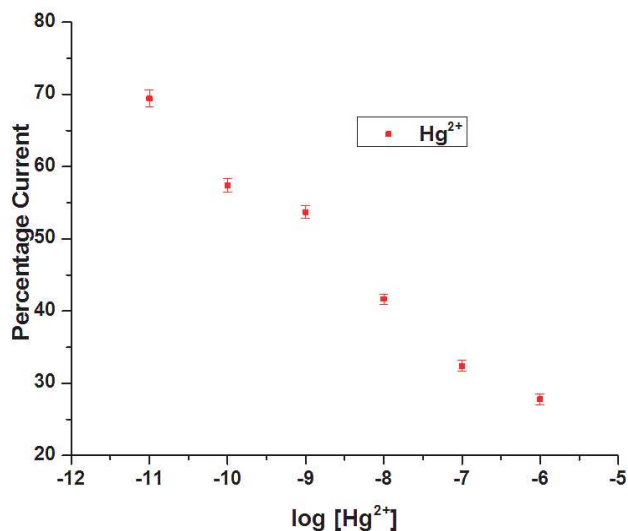


Fig: 5 Calibration curve for mercury ions at a flow of 1.5 mL/min

The continuous flow system was tested for interference from potential interferents like silver, zinc, calcium and sodium. The results obtained, confirmed that the biosensor showed no interference from any of the potential interferents<sup>20</sup>. Hence, it is proposed as an excellent technique for  $\text{Hg}^{2+}$  ion determination without any interference from silver or other heavy metal ions in a flowing stream of aqueous medium.

Table 1: Mercury determination using biosensor in continuous flow system compared with batch samples and atomic absorption spectroscopy

S. No	Sample	AAS ( $10^{-8}$ M)	Batch method determination ( $10^{-8}$ M)	Flow system determination ( $10^{-8}$ M) (Proposed method)
1	Sample 1	$2.5 \pm 0.01$	$2.49 \pm 0.04$	$2.46 \pm 0.07$
2	Sample 2	$1.0 \pm 0.04$	$1.02 \pm 0.02$	$0.99 \pm 0.05$
3	Sample 3	$0.35 \pm 0.05$	$0.36 \pm 0.04$	$0.34 \pm 0.05$

Another experiment was conducted to check consistency of the current response of the biosensor for  $\text{Hg}^{2+}$  ion on successive addition of  $\text{Hg}^{2+}$  ions after regular intervals of time. Chronoamperometry was performed on solution of mercury ions running ( $10^{-11}$  M) at a flow rate of 1.5 mL/min, electrolyte medium was spiked with 100  $\mu\text{L}$  of  $10^{-11}$  M  $\text{Hg}^{2+}$  ion solution after every 120 seconds. The results were plotted (curve 'b' in Fig 6) and compared with those containing no mercury ions (curve 'a' in Fig 6). The curves show a successive decrease in current with successive increase in  $\text{Hg}^{2+}$  ion content in the medium. The current increases almost in same manner in both the plots, but as mercury was injected into the system (b), the decrease in current has been observed, while no such decrease was observed in (a). The same response was observed in each addition of mercury ions and current keeps on decreasing and becomes almost constant in later stages.

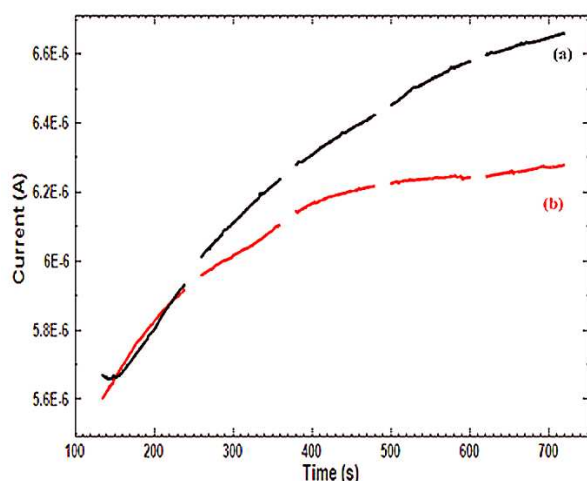


Fig: 6 Response of biosensor electrode (a) Normal flow (b) flow with injected  $\text{Hg}^{2+}$

This shows that the electrode can be used for determination of mercury at any given stage of the experiment and biosensitivity of the biosensor electrode for  $\text{Hg}^{2+}$  ions remain active throughout.

#### Comparison of Modified GC Electrode performance with the reported sensor for mercury

Results of the proposed biosensor technique have been compared with other recently reported methods of mercury determination using various techniques. The comparison is shown in Table 2. Most of the reports have not studied the interference from probable interfering metals like silver and lead ions, while other reports indicate no interference from any metal ions. Most of the methods<sup>27,28,30</sup> shown in Table 2 are ICP based, which is a very expensive technique. Overall, the proposed method is better than other methods considering the selectivity, lower detection limit, ease to use and interference parameters.

Table 2: Comparison of the reported mercury sensors based on different techniques with the proposed method

S.No	Methods for mercury determination	Detection limit (M)	Interference Studies	References
1	Photonic crystal hydrogel	$1 \times 10^{-12}$	No interference from any metal ion	24
2	Homogeneous liquid-liquid extractoin	$1 \times 10^{-9}$	Interference study not reported	25
3	DNA (HS-DNA) was then covalently linked to the surface of the Au-NPs/MWCNTs via Au-S bonds	$3 \times 10^{-11}$	No interference from any metal ion	26
4	Flow Injection-Cold Vapor Generation-Inductively Coupled Plasma Optical Emission Spectrometry	$1 \times 10^{-10}$	Interference study not reported	27
5	flow injection chemical vapor generation inductively coupled plasma mass spectrometry	$9 \times 10^{-8}$	Interference study not reported	28
6	Surface-enhanced Raman scattering (SERS)-based microdroplet sensor	$1 \times 10^{-12}$	Interference study not reported	29
7	inductively coupled plasma mass spectrometry (CVG-ICP-MS)	$6 \times 10^{-12}$	Interference study not reported	30
8	Whole cell bacterial based biosensor	$2 \times 10^{-8}$	Interferences from Cd, Pb and As	31
9	Flow injection-chemical vapor generation atomic fluorescence spectrometry	$4.5 \times 10^{-8}$	Interference study not reported	32
10	Nitro benzoyl diphenylmethylenphosphorane (N-BDMP)Chemically modified electrode with anodic stripping voltmmetry	$8 \times 10^{-9}$	No interference from any metal ion	33
11	Antimony film carbon paste electrode using an anodic stripping square-wave voltmmetry	$1 \times 10^{-9}$	Interference study not reported	34
12	Modified GC Electrode based biosensor	$1 \times 10^{-11}$	Selective for mercury, No interference from any metal ion	Proposed Method

#### Conclusions

The prepared biosensor can be used for effective determination of even trace levels of mercury ions in a flowing system or stream having a moderate flow of 0.5 mL/min - 1.5 mL/min, with a detection limit of  $10^{-11}$  M. The proposed biosensor can also be used for in-situ or online determination and has a great edge over detection and sensitivity of recently reported sensors for mercury<sup>24-34</sup>.

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#### Notes and references

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