A Novel on Chip Analysis of Dissolved Hg(II) in Drinking Water

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ABSTRACT

Determination of contaminants such as mercury, lead and cadmium at low concentration into the sinks such as ground water aquifers and river water always remained a major challenge for analytical scientists. Herein, a miniaturized assay on titanium coated chip for ultra trace determination of dissolved Hg (II) in drinking water is presented. Using alkanethiol self assembled monolayer and surface activation chemistry, the enzyme alcohol oxidase was coupled covalently on the chip. The activity of the enzyme on chip was characterized using chemiluminescence technique with incubation time of 10 minutes. A linear range for Hg(II) was obtained in the rage 250-25pg/mL with imaging based determination. A working range with least interference from Cd(II) and Pb(II) has been determined for Hg(II). The titanium coated biochip has been tested for a number of spots using hand held arrayer. Reusability of the chip was tested by enzyme regeneration.

Keywords: biochip, mercury, chemiluminescence, alcohol oxidase, titanium, inhibition

1 INTRODUCTION

Mercury is one of the most hazardous pollutants. In dissolved form it exists mainly as Hg(II) in water. The concentrations of mercury species in real samples such as aquifers and coastal waters are usually at trace levels [1]. World Health Organization has set a limit for 0.001µg/mL Hg(II) in drinking water for water quality monitoring purposes. Optical detection techniques provide a great platform to analyze toxic metals with higher sensitivity and minimum analysis time [2]. The added advantage is minimum interference in real sample analysis. Monitoring of trace Hg(II) based on *in-situ* sensing devices is of immense interest [3]. The need for on-site monitoring of trace metals in a variety of environmental matrices has led to the adaptation of on chip analysis techniques. Development of chip based systems will facilitate field deployable technique capable of monitoring metal contaminants. Gold coated chips have been extensively reported for biochip development [4, 5]. Herein, we report, development of a titanium (Ti) chip and its application for determination of Hg(II) in drinking water using immobilized alcohol oxidase(AlOx). The chip provided very low detection limit. The biochip exhibited good reproducibility when used for drinking water analysis.

2 EXPERIMENTAL

2.1 Materials

Mercury AA/ICP Calibration/Check Standard for environmental analysis, 5-amino-2, 3-dihydro-1,4-phthalazinedione (luminol) Peroxidase (1.11.1.7) from *Horseradish* and Alcohol Oxidase (1.1.3.13) from *Pichia pastoris* were purchased from Sigma Chemical Company MO (USA). Lead, Cadmium standards, Methanol (99.9%), ethanol (99%), N-hydroxysuccinimide and Hydrogen peroxide (30%) were from Merck (Germany), whereas 4-Iodophenol was purchased from Aldrich. A 1.0 mmol L^{-1} luminol solution was prepared by dissolving 4 mg of luminol in 2 ml of 0.1 M NaOH and making up the volume to 20 mL by 0.1 M phosphate buffer pH 7.5. Other reagents were of analytical reagent grade.11-Mercaptoundecanoic acid (MUA) was purchased from Aldrich (USA), Ethyl alcohol (Absolute, 200 proof) was purchased from TEDIA (USA), 1-ethyl-3(3/-dimethylaminopropyl) carbodiimide, HCl (EDCl) was purchased from Calbiochem (Germany).

2.2 Fabrication of chip

The chip was fabricated using chemical vapour deposition technique. Ti chip consists of glass coated with gold and subsequent deposition of Ti.

2.3 Preparation of self assembly on chip

Ti chips were washed with ethanol, and dried under a stream of high-purity nitrogen before use. These samples were immersed into 5mM ethanol solutions of 11-MUA for 24 h (assembly time), rinsed in ethanol followed by

distilled deionized water and dried with a stream of dry nitrogen.

2.4 Enzyme immobilization on chip

For enzyme coupling on chip, the carboxylic acid-terminated SAMs were immersed into an aqueous solution (100mM EDC /100mM NHS) for 3 hrs. The resultant NHS ester monolayers were reacted for 3 hrs in a solution of AlOx (0.003U/1µL) in PB, 0.1M, pH7.5at 4ºC. After removal of the SAM from enzyme solution, the surface was rinsed exhaustively with PB and used immediately for enzyme activity measurement. Enzyme coupled Ti-chip is presented (see Fig. 1).

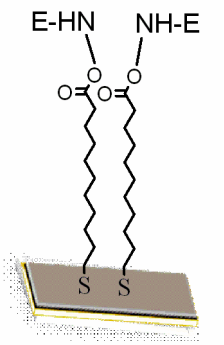

Fig. 1. Image showing self assembly of 11-MUA on Ti-chip coupled with AlOx.

2.5 Principle

The property of inhibition of enzyme by heavy metals such as Hg(II) was utilized in the development of bio-analytical technique [6]. The presented on chip assay also exploit the principle of enzyme inhibition for Hg(II) determination. The activity of enzyme before and after inhibition was measured and the difference is calculated as;

$$\mathbf{I\ \% = 100\ (h\upsilon_1 - h\upsilon_2)\ /\ h\upsilon_1}$$

$\mathbf{h\upsilon_1}$ = Photon count in absence of Hg(II) compound.

$\mathbf{h\upsilon_2}$ = Photon count in presence of Hg(II) compound

The principle of chemiluminescence determination of Hg(II)using AlOx/peroxidase enzyme is presented as Scheme 1.

Scheme 1**.** bi -enzymatic (AlOx/HRP) reaction involved in the proposed assay.

$$CH_3OH + O_2 \xrightarrow{AlOx} HCHO + H_2O_2 \quad (1)$$

$$C_8H_7N_3O_2 \xrightarrow[HRP]{2H_2O_2} C_8NO_4H_5^{2-} + N_2 + O_2 + 2H_2O + h\upsilon \quad (2)$$

$$AlOx_{(Active)} + Hg(II) \xrightarrow{Incubation} AlOx_{(Inhibited)} + h\upsilon \quad (3)$$

AlOx activity was observed in terms of liberated photons (hυ).

2.6 Sample preparation

Drinking water samples were collected fresh prior to analysis. Samples were filtered through a 0.45 micron pore size filter (Whatman, USA) and the filtrated samples were adjusted to pH 7.5.

3 RESULTS AND DISCUSSION

3.1 Assay development and miniaturization

The miniaturization of assay provides several advantages such as increased throughput, reduced reagent consumption and reduction in consumables. The reduction in assay volume in 1536 well plates provides opportunity for inhibition studies in closer proximity

Prior to developing an effective miniaturized AlOx assay, all the necessary parameters such as temperature of the assay, pH of the buffer and concentration of substrate enzyme were optimized. For use in inhibition experiments, the AlOx was incubated with Hg(II) solution for 10 minutes at 35°C.

3.2 Assay on chip

Initially the activity of immobilised AlOx was tested on chip and compared with free enzyme. Various optimized experimental parameters (substrate, enzyme loading, pH, temperature etc) for AlOx/methanol and AlOx/Hg(II)/methanol assay were tested. Performance on gold and Ti surfaces were compared. Experiments with AlOx assay on chip demonstrate the ability to further miniaturize the developed assay.

3.2.1 Comparative study of enzyme –substrate assay in 1536 well plate and Chip

The activity of free and immobilized AlOx was compared using different methanol concentration. The activity of enzyme AlOx in free solution and after immobilization on Ti-chip was measured. The results are presented (see Fig. 2). A comparable light signal is

observed for on chip immobilized enzyme. The signal is sufficient to carry out further studies for inhibition.

Initially, assay was carried out to determine methanol concentration in 1536 well plates and followed by measurements on immobilized AlOx chip. After adding the substrate and luminal. Chip was mounted on to the stage of an inverted semi-confocal microscope attached with an electron multiplier charge couple devise (EMCCD) camera (Andor, USA). A representative recorded image of the chip is presented (see Fig. 3). For methanol sensing the reusability of chip was tested for consecutive 25 runs. Chip was washed with PB between two runs. From the results obtained it is clear that AlOx chip gives good reproducibility for 25 runs.

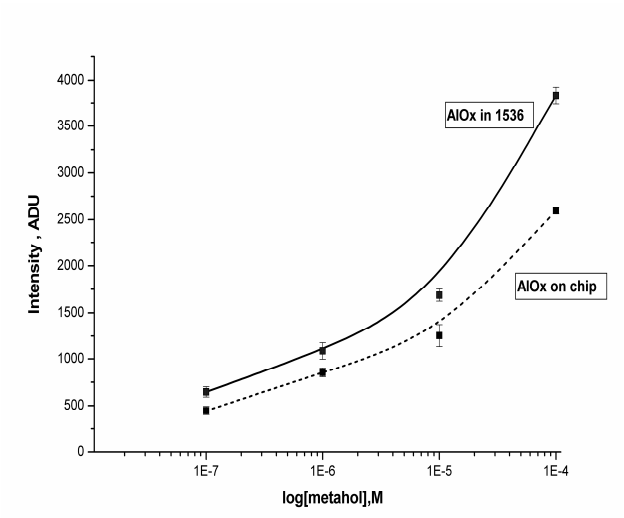

Fig. 2. Activity of AlOx in free and immobilized form on chip using 0.5mM methanol. Upper curve represent response of free AlOx in 1536 well plate. Lower curve represent response signal for on chip measurements.

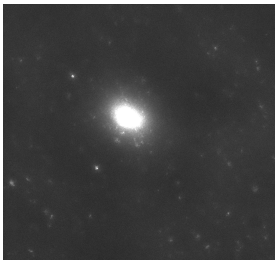

Fig. 3. On chip chemiluminescence image obtained using an EMCCD camera on inverted microscope with 40 x magnification

3.2.2 Comparative study of enzyme inhibition assay in 1536 well plate and Chip

Inhibition studies for AlOx were carried out using Hg(II) as inhibitor. Two different formats were used to study inhibition, micro-well plate and chip. 1536 well plate format with assay volume 8.5 µL and with Ti- chip 7.5 µL.The AlOx was incubated for 10 min. with Hg(II). The linear range for Hg (II) in 1536 well plate was found to be 25-500 ng/L and that on chip 25-500ng/L. Sensitivity of assay is as low as 25pg/mL Hg(II) on chip is one of the most remarkable features of our assay.

3.3 Mixture of heavy metal

Co-existence of Hg(II), Cd(II) and Pb(II) are commonly encountered in the food, water and other part of the environment as a result of human and natural activity. During the metal ion analysis, presence of other metal ion may elicit antagonistic, additive or synergistic effect. In order to understand the toxic effect of these chemicals in totality, it is essential to study inhibition at various concentration levels. Experiments were carried out to determine the relative inhibition of AlOx by Hg(II). The 10 fold difference observed (data not shown) in the linear range of Hg(II) and Pb(II), Cd(II) proves that these two ions do not interfear in Hg(II) analysis.

3.4 Analysis of real samples

The proposed on chip method was applied for determination of Hg(II) in three water samples. Since the mercury species in real water samples was below detection limit, standard Hg(II) solutions were spiked. The results indicated that the recoveries were in the range of 95–106% in different water samples.

3.5 Repeatability and stability

Stability of enzyme on Ti-bio-chip assay in terms of repetitive uses was performed for successive assays with 0.5mM of methanol per day over the period of one week. Excellent storage stability was observed for immobilized AlOx in 0.1M PB at pH 7.5 when AlOx aliquots were immobilized on Ti chip and stored at 4ºC. From Fig.3, it is clear that for the first three assay only 10 % of enzyme activity was lost which increases up to 20% during first 8 assay. For twenty repetitive measurements AlOx gives good reproducibility. It is evident that AlOx is highly stable enzyme and retain its activity (around 80%) on chip when stored at 4 ºC. The activity profile of immobilized enzyme on chip, measured over a period of seven days is presented (see Fig. 4).

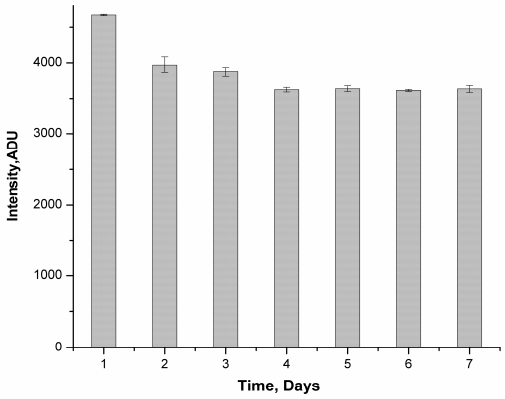

Fig. 4. Repeatability profile of Ti-chip coupled with enzyme AlOx over seven days

CONCLUSION

A novel Ti-biochip is demonstrated for use in Hg(II) biosensing. Enzyme (AlOx) coupling on Ti surface using thoil self assembly is also demonstrated successfully. Activity of the free enzyme in 1536 well plates against the on-chip immobilized enzyme is determined and found comparable. On chip inhibition studies for demonstration of Hg(II) is demonstrated successfully. On chip measurement were found highly sensitive with lower limit of detection 25pg/mL. Such system will facilitate sensitive *in-situ* measurement of trace contaminants in ground water samples. Besides the drastic reduction in the size of the analytical system, such miniaturization should lead to increased speed, minimal reagent consumption and waste generation. A high repeatability is observed without significant change in response signal over 25 continuous measurements.

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