ENZYME BASED BIOSENSOR FOR HEAVY METAL IONS DETERMINATION

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ABSTRACT
Sol-gel-immobilized urease conductometric biosensor on a thick film interdigitated electrode was developed. The biosensor can be used for heavy metal ions determination in liquid samples. The biosensor exhibited good response to changes in urea concentration within the range of 1mM to 15 mM. After standardizing the sensor for Urea, the biosensor has been used to determine the heavy metal ions of different concentrations. The concentration range of Urea that can be detected by using this sensor is 1mM to 15mM. The heavy metals range is from 0.1mM to 10mM. Among the three metals used, the amount of inhibition is found to be more in Cadmium, then Copper and then Lead. The sensitivity is 1 mM in spectrophotometric technique and 5 mM in electrical method. The described sensor is tested for synthetic effluents in laboratory conditions. By further refinement, it can be used to test real industrial samples.

Introduction
The accumulation of toxic substances in the environment continuously increases due to diverse pollutants from the industries. Contamination of land and water due to disposal of industrial effluents is the most significant problem. Heavy metal ions are regarded as one of the most toxic substances affecting the environment (1). The presence of heavy metals in excess affects air, water as well as soil. The plants grown in such areas can accumulate heavy metals like cadmium, zinc, lead and copper. These metals have certain threshold levels for essential functions of living organisms and man, which turn into toxic actions if the respective tolerance level for the respective organism is exceeded. Due to the high toxicity caused by the heavy metal ions there is an obvious need to determine them rapidly on site at trace levels. The present investigation aims at the development and evaluation of a sol-gel based biosensor for quantitative determination of heavy metals.

In recent years, biosensors are gaining importance as suitable detectors for heavy metal ions. They prove very promising for environmental monitoring, since the system is simple, rapid and selective. Several techniques based on spectroscopy, ion-selective electrodes, polarography and voltametry have been described in the past (6). Zhylyak et al. (9) developed a urease based conductometric biosensor for the determination of heavy metal ions in wastewater. The enzyme was immobilized by cross-linking urease with bovine serum albumin, which forms a biologically sensitive membrane. An interdigitated gold electrode was used as the transducer. The response of the
sensor for varying concentrations of heavy metal ions was evaluated by measuring the urease activity after incubating the electrodes in sample solutions of heavy metal ions. Li et al. (7) used horseradish peroxidase and developed an amperometric enzyme electrode for peroxide determination. Urease isolated from pigeon pea was immobilized in poly acrylamide gel and calcium alginate beads analyzed for various performance factors (4). Ho et al. (5) found that an enzyme-catalyzed polymer transformation when effectively combined with a transducer could be used effectively as a sensor. Srivastava et al. (8) immobilized urease enzyme on gelatin beads via cross-linking with glutaraldehyde. They analyzed the immobilized enzyme for various performance factors. Chern et al. (3) designed a biosensor that contained two metacrylic-acrylic membranes: One consists of proton ionophore and the other contains the enzyme urease for the detection of urea. When exposed to urea sample, the change in pH at the membrane was detected by the selective ionophore membrane and this was converted into an electrical signal (mV) by Ag/AgCl electrode.

Literature reports indicate that spectroscopic methods are expensive, as they require very sophisticated equipment, which cannot be used for field monitoring. Both polarographic and voltametric techniques lack selectivity. Since ion selective electrodes were based on the measurement of the potential at an electrode surface caused by a selective ion exchange reaction, the design of ion-selective membrane was a major difficulty in the development of this type of sensor. Taking into consideration the drawbacks mentioned above, there is a need for the development of a cheap, simple and portable detector for heavy metal ions.

Materials and Methods
The determination of heavy metal ions using the urease-immobilized biosensor is based on the measurement of the urease enzymatic activity, which is inhibited by heavy-metal ions. The enzyme urease was immobilized using the sol-gel process on a screen-printed electrode and used as the bio-recognition element in the biosensor. The enzymatic reaction was converted into an electrical signal using a transducer whose impedance change was the measure of the activity of the enzyme. The change in impedance was converted into a voltage signal by using a suitable circuit.

Construction of Biosensor
The sensor consisted of two electrodes separated by a known distance was shown (Fig. 1). The conductometric sensor considered for fabrication consists of two sets of interdigitated fingers deposited on an insulated substrate with each set having four fingers. Each set of fingers is connected to an electrical conductor that can be constructed of gold, platinum, iridium, carbon, copper or several other conductive materials which are inert in the medium and upon which coating containing urease remain adherent. It is fabricated by using PCB manufacturing technique.

Enzyme immobilization
A homogeneous stock sol-gel solution was prepared by vigorously mixing 570 μl of methanol, 50 μl of Tetra methoxy silicate (TMOS), 10 μl of 3.8 % Cetyl trimethyl ammonium bromide solution in a small test tube at room temperature. This stock gel solution was then cooled to 4 °C immediately after mixing. Enzyme stock solution was prepared by dissolving a known quan-
tity of urease in 50 ml of 0.02 mM phosphate buffer (pH 7.0). Enzyme solution was then stored at 4 °C in a refrigerator. 50 μl of enzyme stock solution along with 5 μl of glycerol was pipetted onto the surface of the electrode and distributed gently over the entire surface of the electrode with help of a capillary tube. The electrode was allowed to dry in ambient conditions for 1 h. Equal amount of stock sol-gel solution was pipetted to cover the enzyme layer formed over the surface of the electrode. The electrode was allowed to polymerize and dried for 1 h in ambient temperature. The enzyme electrode was immersed in a phosphate buffer and kept at 4 °C in a refrigerator overnight.

**Measuring circuit**

The impedance change was converted into voltage using the circuit shown (Fig. 2).
Fig. 3. Concentration of Urea Vs Voltage.

The response of the sensor was measured by applying a sinusoidal ac voltage of 1V and 10 kHz frequency. The output voltage was computed using the following equation:

\[ V_o = \left[ \frac{Z_L}{Z+Z_L} \right] V_i \]

The output voltage is acquired for further analysis using LabView 7.1. A data logger was designed using LabView to acquire voltage and store it in the computer to compute time response.

**Urea and urease activity**

Urea was determined by the diacetylmonoxime reaction as reported earlier (2). All the measurements were carried out in 25 ml beaker filled with 15-ml of test solution at room temperature. Standard plot for various concentrations of urea in the range of 1-15 mM were constructed. The immobilized electrode was dipped in 25 ml beaker which consisted of a known concentration of urea for 10 minutes. The output of the sensor was acquired by virtual instrumentation. The sample was taken and analyzed for urea concentration. The extent of urea hydrolysis was determined by comparing the obtained absorbance value with the standard absorbance plot drawn for the urea. The experiment was repeated to standardize the sensor for various concentrations of urea. The response of the electrode for 2 mM concentration was chosen for further evaluation. The electrode was incubated in a test solution containing heavy metal ion for 10 minutes. The heavy metal ions selected were copper, cadmium and lead. After incubating in heavy metal solution, the electrode was then washed with phosphate buffer and dipped in 2 mM of urea solution in 25 ml beaker. The procedure was repeated by changing the concentrations of heavy metal ions for different metals. For each sample the output voltage was acquired for further analysis and the samples were subjected to Spectrophotometric analysis to determine the % inhibition caused by heavy metals.

**Results and Discussion**

The steady state response of the biosensor as a function of urea concentration under the specified conditions was examined. As depicted in Fig. 3, the biosensor exhibited good response to changes in urea concentration within the range of 1 mM to 15 mM. The response was found to be linear.

The time response of the sensor was studied by using acquired voltage at various times for a known concentration of urea. The response shows gradual increase and reaches the steady state value after 3 min. The time response for 2 mM urea is shown (Fig. 4).

**Testing with metal ions**

The rate of enzyme inhibition by an inhibitor (heavy metal ion in this study) is
rather slow. Therefore, the biosensor to be tested should be pre-incubated for a certain period of time in the test solution containing an inhibitor in order to obtain the measurable inhibition. A measurable inhibition is obtained within 5 to 10 minutes pre-incubating period. Although it is clearly dependent on the specific metal ion and its concentration. An incubation time of 10 minutes is shown for further experiments. After standardizing the sensor for Urea, the biosensor has been used to determine the heavy metal ions of different concentrations. The concentration of heavy metal ions is measured in terms of % Inhibition and it is given by:

\[
\% \text{ Inhibition} = \left( \frac{A_0 - A}{A_0} \right) \times 100\% 
\]

\( A \) - absorbance of urea obtained before incubation in metals
\( A_0 \) - absorbance of urea obtained after incubation in heavy metals

The sequence of inhibition to the urease activity is \( \text{Cu}^{2+} > \text{Cd}^{2+} > \text{Pb}^{2+} \), which is in good agreement with the results observed in previous reports (6). The plots for various concentrations of all the three metals against % Inhibition are shown (Figs. 5, 6, 7). All the responses are for 2 mM of Urea.

**Response curves for Metal ion concentration Vs % Inhibition**

The Inhibition by Copper shows linear increase in lower concentration range (0.1 mM to 1 mM) and the increase in inhibition becomes less as the concentration increases (1 mM to 10 mM).

The Inhibition by Cadmium shows almost linear increase in the concentration range (0.1 mM to 10 mM). The Inhibition by Lead shows rapid increase in lower concentration range (0.1 mM to 1 mM) and as the concentration increases the increase in inhibition becomes less but shows a linear rise.

**Performance factors**

The storage time of the sensor is found to be 6 to 7 days. It can be used 4 to 5 times when it is used to measure Urea and only one time if it is used to measure heavy metals concentration. The concentration range of Urea that can be detected by using this sensor is 1 mM to 15 mM. The heavy metals range is from 0.1 mM to 10 mM. Among the three metals used, the amount of inhibition is found to be more in Cadmium, then Copper and then Lead. The sensitivity is 1 mM in spectrophotometric technique and 5 mM in electrical method.
The described sensor is tested for synthetic effluents in laboratory conditions. By further refinement, it can be used to test real industrial samples. Future development will include adopting suitable methods to achieve selective detection of heavy metal ions.

Conclusions
The studies conducted in this project show that the Sol-gel-immobilized-urease conductometric biosensor on a thick film interdigitated electrode can be used as a reliable sensor for heavy metal ion determination in liquid samples. It has several advantages like easy production of the sensor, low cost, sensibility and ease of operation.

REFERENCES