Microbial Electrode Sensor for Heavy-metal Ions

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A whole-cell-based amperometric biosensor was fabricated using Escherichia coli cells immobilized on a glassy carbon electrode to detect mercury, cadmium, and zinc ions. E. coli cells were immobilized by the cross-linking method using bovine serum albumin (BSA) in glutaraldehyde (GA) vapor. The principle of the microbial electrode sensor is the inhibition of alkaline phosphatase (AP) enzyme activity by heavy metal ions. Therefore, by monitoring the oxidation current of the product generated by AP in a metal ion solution, its concentration was determined. The Michaelis–Menten constant (Km) for AP was evaluated to be 2.23 mmol/L. The E. coli-based biosensor has detection limits of $5.58 \times 10^{-11}$ mol/L for mercury ion, $5.10 \times 10^{-10}$ mol/L for cadmium ion, and $1.38 \times 10^{-9}$ mol/L for zinc ion. The prepared biosensor can respond steadily for seven days.

1. Introduction

The rapid and sensitive detection of heavy metals at trace levels in fresh water sources becomes a very important issue because heavy metal ions have serious toxic effects on living organisms. Conventional techniques, such as flame spectrometry, atomic absorption spectrometry (AAS), and anodic stripping voltammetry, have high sensitivity and accuracy, but require high cost, skilled personnel, and long measurement time. Biosensors have drawn much attention in the toxicity assay of heavy metal ions owing to their high selectivity, low cost, rapid measurement, and applicability to on-line monitoring. Enzyme-based biosensors have been used in the determination of toxic heavy metal ions since a particular enzyme has a high specificity for many inhibitors such as heavy metals and pesticides. However, commercially available enzymes are usually expensive. Furthermore, when using several enzymes simultaneously for multitarget detection, biosensor stability becomes an issue as each enzyme requires specific reaction circumstances. Microbial-cell-based biosensors are good alternatives for enzyme-based biosensors. Although microbial sensors do not have a high selectivity, they have some advantages compared with enzyme biosensors. Since a microorganism cell is a highly integrated system consisting of
many enzymes and can be massively produced by cell culture, microbial biosensors are cost-effective and can be used as stable multienzyme arrays for the simultaneous determination of toxicants. In previous reports, the inhibition of alkaline phosphatase (AP) enzyme activity was widely studied for the detection of heavy metals.\(^{6,9,12,21}\) AP was found in *Escherichia coli*. 

*p*-Nitrophenyl phosphate (pNPP) is widely used as a substrate of AP to dephosphorylate into *p*-nitrophenol (pNP) and phosphate ions. Its activity is known to be inhibited by heavy metal ions.

A conductometric biosensor has been developed using immobilized *Chlorella vulgaris* to detect cadmium and zinc ions that inhibit the AP activity,\(^{22,23}\) whilst an amperometric biosensor with a detection limit of 0.1 ppb for zinc and cadmium ions was developed using immobilized *C. vulgaris* on a diamond electrode.\(^{24}\) The whole-cell-based amperometric biosensor was fabricated to detect mercury and zinc ions by immobilized microalgae *Chlorella sp*.\(^{25,26}\) Cells of the cyanobacterium *Arthrospira platensis* were directly immobilized on the ceramic part of gold interdigitated transducers to obtain an ultrasensitive conductometric biosensor for cadmium and mercury ions.\(^{27}\) The purpose of our study is to develop a microbial biosensor based on the inhibition of the enzyme inside *E. coli* for the detection of heavy metal ions.

*E. coli* cells were immobilized on a glassy carbon electrode to detect mercury, cadmium, and zinc by analyzing the inhibition behavior of the AP enzyme. Currents generated by the oxidation of enzymatically produced pNP were monitored amperometrically. The prepared biosensor may have potential in the detection of the total toxicity of water.

## 2. Materials and Methods

### 2.1 Materials

Bovine serum albumin (BSA) was purchased from Sigma (China). A 50% aqueous solution of glutaraldehyde (GA) was purchased from Tianjin Bodi Chemicals (China). pNPP from Aladdin (China) was used as a substrate to determine the AP activity. Mercury chloride (HgCl\(_2\), 99.0%) was purchased from Tianli Chemicals (China). Cadmium chloride (CdCl\(_2\), 99.0%) and zinc sulfate (ZnSO\(_4\), 99%) were purchased from Tianjin Kernel Chemicals (China). All reagents were of the analytical grade. Ultrapure water (18.2 M\(\Omega\)cm) was used in all experiments.

### 2.2 *E. coli* culture

*E. coli* DH5\(\alpha\) from Northeast Agricultural University was cultured in Luria broth (LB) medium at 37 °C for 20 h each day. *E. coli* was harvested by centrifugation at 6000 r/min for 5 min and washed twice with Tris-HCl buffer (pH 8.0). Cells were resuspended in Tris-HCl buffer and adjusted to the desired cell density. All cell suspensions were kept at 4 °C and used in the experiment on the day of harvesting.
2.3 Biosensor preparation

*E. coli*-BSA was immobilized on the electrode surface using GA as a cross-linker. 5 μL of a mixture of *E. coli* solution and 1% (w/v) BSA was deposited on the surface of the glassy carbon electrode surface by the casting method. Then, the electrode was placed in saturated GA vapor and then dried for 30 min at room temperature (25 ℃). Another mixture of 5 μL culture medium and 1% (w/v) BSA was deposited on another electrode used as a reference for differential measurements. The electrode surface area was 0.0314 cm². The prepared biosensor was stored in LB medium at 4 ℃.

2.4 Instrument and electrodes

The cell optical density was measured at 600 nm using a Cary 500 Scan UV–Vis–NIR spectrophotometer. Chronoamperometric experiments were performed using a potentiostat (Autolab, PGSTAT128, Netherlands). A glassy carbon electrode, a Au electrode, and a saturated Ag/AgCl electrode were used as the working, counter, and reference electrodes, respectively.

2.5 AP activity measurement

For chronoamperometric study, an *E. coli*-modified glassy carbon electrode was immersed in 0.1 mol/L Tris–HCl buffer (pH 8.0) and 1 mmol/L MgCl₂ (enzyme activator) solution with constant stirring at 100 r/min. After stabilizing the output signal for 15 min, different aliquots of 20 mmol/L pNPP as a substrate were added to the electrochemical cell. A coamperometric scan was taken by biasing the electrode at 1.05 V vs the gCl reference electrode. The oxidation current of pNP generated from pNPP enzymatically was detected.

2.6 Heavy metal ion measurement

Some heavy metal ions, such as mercury, cadmium, and zinc ions, were detected in this work. Different aliquots of heavy metal solution were made by diluting 0.1 mol/L stock solutions before the experiment. After stabilizing the output signal for 15 min, pNPP as a substrate was added. The oxidation currents were stabilized for a further 10 min; then, an aliquot of heavy-metal solution was added into the electrochemical cell.

3. Results and Discussion

3.1 AP activity

Even a trace amount of heavy metal would inhibit the AP activity because the enzyme forms a stable metal enzyme complex with a heavy metal. The most widely used substrate for AP is pNPP. AP in microbial cells modifying the electrode causes the generation of electroactive pNP from electroinactive pNPP, leading to a current signal (Fig. 1). When the biosensor is exposed
to a heavy metal ion solution, the decrease in current would be related to the heavy metal ion concentration. Chronoamperometry was used to monitor the oxidation current of pNP.

Figure 2 shows the calibration curves for the substrate pNPP responding to the AP-immobilized E. coli cells. The response current increased with the pNPP concentration and leveled off at 4.8 mmol/L. The enzymatic activity follows a typical Michaelis–Menten behavior. The Michaelis–Menten constant (Km) for AP was evaluated to be 2.23 mmol/L. This value is about three times larger than that in previous studies, which is probably due to the difficulty in the diffusion of the substrate from the bulk to enzymes in cells. The biosensor showed a sensitivity of 10.95 μA mol⁻¹ cm⁻² with a relative standard deviation of less than 4%.

3.2 Parameter optimization of E. coli biosensor

Figure 3 shows SEM images of prepared E. coli–BSA membranes. Figure 3(a) indicates that E. coli cells (white spots in yellow circles) were uniformly distributed in the BSA membrane matrix. The maximum length of the white spots is about 2 μm, which is in accord with the length of E. coli cells. In the image, many spots smaller than 2 μm can be seen because part of the E. coli cell is entrapped in the immobilization membrane matrix. In the image of the membrane without E. coli cells, there were no white spots [Fig. 3(b)].

Exposure time to GA vapor is an important parameter in the immobilization of microbial cells. Too short a GA vapor exposure results in the poor formation of E. coli–BSA membranes, whereas an excess contact of GA with microbial cells leads to a decrease in enzymatic activity in the cells because GA is toxic to the microbial cells. As shown in Fig. 4(a), an optimal signal was observed for 30 min of exposure. In addition, no significant signal decrease was observed over a period of 4 h. These results confirm the stability of the biosensor obtained from 30 min of GA vapor exposure.

The thickness of microbe–BSA membranes has a direct effect on the performance of the microbe-immobilized biosensor. The thickness of microbe–BSA membranes can be adjusted...
by varying the BSA concentration in the cross-linking mixture. A low BSA concentration results in the poor formation of microbe–BSA membranes, probably owing to a smaller degree of *E. coli* immobilization. A high BSA concentration also causes a low signal because of the poor electrical conductivity. As shown in Fig. 4(b), the biosensor response suddenly increased at BSA concentrations of 0.05 to 1% maximum and then gradually decreased. It showed no further change after 7.5% BSA. In addition, at 0.05% BSA, the response decreased to

Fig. 3. (Color online) SEM images of membrane containing (a) BSA and *E. coli*, and (b) BSA only.

Fig. 4. (Color online) Response characteristics of *E. coli* biosensor immobilized under different parameter conditions: (a) cross-linking time, (b) BSA concentration, (c) *E. coli* cell density, and (d) pH. Measurements were performed in 0.1 mol/L Tris–HCl and 1 mmol/L MgCl₂.
about zero in 1 h, which indicates that the immobilization membrane falls from the surface of the electrode owing to the poor cross-linking since the amount of BSA is very small. The best biosensor performance was with 1% BSA; therefore, 1% was chosen in the following experiments. The thickness of the immobilization membrane obtained with 1% BSA was about 8 μm.

The density of immobilized cells is directly related to the signal magnitude of microbial sensors. A lower \textit{E. coli} cell density in membranes yields a lower current because fewer substrate molecules are transformed on the surface of the electrode. On the other hand, an overloading of cells on the electrode will restrict the diffusion of substrate molecules to the electrode surface.\(^{22,23}\) Such a trend has also been observed for pure enzyme membranes.\(^{30}\)

The signal responses for biosensors modified with different \textit{E. coli} cell densities are shown in Fig. 4(c). The density of microbial cells in the immobilization membrane was controlled by changing the \textit{E. coli} cell concentration in the immobilization mixture solution. The highest oxidation current was obtained at \(1 \times 10^9\) cells/mL. However, it has been decided to use a microbial concentration of \(5 \times 10^8\) cells/mL as a compromise between the signal amplitude and a significant inhibition level.

The enzymatic activity is known to depend on the pH of the solution. It has been reported that the optimum pH for the AP activity is 9.\(^{9}\) Similar results are reported for algal biosensors. However, our results show that the highest current response of the \textit{E. coli}-modified electrode was at pH 8 [Fig. 4(d)]. The reason is that \textit{E. coli} cells are more stable in a less alkaline medium.

### 3.3 Heavy metal ion detection

Under the optimized immobilization conditions, the prepared biosensor was used for the detection of some heavy metal ions, including mercury, cadmium, and zinc ions. Since the AP activity is inhibited by heavy metal ions, the oxidation current of pNP, an enzymatic reaction product, decreases with increasing heavy metal ion concentration. Figure 5 showed the responses of the \textit{E. coli} biosensor to various mercury, cadmium, and zinc ion concentrations. The prepared biosensor responded quantitatively to the concentration ranges of \(4.99 \times 10^{-10}\) to \(4.99 \times 10^{-3}\) mol/L for mercury, \(8.89 \times 10^{-10}\) mol/L to \(8.89 \times 10^{-3}\) mol/L for cadmium, and \(15.29 \times 10^{-10}\) mol/L to \(15.29 \times 10^{-3}\) mol/L for zinc. The detection limit of the biosensor was evaluated as 10% of the degree of inhibition.\(^7\) The prepared biosensor exhibited detection limits of \(5.58 \times 10^{-11}\) mol/L for mercury, \(5.10 \times 10^{-10}\) mol/L for cadmium, and \(1.38 \times 10^{-9}\) mol/L for zinc. Although these values are not better than those for other whole-cell-based biosensors,\(^{26,27}\) they are well below the maximum contamination limits for heavy metals set by the World Health Organization and US for drinking water (1 ppb for mercury, 3 ppb for cadmium, and 5 ppm for zinc). These results show the potential of whole-cell biosensors based on \textit{E. coli} cells for the detection of heavy metal ions in water samples. If the genetic engineering of \textit{E. coli} cells can increase the amount of internal AP, the detection limit of biosensors for heavy metal ions will be improved.
3.4 Stability of microbial biosensor

In order to evaluate its stability, the prepared biosensor was stored under the storage conditions described in Sect. 2.3 and used to measure the AP activity every day. As shown in Fig. 6, the biosensor response remained stable for five days and suddenly decreased to below 50% of the original response after seven days. This indicates that the microbial cells in the immobilization membrane are in a stable phase for five days and then transition to the decline phase.
4. Conclusions

We developed an *E. coli* whole-cell biosensor for the detection of some heavy metal ions with the inhibition of the AP activity. *E. coli* cells were immobilized on a glassy carbon electrode using BSA as the matrix and GA as the cross-linker. The conditions of immobilization, such as cross-linking time, the pH of the medium, microbial cell density, and BSA concentration, were optimized. The prepared biosensor responded quantitatively to the concentration of mercury, cadmium, and zinc ion concentrations. The detection limits of the biosensor were $5.58 \times 10^{-11}$ mol/L for mercury ion, $5.10 \times 10^{-10}$ mol/L for cadmium ion, and $1.38 \times 10^{-9}$ mol/L for zinc ion. Compared with the enzyme sensor, the *E. coli*-based sensor has the advantages of low cost and easy availability.

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References