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Detecting total toxicity in water using a mediated biosensor system with flow injection

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HIGHLIGHTS

- This paper provides a biosensor for toxicity monitoring.
- This method is a simple, rapid and low cost means.
- This constructed biosensor system will easier to be applied and commercialized.

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ABSTRACT

A novel total toxicity detection method based on a mediated biosensor system with flow injection (MB-FI) was developed to rapidly and reliably detect respiration inhibitors (i.e., As_2O_3, KCN, salicylic acid (SA), 2,4-dintirophenol (DNP)) in water. The mediated biosensor toxicity assessment using microorganisms immobilized in calcium alginate filaments can greatly simplify the testing process and save time. In the MB-FI system, ferricyanide together with a respiration inhibitor was injected into the bioreactor, inhibiting the respiration of the immobilized microorganisms. The degree of inhibition was measured by determining the ferrocyanide generated in the effluent, expressed as the 50% inhibition concentration (IC50). The IC50 values for the four respiration inhibitors obtained using this method were comparable to those obtained using the classic method, confirming that this approach is an alternative alert method. More importantly, this constructed biosensor system with flow injection will facilitate the application and commercialization of this toxicity monitoring technology.

1. Introduction

Chemical pollution is a global problem, and many scientists have focused on the development of technologies to enforce environmental monitoring and homeland security (Yeates et al., 1994). However, the conventional analytical detection of specific pollutants is not sufficient to assess environmental risk because toxicity is a biological response (Hernando et al., 2005). Furthermore, the synergistic toxicity of pollutants induced by the toxicity of multiple pollutants is greater than the sum of the toxicities of the individual pollutants (Eaton, 1973; Fernandez-Alba et al., 2002; Hsieh et al., 2006). Therefore, developing an effective means to evaluate total toxicity on living organisms is highly desirable and remains a great challenge.

In recent decades, various biological models have been developed to study toxicity and measure the risk of environmental pollution. Existing conventional techniques that rely on changes in physiological characteristics or motility, such as in *Daphnia magna* or fish, are typically time consuming, costly and involve specialized equipment and trained personnel (Nancharaiah et al., 2007; Drost et al., 2007; Burýšková et al., 2006; Arufe et al., 2004; Levy et al., 2007). Among them, the most common bacterial tool for toxicity measurements is MICROTOX, which can be used to assess the toxicity of water samples by measuring the decrease in bioluminescence when the water samples contain toxic chemicals (Hassan and Oh, 2010). However, this method has several disadvantages and limiting characteristics. Luminescence tests that depend on optical detection are restricted by cell populations and are not suitable for samples with high turbidity, which can cause fluorescence scattering (Johnson, 2005). Additionally, luminous bacteria use 3% NaCl to provide the osmotic pressure to maintain their activities (Liu et al., 2007), and this decreases the solubility of some organic chemicals and limits its practical application.

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Recently, microbial toxicity sensors utilizing redox mediators have received much attention. The mediator acts as an electron acceptor in place of oxygen in the microbial respiratory pathway and shuttles electrons from the microbes to the electrode surface (Emde et al., 1989; Hadjipetrou et al., 1970; Patchett et al., 1989). Ferricyanide is the most widely studied electron acceptor and has significant advantages over oxygen due to its high solubility (Pasco et al., 2000). The significance of this feature is that a denser microbial population that will deplete the electron acceptor more. Using larger microbial populations accelerates the reaction and produces a larger analytical signal. Potassium hexacyanoferrate (HCF(III)) is an efficient mediator, and the HCF(III)-mediated toxicity determination has been extensively studied (Tizzard et al., 2004; Liu et al., 2009a,b; Pasco et al., 2008; Catterall et al., 2010a,b; Yong et al., 2011). The principle behind the microbial toxicity sensor is shown in Scheme 1B. HCF(III) acts as an electron acceptor instead of O_2 in the biochemical incubation and is reduced to HCF(II) (Eq. (1)):

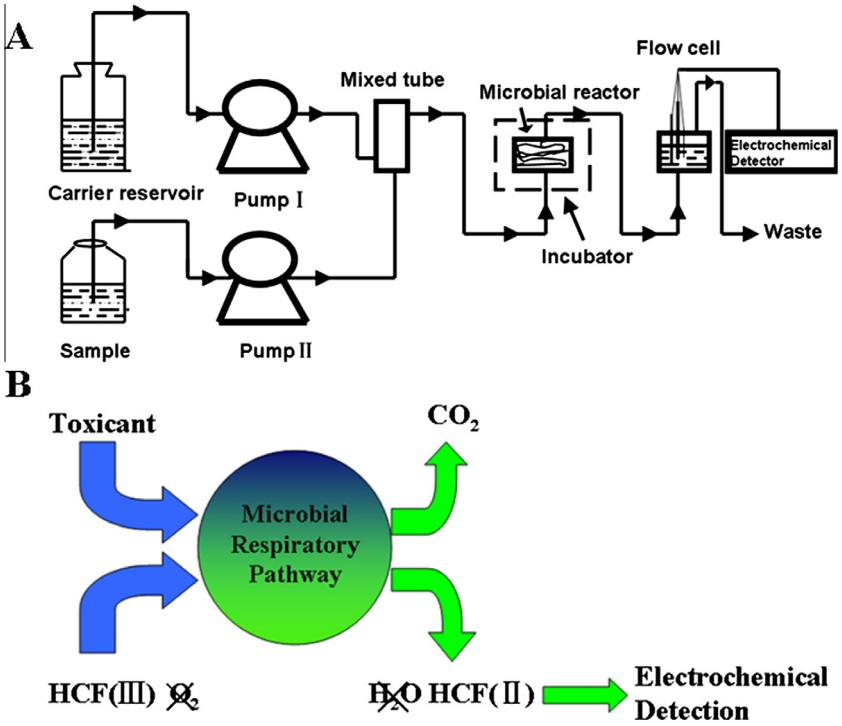
$$CH_2O + H_2O + 4[Fe(CN)_6]^{3-} \rightarrow CO_2 + 4H^+ + 4[Fe(CN)_6]^{4-} \quad (1)$$

Chronoamperometry in the microelectrode array measured the quantity of the reduced mediator (HCF(II)) produced in the terminal of the biochemical incubation. In general, the current obtained is proportional to the activity of the microbial cells, which can easily be detected electrochemically. In the presence of toxicants, the bioactivity of the microbial cells was inhibited and thus decreased the limiting current. In electrochemistry, the limiting current is the limiting value of a faradaic current that is approached as the rate of charge transfer to an electrode increases. Therefore, the degree of toxicity was estimated by comparing the differences in the limiting currents obtained. This method directly measures the amount of electron transfer in the biochemical incubation; therefore, it determines toxicants with high sensitivity.

However, there are many disadvantages of the mediator-based toxicity assay, such as tedious preparation and complicated operation. Microbial cultivation is an indispensable procedure for most established methods, which takes a considerable amount of time (Tizzard et al., 2004; Liu et al., 2009a; Pasco et al., 2008; Catterall et al., 2010a, 2010b; Yong et al., 2011). A simple method of immobilizing the microorganisms on the calcium alginate beads has been proposed in many studies. Sodium alginate is a water-soluble polymer that produces highly viscous solutions that stabilize the suspension of microorganisms in the alginate matrix. This immobilization method is manoeuvrable and can provide biocompatible conditions to maintain bioactivity. However, the diffusion barrier of the matrix needs improvement. The improved immobilization of the alginate matrix in the mediator-based biosensor toxicity assessment will greatly simplify the testing process, shorten the operation time and reduce the cost.

On the other hand, flow injection analysis (FIA) is a well-established automated technique (Ruzicka and Hansen, 1975). This methodology has gained widespread use due to its great potential for routine analysis and high reproducibility. Its excellent versatility allows it to successfully couple with a variety of detection systems (Anthemidis and Miró, 2009; Tzanavaras and Themelis, 2007; Wang et al., 2009; Blasco et al., 2007). This methodology also increases the environmental safety of the process because of its inherent miniaturization, a considerable decrease in reagent consumption and waste disposal; moreover, the closed-system chemistry prevents the operator from coming into contact with hazardous chemicals. FIA is also simple, low cost, and rapid, which are all attractive features in environmental analysis (Wang et al., 2009; Xu et al., 2005; Miró and Frenzel, 2004). Thus, the development of FIA in mediator-based toxicity sensor systems is promising.

Here, we describe a new design for total toxicity monitoring in water with immobilized microorganisms. To demonstrate the feasibility of this proposal, *Escherichia coli* (*E. coli*) was immobilized on calcium alginate filaments as the biological recognition element. A flow cell with a three-electrode configuration was assembled and used as a transducer. A flow injection sensor system was constructed by arranging the reactor and flow cell separately. Four



Scheme 1. Scheme of the mediated biosensor system of flow injection mode and principle of mediated biosensor method. (A) Scheme of the mediated biosensor system of flow injection mode. (B) Principle of mediated biosensor.

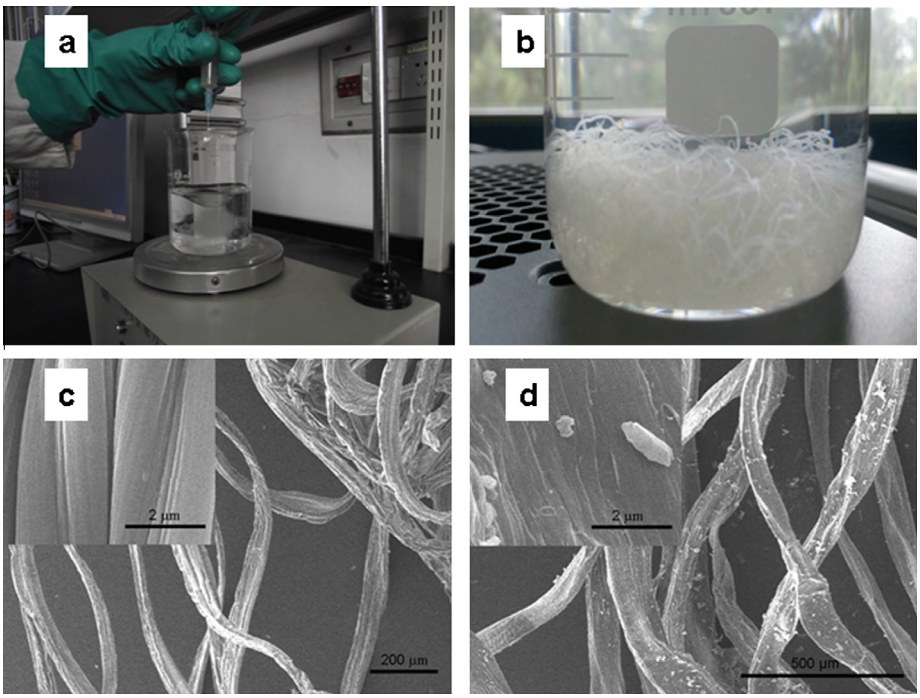


Fig. 1. Digital photos and SEM images of calcium alginate filaments. (a) The digital photo of process of *E. coli* immobilization. (b) The digital photo of calcium alginate filaments containing entrapped *E. coli*. SEM images of calcium alginate filaments (c) and calcium alginate filaments containing entrapped *E. coli* (d).

respiration inhibitors as model toxicants were examined by this system. Comparing our results with that of MICROTOX confirmed that our method provides a simple and rapid alternative to chemical toxicity screening and is especially advantageous for monitoring total toxicity in water. This method can greatly simplify the testing process and save time; it is simple, rapid, and inexpensive, and there is no need to train specialized personnel, which make this toxicity monitoring technology easier to apply and commercialize. This method can detect inhibitors, in addition to other chemicals that directly or indirectly influence respiration, such as membrane disruptors, disrupting a specific microbial process and oxidizing vital cellular structures or components.

2. Materials and methods

2.1. Chemicals and solutions

KCN, As_2O_3 and 3,5-dichlorophenol (DCP) were purchased from Sigma. Salicylic acid (SA) and 2,4-dintirophenol (DNP) were obtained from the Beijing Chemical Reagent Co., Ltd. *Escherichia coli* (*E. coli*) DH 5α was purchased from the Beijing Dingguo Changsheng Biotech. Co., Ltd. *Pseudomonas putida* (*P. putida*) was obtained from the China General Microbiological Culture Collection Center (CGMCC).

The Luria–Bertani (LB, 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) broth was adjusted to the desired pH with 2 M HCl and sterilized in high-pressure steam at 120 °C for 20 min. $K_3[Fe(CN)_6]$ was freshly prepared before use. All chemicals used in this study were of analytical grade, and all solutions were prepared with deionized water, unless otherwise stated.

2.2. Microbial cultivation and immobilization

LB medium was inoculated with *E. coli* DH 5α, which was then grown aerobically in a shaker (180 rpm) for 16 h overnight at 37 °C. *P. putida* was grown aerobically for 12 h at 30 °C. Cells were harvested by centrifugation at 6000 rpm for 5 min at room temperature and washed twice with phosphate buffer solution (PBS). The cells were then re-suspended in PBS to obtain a microbial cell slurry, the concentration of which was adjusted by measuring the absorbance at 600 nm using a Cary 500 Scan UV–Vis–NIR Spectrophotometer (OD_{600}). The OD_{600} value for the original

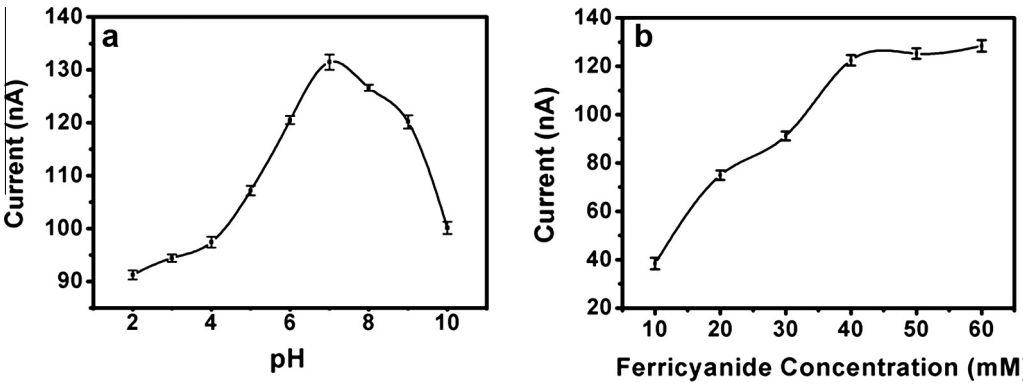


Fig. 2. The different reaction conditions effect on current responses. (a) Effects of carrier solution pH on the sensor response. (b) Effects of the concentration of ferricyanide on the limiting current response. The values represent the mean of three independent experiments (±SD).

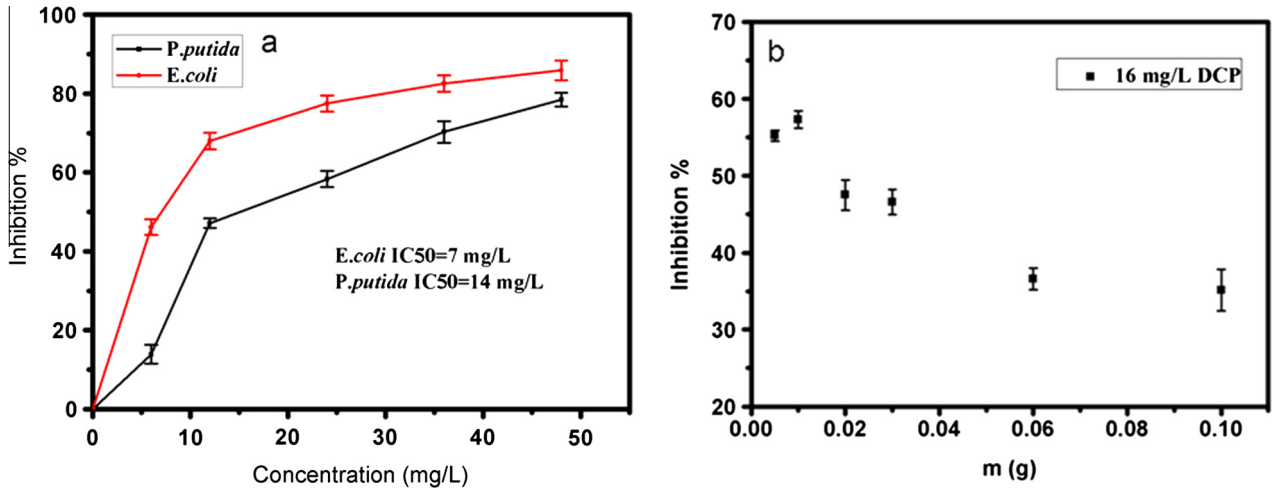


Fig. 3. The kind and the amount of microorganism strain on sensitivity. (a) Effects of the kind of microbial strain on sensitivity. Two microbial strains are *E. coli* and *P. putide*, respectively. (b) Effects of microbial amount on sensor sensitivity (45 mM ferricyanide and 16 mg/L DCP). The values represent the mean of three independent experiments (±SD).

bacterial suspension was 90, calculated by dilution of the actual absorbance measurement of 0.9; the diluted value was used in the subsequent experiments on the day of harvesting. The cell suspension was fully mixed with a 2% (w/v) sodium alginate solution under aseptic conditions. As shown in Fig. 1a, the mixture was immobilized by extruding the sodium alginate filaments into a 5% (w/v) $CaCl_2$ solution using a syringe. Due to the formation of a hydrogel, the calcium alginate filaments containing entrapped *E. coli* were obtained instantly (Fig. 1b). The filaments containing the entrapped cells were maintained in a 5% (w/v) $CaCl_2$ solution for 12 h at 4 °C and then filtered and washed with 0.7% (w/v) NaCl for further use.

2.3. Instrumentation and procedure

A schematic diagram of the flow injection biosensor system is illustrated in Scheme 1. The system consisted of two peristaltic pumps (model BT100-1J, Longer pump Co., Hebei, China), a mixing tube, a microbial reactor, and a flow cell. Silicone tubes that were 2.5 mm in diameter connected these parts. Under the same flow rate, the sample container was connected to pump II when measuring the response to the sample, and the deionized water container was connected to pump II when checking the sensitivity of the electrode. After the immobilized microbe filaments were packed into a cell holder, the packed bed was thoroughly washed by passing a sterilized 0.7% NaCl solution through at 10 rpm overnight. PBS and 90 mM HCF(III) solutions were used as the carriers. The carrier reservoir was held in an incubator at 37 °C, and the flow rate was set at 10 rpm.

2.4. Electrochemical detection and toxicity calculation

Toxicity was monitored by measuring the changes in the limiting currents (CHI 832B electrochemical workstation (Chen Hua, Shanghai)) before and after injection of the sample solution. Amperometric measurements were carried out by applying a given potential (+450 mv) to the working electrode. An ultramicroelectrode array (UMEA) fabricated with either 20 pieces of single 25 μm Pt ultramicroelectrodes or a single Pt ultramicroelectrode (25 μm) was used as the working electrode; Pt gauze was used as the auxiliary electrode, and Ag/AgCl (saturated KCl) was the reference electrode. The inhibitory effect (I) of the inhibitors on *E. coli* was calculated using the following equation:

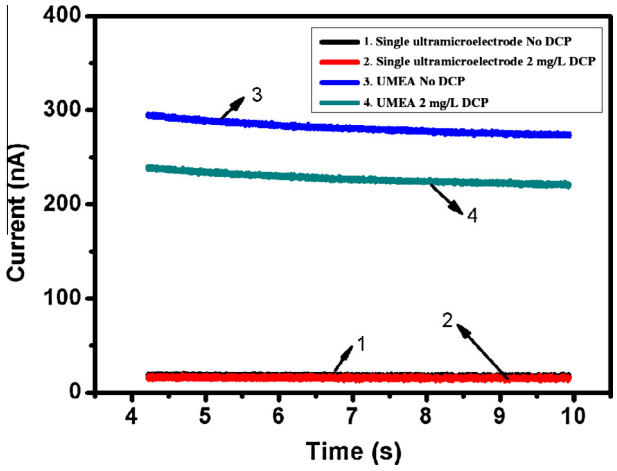


Fig. 4. The variation of limiting current of single ultramicroelectrode and UMEA depending upon time under the same conditions (45 mM ferricyanide, 1 h incubation and 2 mg/L DCP).

$$I\ (\%) = \frac{i_{\text{lim control}} - i_{\text{lim sample}}}{i_{\text{lim control}}} \times 100\% \qquad (2)$$

where I is the percentage of inhibition after 1 h incubation, and $i_{\text{lim control}}$ and $i_{\text{lim sample}}$ are the limiting current for the control and sample after 1 h incubation, respectively. The effective concentration (IC50) responsible for 50% growth inhibition of *E. coli* was calculated by using OriginPro 8 (graphing and data analysis software).

3. Results and discussion

3.1. Characterizations of blank filaments and filaments containing entrapped E. coli

The morphologies of the blank filaments and filaments containing entrapped *E. coli* were investigated by scanning electron microscopy (SEM). All samples were frozen at −20 °C overnight and then desiccated in the chamber of a freeze-drier. Typical SEM images are shown in Fig. 1. Remarkably, the shape of the filaments after freeze-drying was linear with diameters of approximately 100 μm. The high specific surface area is beneficial for immobilizing microorganisms. Fig. 1c and d presents images of the blank filaments and filaments containing entrapped *E. coli*, respectively; *E.*

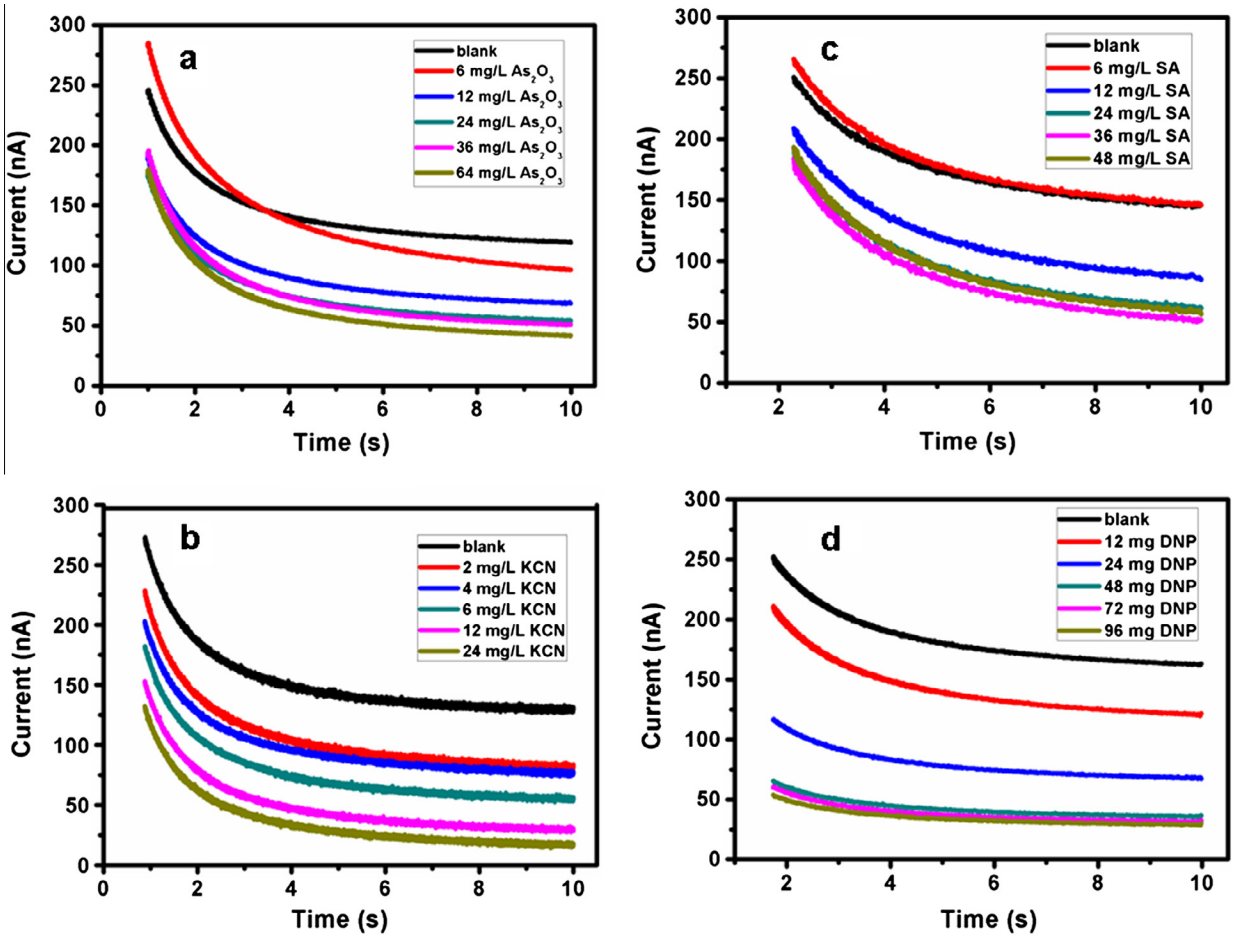


Fig. 5. The currents obtained in different concentration of four inhibitors. (a) As_2O_3 and (b) KCN. (c) SA. (d) DNP.

coli had clearly been immobilized in the filaments. However, we also observed some small pieces containing entrapped *E. coli* on the external surface of the filaments due to cytoplasm that had leaked out of cell damaged in the freeze-drying process.

The abilities of the balls and filaments containing entrapped *E. coli* with the same weight to reduce HCF(III) were investigated (data not shown), using the limiting current to quantify HCF(II). The limiting current produced by the filaments was approximately twice as large as that of balls of the same weight mainly because the filaments have higher mass transfer efficiency than the balls. In particular, different batches of filaments with the same weight had the same ability to reduce HCF(III), and the limiting currents produced by them were similar (data not shown). This result ensured that different batches of filaments containing entrapped *E. coli* yielded reproducible results. Furthermore, the calcium alginate filaments are very inexpensive; therefore, their recovery and re-use does not need to be considered.

3.2. Optimization of the biosensor system

3.2.1. Carrier solution pH and concentration of HCF(III)

Effects of the carrier solution pH on the sensor response were investigated using buffer solutions with different pH levels. Buffer solution containing HCF(III) was passed through the microbial reactor, and, after 1 h of incubation, the biosensor responses to the different pH levels were converted to limiting current values and are shown in Fig. 2a; the optimum pH of the system was therefore between 2.2 and 10.0. The largest response was observed at pH 7.0. These results may indicate that the metabolism of the microbe becomes more active in a neutral pH range. The pH of the samples was subsequently adjusted to 7.0 using phosphate buffer.

The dependence of HCF(III) concentration on the sensor response was also examined. As shown in Fig. 2b, the sensor response increased proportionally to the concentration of HCF(III) from 10 mM to 60 mM. In particular, the limiting current values increased steadily with increasing concentrations of HCF(III) up to 40 mM, and it then stabilize from 40 mM to 60 mM. Greater than 50 mM HCF(III) harmed the microorganisms, which was consistent with a previous report (Liu et al., 2009a,b). Therefore, 45 mM ferricyanide was used in following experiments.

3.2.2. Microbial strain and amount of microorganisms

Two types of bacteria (*E. coli* and *P. putida*) were immobilized in calcium alginate filaments to investigate the effects of different microbial strains on the sensor sensitivity. DCP was chosen as the reference toxicant because it had been widely studied in previous toxicity assays. The OECD 209 Activated Sludge Respiration Inhibition Test (ASRIT) also advised the use of DCP as a reference toxicant (Dos Santos et al., 2002). The toxicities of DCP on *E. coli* and *P. putida* are presented in Fig. 3a. Through comparison of the two inhibition curves, we find that these two bacteria have different responses to DCP. IC50 values of *E. coli* and *P. putida* were 7 mg/L and 14 mg/L, respectively. *E. coli* had a lower IC50 value than that of *P. putida*, indicating a more sensitive response to DCP. This result demonstrates that different types of microorganisms can have different responses to the same toxicant. *E. coli* DH 5α was used as the microbial reactor to demonstrate the feasibility

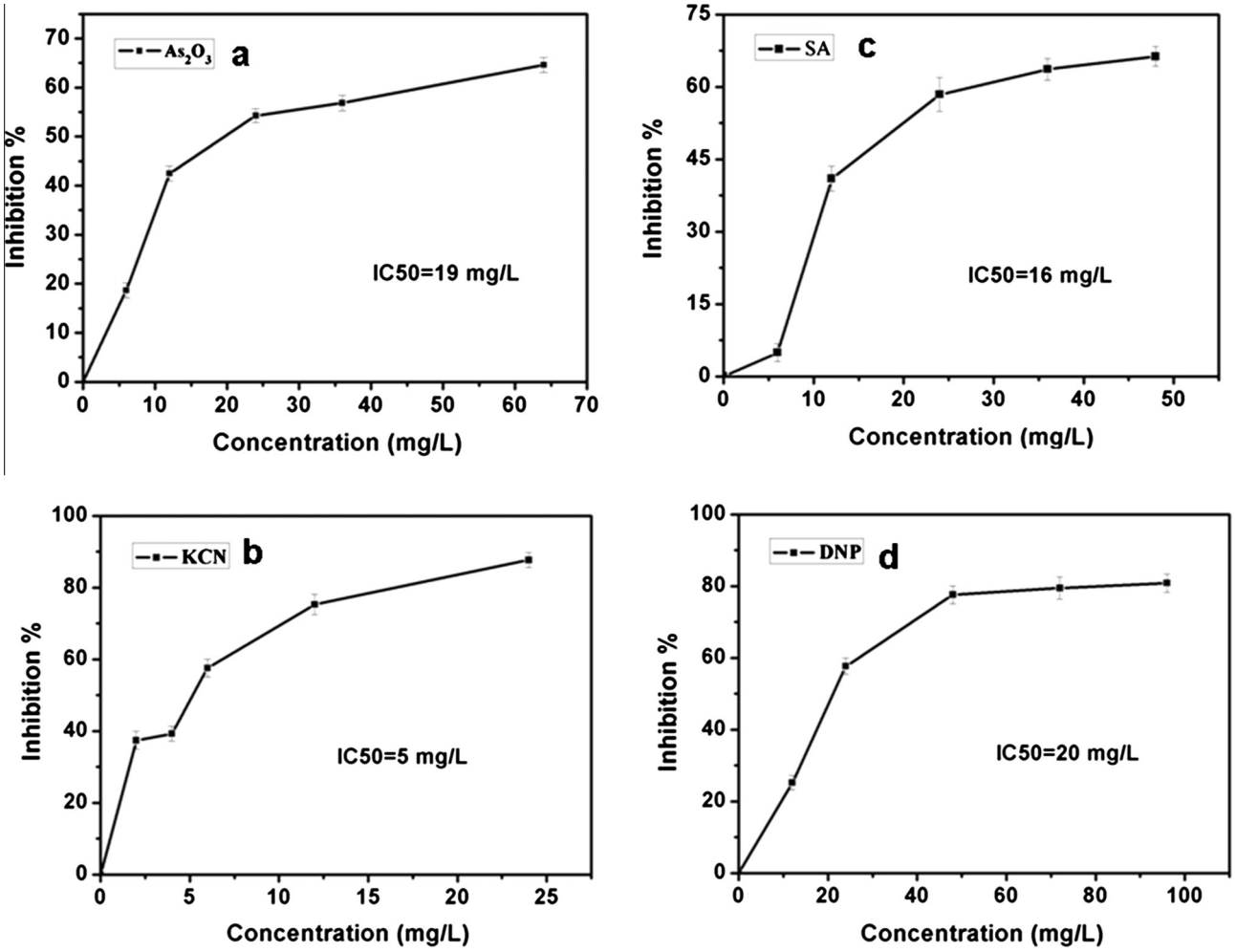


Fig. 6. The relationship between the inhibitory percentages and concentrations of four respiration inhibitors. a. As_2O_3. b. KCN, c. SA. d. DNP.

Table 1
Comparison of IC50 values by using two methods (MB-FI method and MICROTOX test).

Respiration inhibitors	MB-FI (mg/L)	Microtox (mg/L)
As_2O_3	19	43
KCN	5	4
SA	16	30
DNP	20	22

of this method because *E. coli* is ubiquitous in soils and freshwater. The amount of microorganisms was expected to seriously affect the sensitivity of the sensor system. Fig. 3b shows the effect of the amount of microbes on sensor sensitivity. Under the same immobilization conditions, the amount of microbes was proportional to the total weight of the calcium alginate filaments with immobilized microorganisms. Different weights (0.005 g, 0.010 g, 0.020 g, 0.030 g, 0.060 g, 0.100 g) of calcium alginate filaments with microorganisms were used as microbial reactors. As shown in Fig. 3b, as the weight increased, the inhibition of *E. coli* by DCP decreased, indicating that the sensor sensitivity was improved as the weight decreased. The reason for this could be that when different amounts of microorganisms are faced with the same concentration of toxicant, fewer microorganisms fully contact the toxicants; in this case, more microbes are destroyed, which leads to lower IC50 values. However, the current obtained at 0.005 g was too small to be measured by the UMEA. To improve the stability and reproducibility, 0.010 g of calcium alginate filaments with *E. coli* was used in this study.

3.2.3. Working electrode

The working electrode of the sensor was held at a sufficiently high electric potential to reoxidize the HCF(II) produced by the reduction of HCF(III) during microbial respiration. After incubation, HCF(II) had accumulated, and the anodic current had increased in the 400–600 mV range. Simultaneously, the cathodic current decreased rapidly in range of 0–150 mV. Therefore, the operating potential was maintained at +450 mV (Liu et al., 2009a). The UMEA was used as the working electrode in this study. The advantage of using the UMEA is that its current in the diffusion-limiting region is independent of both time and potential. As expected, the UMEA provides a steady and large limiting current in less than 10 s and can distinguish the difference between a blank and a low amount of added toxicant. Fig. 4 shows the variation in the limiting current of a single ultramicroelectrode and the UMEA depending on time under the same conditions. The current generated at the single electrode was much smaller than that of the UMEA, and it could not clearly distinguish between 2 mg/L of added DCP and a blank (no DCP).

3.3. Application of the sensor to determinate the toxicity of four respiration inhibitors

Toxicity determinations using four respiration inhibitors were carried out using both the MB-FI and MICROTOX methods, and

the results obtained by the two methods were compared. Fig. 5 shows the currents obtained at different concentrations of four inhibitors. Among them, KCN was the most toxic; the current decreased rapidly after injection of 2 mg/L KCN, and 90% inhibition was observed after injection of 24 mg/L (Fig. 5b). SA was less toxic than KCN; the current slightly decreased when 6 mg/L SA was injected. However, the current decreased rapidly after injection of 12 mg/L SA, and 60% inhibition was observed after a 24 mg/L injection (Fig. 5c). The toxicities of DNP and As_2O_3 were similar, and they were the least toxic among the four respiration inhibitors. The injection of 6 mg/L and 12 mg/L As_2O_3 decreased the current by 20% and 45%, respectively (Fig. 5a). However, injection of 12 mg/L DNP only decreased the current by 20% (Fig. 5d).

Fig. 6 shows the relationship between the inhibitory percentages and concentrations of the four respiration inhibitors. The inhibitory percentages were calculated using Eq. (1), where $i_{\text{lim control}}$ and $i_{\text{lim sample}}$ were obtained from Fig. 5. According to Fig. 6, the IC50 values of As_2O_3, KCN, SA and DNP were 19 mg/L, 5 mg/L, 16 mg/L and 20 mg/L, respectively. Table 1 shows the comparison of the IC50 values using two methods (MB-FI method and MICROTOX test). The EC50 values for As_2O_3, KCN, SA and DNP were obtained from the MICROTOX test as 43 mg/L, 4 mg/L, 30 mg/L and 22 mg/L, respectively. The results obtained from the two methods were similar. The IC50 values obtained from the MB-FI method and MICROTOX test were similar because they have similar principles. MB-FI measures the current generated by the process of respiration, and the MICROTOX test measures the light intensity caused by respiration. Therefore, these values can be detected and produce similar results using both the MB-FI method and the MICROTOX test. These results demonstrate that the MB-FI method with good potential can be used as an initial toxicity-screening tool.

4. Conclusions

This paper clearly demonstrated the efficacy of a mediated biosensor system with flow injection in toxicity monitoring using model toxicants. The mediated biosensor was a rapid measurement method and a good alternative to the conventional method. A simple method of immobilizing the microorganisms in calcium alginate filaments was proposed. The application of this immobilized microorganism in the mediated biosensor toxicity assessment can greatly simplify the testing process, shorten the operation time and reduce the cost. The method using the flow injection biosensor system constructed in this work correlates well with other bioassay methods using higher organisms. Furthermore, it is a simple, rapid, and inexpensive technique that does not require training of specialized personnel, which make this toxicity monitoring technology easier to apply and commercialize. This method can detect inhibitors, in addition to other chemicals directly or indirectly influencing respiration, such as membrane disruptors that disrupt specific microbial processes and oxidize vital cellular structures or components. Considering that biosensors produce a biological response to toxicity, the response to the additive effect of multiple contaminants is likely a better predictor of overall toxicity than the measurement of individual chemicals. Further research efforts are clearly necessary to detect the toxicity of other individual chemicals and the overall toxicity of multiple chemicals before such a device can be put into full-scale field tests.

Acknowledgements

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