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An unexpected discovery of 1,4-benzoquinone as a lipophilic mediator for toxicity detection in water†

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Since most toxicological risk assessments are based on individual single-species tests, there is uncertainty in extrapolating these results to ecosystem assessments. Herein, we successfully developed a mediated microbial electrochemical biosensor with mixed microorganisms for toxicity detection by microelectrode array (MEA). In order to fully mobilize all the mixed microorganisms to participate in electron transfer to amplify the current signal, 1,4-benzoquinone (BQ) was used as the lipophilic mediator to mediate the intracellular metabolic activities. Hydrophilic $K_3[Fe(CN)_6]$ was employed as an extracellular electron acceptor to transport electrons from hydroquinone (HQ) to the working electrode. Under the optimal conditions of 50 mM phosphate buffer solution (PBS), 0.4 mM BQ, 10 mM $K_3[Fe(CN)_6]$ and OD_{600} = 0.5 bacteria concentration, the half-maximal inhibitory concentration (IC_{50}) values measured with the composite-mediated respiration (CM-RES) of BQ–$K_3[Fe(CN)_6]$ for Cu^{2+}, Cd^{2+} and Zn^{2+} were 5.95, 7.12 and 8.86 mg L^{-1}, respectively. IC_{50} values obtained with the single mediator $K_3[Fe(CN)_6]$ were 2.34, 5.88 and 2.42 mg L^{-1} for the same samples. The results indicate that the biosensor with the single mediator $K_3[Fe(CN)_6]$ had higher sensitivity to heavy metal ions than the biosensor with composite mediators. After verification, we found that the addition of BQ cannot amplify the current. The IC_{50} value of 0.89 mg L^{-1} for BQ was obtained using $K_3[Fe(CN)_6]$ as the single mediator. This suggests that BQ is highly toxic, which explained why the sensitivity of the biosensor with the combined mediator BQ–$K_3[Fe(CN)_6]$ was lower than that of the biosensor with the single mediator $K_3[Fe(CN)_6]$. At the same time, this also implies that toxicity itself cannot be ignored when it is used as an electronic mediator in a mediated microbial electrochemical biosensor.

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Introduction

Numerous biological methods for detecting wastewater containing heavy metal ions have been proposed to study toxicity and evaluate the risk of environmental pollution by monitoring the physiological response of living organisms such as daphnia,[1] algae,[2] mice,[3] fish[4] and bacteria.[5,6] However, in addition to ethical concerns, the methods for monitoring pollutants using higher organisms also suffer from technical limitations such as low sensitivity, time-consuming and complicated detection procedures, the need for well-trained personnel and the inability to provide real-time alerts in the event of accidents affecting the ecosystem. In contrast, toxicity assays using bacteria and other microorganisms are regarded as desirable both in ethical terms and for their simplicity, short life cycle, rapidity, low cost and flexibility. The sensing mechanisms of these methods normally rely on monitoring certain physiological changes of microbes under the stimulus of environmental pollutants. Based on the changes in the respiratory chain activity,[7] optical properties[8] or morphologies of microorganisms[9] induced by toxicants, numerous toxicity bioassays have been designed and developed.[10–12] Optical toxicity bioassays based on luminous bacteria, such as *Photobacterium phosphoreum*, *Vibrio fischeri*, and *Vibrio qinghaiensis* sp.-*Q67*, have been successfully used to assay the toxicity of a large number of chemicals.[13–16] However, these bioassays are not suitable for the measurement of turbid solutions because of the low level of light intensity.[17]

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Many efforts have been made to develop an alternative method for toxicity assessment.[6,12] Electrochemical biosensors based on whole-cell assay have been proposed as rapid and reliable methods. Moreover, in addition to these advantages, electrochemical biosensors possess the merits of easy operation, low cost and insensitivity to turbid and colored samples.[18,19] Thus, the fabrication of electrochemical biosensors with high sensitivity and stability has become a research hotspot in recent years. Most commonly, the respiratory chain activity is used to reflect the toxicity. Among these methods, cell sensing assays have been developed that can respond to toxicants using *Pseudomonas putida* immobilized on screen-printed electrodes. However, the time spent on pretreatment and immobilization is unacceptably long.[20] The main drawback of this kind of microbial electrochemical biosensor is the slow electron transfer between the microbial cell wall and the electrode surface. Therefore, it has become a development trend by using suspended microorganisms with advantages of uniform dispersion and high sensitivity as test microorganisms to construct toxicity biosensors.[21–23]

To accelerate the electron transfer, many efforts have been devoted to overcome the kinetic barriers. Microarray electrodes used in this study have been proved to have the advantages of fast mass transfer of microelectrodes and large current of millimeter electrodes.[24–26] Several artificial electron mediators have been widely used to shuttle the electrons by replacing dissolved oxygen as the electron acceptor during the microbial respiratory chain activity between the microbe and the electrode. Some mediators, such as ferrocene, ferricyanide, and quinones have been widely used in enzymatic[27] or microbial biosensors.[28,29] The mediators that were commonly used can be classified into two categories, *i.e.*, hydrophilic mediators (such as potassium ferricyanide ($K_3[Fe(CN)_6]$) and lipophilic mediators (such as 1,4-benzoquinone (BQ), menadione and neutral red). BQ is also known as *p*-benzoquinone (*p*-BQ) and is widely used in industrial organic synthesis processes. In previous works, as neutral mediators, BQ was often used as a mediator in the enzymatic reaction. Yusuke Ayato *et al.* reported that BQ is one of the promising mediators for the biofuel cell (BFC) anode with glucose oxidase (GOx).[30] Jiahui Sun *et al.* reported a colorimetric- and electrochemical-based bioassay for specific, sensitive, and economical detection of *E. coli*, in which BQ was used to monitor the bacterial concentration and specifically distinguish *E. coli* from four other common clinical bacteria.[31] BQ was linked to a number of adverse effects observed both *in vivo* and *in vitro*, such as sister chromatid exchange (SCE) and micronuclei (MN) formation in V79 cells.[32] F. Yang *et al.* suggest that exposure to low concentrations of *p*-BQ induced a concentration-dependent cellular toxicity, and excess production of ROS might be a possible mechanism underlying the DNA damage attributed to the *p*-BQ-mediated cellular toxicity.[33] Although BQ is known to be involved in toxicity, but its own toxicity and its effect on the test results as a mediator have not been fully elucidated. BQ has been widely used as a single mediator to construct a microbial toxicity biosensor due to its excellent electrochemical reversibility and 2 electron transfer. Dengbin Yu *et al.* reported an improved mediated microbial toxicity bioassay using 0.625 mM BQ as the articial electron mediator and OD_{600} = 9.6 *E. coli* as the test organism, at which the largest inhibition ratio was obtained.[25] Jiuming Li *et al.* reported that a simple BQ mediated biosensor was fabricated by immobilizing *E. coli* on a glassy carbon electrode with gelatin solutions. The neutrality and lipophilicity rendered BQ with better efficiency than $K_3[Fe(CN)_6]$ in the mediated microbial system.[34] Xuejiang Wang *et al.* developed a BQ-mediated amperometric microbial biosensor "ToxTell" for real-time monitoring of the inhibition effect of the metabolism by toxicants with microbial immobilization, which adopted *Psychrobacter* sp. as the biocomponent and *p*-benzoquinone as the mediator.[35] In the above studies, single bacteria including *E. coli* or *Psychrobacter* sp. were used as test organisms, therefore, errors would be inevitable using these bacteria with selective recognition characteristics to detect the actual wastewater.

The construction of *in situ* and rapid microbial toxicity detection biosensors is of great practical significance for early warning of water quality safety. It is more scientific to prepare a electrochemical biosensor for toxicity detection by using *in situ* culture of a broad spectrum of mixed bacteria as test organisms. In this work, we adopted two strategies to achieve rapid and sensitive detection of water toxicity containing rapid detection. One was to use an as-prepared MEA as the working electrode to realize rapid detection; and the other is BQ compounded with $K_3[Fe(CN)_6]$ as the artificial electron mediator to amplify the current signal. The feasibility of toxicity detection was demonstrated with Cu^{2+} as the reference toxicant, which has been widely researched.[36–38] Three heavy metal ions Cu^{2+}, Cd^{2+} and Zn^{2+} were measured using the biosensor with composite-mediated respiration (CM-RES) of BQ–$K_3[Fe(CN)_6]$ and the single mediator $K_3[Fe(CN)_6]$ under the optimal conditions. The currents were directly converted into inhibitory percentages through simple signal treatment. Finally, the half-maximal inhibitory concentration (IC_{50}) was obtained from the inhibition curve. This study afforded a practical method for toxicity detection of wastewater.

Experimental details

Chemicals and materials

Peptone was purchased from Beijing Aoboxing Bio-tech Co., Ltd. Yeast extract was provided by Oxoid Co. Ltd, UK. Glycerol was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. $CuSO_4{\cdot}5H_2O$, $CdCl_2{\cdot}2.5H_2O$, $ZnCl_2$, $K_3[Fe(CN)_6]$, KCl, NaCl, KH_2PO_4, Na_2HPO_4, and NaOH were bought from the Beijing Chemical Plant. LB broth (10 g peptone, 10 g NaCl and 5 g yeast extract per liter) and phosphate buffer solution (PBS, 0.12 M Na_2HPO_4 and 0.08 M KH_2PO_4) were prepared with deionized water and sterilized at 121 °C for 15 min. The toxicant solutions (Cu^{2+}, Cd^{2+} and Zn^{2+}) and PBS with desired concentration were prepared by diluting high-concentration stock solutions (1000 mg L^{-1} toxi-

cant solutions, 200 mM PBS). The toxicant solutions were replaced by deionized water in the control solution. If not stated specifically, all chemicals were of analytical grade and used as received without further purification. All solutions were prepared with deionized water (>18.0 MΩ cm^{-1}) using a Milli-Q Plus system (Millipore) throughout the experiments.

Instrumentation

The optical density (OD_{600}) of mixed microorganism suspension was measured using a Cary 500 UV-vis-NIR spectrometer (Varian) at 600 nm. All electrochemical experiments were performed using an electrochemical workstation (CHI 832B, Shanghai Chen Hua Instrument Co., Ltd).

Microorganism cultivation

Microbial strains were obtained from the South Lake as the parent strains. A 500 mL triangle flask containing 330 mL of autoclaved LB broth medium was inoculated with the microbial strains at 37 °C for 15 h on a rotary shaker at 220 rpm to allow the cells grow into a stationary phase. The microorganism cells were harvested by centrifugation (Eppendorf, Centrifuge 5804 R, Eppendorf Co. Ltd) at 6000 rpm for 5 min at room temperature, and then washed twice with PBS and resuspended in PBS. The final concentration of mixed microorganism cell suspension was adjusted to the desired optical density at 600 nm. The bacterial suspension was stored at 4 °C until required and used for further experiments on the day of harvesting.

Fabrication of the biosensor

A total volume of 1 mL of incubation suspension was prepared for each test. The standard incubation suspension comprises: 0.5 mL of cell suspension with the desired concentration, 0.4 mL of mediator solution in optimized concentration, 0.1 mL of toxicant solutions or deionized water. The above suspensions were thoroughly mixed and incubated at 37 °C for 1 h. The incubated cell suspensions were centrifuged at 10 000 rpm for 10 min at room temperature. After following the above procedure, the biosensor was fabricated by dipping a three electrode system composed of a Pt plate as an auxiliary electrode, an Ag/AgCl electrode (saturated KCl, CHI 111, Shanghai Chen Hua Instrument Co., Ltd) as the reference electrode, and an as-prepared Pt MEA (50.0 μm diameter Pt wire, 32 pieces) as the working electrode into the supernatants for electrochemical detection.

Toxicity assessment

Cyclic voltammetry (CV) and chronoamperometry were performed using an electrochemical workstation with a three-electrode system. All measurements were carried out at room temperature. Chronoamperometry was applied to monitor the changes of anodic current of the cultured supernatants, which were collected by centrifugation before electrochemical assay in the toxicity detection procedure. The potential was set as 0.45 V *vs.* the Ag/AgCl reference electrode (saturated KCl), and the data were shown as plots of current against time. Without considering the endogenous respiration of microorganisms, for each toxicant concentration, the anodic currents were converted to equivalent inhibitory percentage values according to eqn (1).

$$\text{Inhibition } \% = 100 \times (i_{con} - i_{tox})/i_{con} \quad (1)$$

where i_{tox} and i_{con} are the steady oxidation currents of $K_4[Fe(CN)_6]$ reduced in the presence and absence of toxicants, respectively.

Results and discussion

Principle of toxicity assay

The basic principle of the present toxicity detection is based on the changes in the respiratory chain activity of microorganisms induced by toxicants. A schematic illustration of toxicity assessment of water is shown in Fig. 1. Microbial respiratory and metabolic activity can produce a large number of electrons. As clearly shown in Fig. 1a, a large amount of mediators were reduced from the oxidized form in the control sample (*i.e.*, without a toxicant). Then, the reduced mediators were re-oxidized on the surface of the MEA, and a large oxidation current was detected. Meanwhile, as shown in Fig. 1b, when a toxicant was added to the sample, the respiration activity of the microorganisms was inhibited, so that the number of generated electrons decreased, and the amount of reduced mediators obtained by reducing the oxidized mediators also decreased. Therefore, the oxidation current also decreased, and the inhibition ratio of toxicity was calculated through the difference in the oxidizing currents of the reduced mediators between the damaged microorganisms (toxicant sample) and non-damaged microorganisms (control sample). In general, the current decreased with greater toxicity and/or greater concentration of toxicants added.

To demonstrate the feasibility of toxicity detection with the biosensor, the anodic changes in the BQ–$K_3[Fe(CN)_6]$-mediated

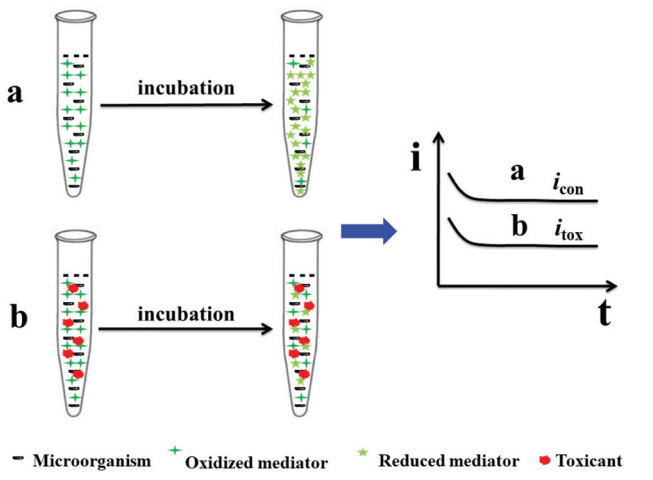

Fig. 1 Schematic illustration of toxicity detection of water.

respiratory activity of bacteria were measured, as shown in Fig. S1.†

Optimization of concentrations of PBS, BQ and $K_3[Fe(CN)_6]$

Fig. 2A shows the anodic currents of the mixtures containing BQ, $K_3[Fe(CN)_6]$ and mixed microorganisms after 1 h incubation at different PBS concentrations. On this basis, the final concentrations of BQ, $K_3[Fe(CN)_6]$ and mixed microorganisms were chosen to be 10 μM, 18 mM and OD_{600} = 3, respectively. The incubation temperature was kept at 37 °C. From Fig. 2A, it is clear that the anodic current significantly increased in the range of 2.5–50 mM PBS. However, the current decreased significantly in the range of 50–100 mM PBS. This clearly indicates that the respiratory activity of the mixed microorganisms is sensitive to variation of PBS concentration. Therefore, in the following experiments, 50 mM was chosen as the optimal PBS concentration, at which the largest anodic current was obtained.

Fig. 2B shows the curves of anodic current *versus* time for the mixture solutions containing 50 mM PBS, 20 mM $K_3[Fe(CN)_6]$ and mixed microorganisms of OD_{600} = 3 for 1 h incubation at different BQ concentrations. As shown, the anodic current increased almost linearly as the BQ concentration increased in the range of 0.01–0.4 mM, then it reached a peak and remained almost unchanged at higher concentrations. Thus, the electron transfer efficiency was maximized at 0.4 mM BQ, and this concentration also caused minimal damage to the mixed microorganisms. As a result, 0.4 mM BQ was used as the optimal condition in the following experiments.

It has been reported that a low concentration of $K_3[Fe(CN)_6]$ can stimulate cell growth, whereas a high concentration of $K_3[Fe(CN)_6]$ would injure cells.[39] Therefore, the concentration of $K_3[Fe(CN)_6]$ was optimized similar to BQ concentration optimization. The mixed microorganism suspension of OD_{600} = 3 was incubated with 0.4 mM BQ and 50 mM PBS at 37 °C for 1 h, while the concentration of $K_3[Fe(CN)_6]$ varied from 1 to 60 mM. The anodic currents of the resulting cell supernatants were recorded by chronoamperometry. As shown in Fig. 2C, the anodic current increased with the concentration of $K_3[Fe(CN)_6]$ in the range of 1.25–10 mM, and then it increased only slightly with the concentration of $K_3[Fe(CN)_6]$. Thus, 10 mM was chosen as the optimal concentration of $K_3[Fe(CN)_6]$ for the following experiments.

Optimization of cell concentration

The sensitivity of a biosensor is a crucial factor for toxicity detection. The parameter of cell concentration was also optimized to improve the detection sensitivity. The mixed microorganism suspension containing 0.4 mM BQ and 10 mM $K_3[Fe(CN)_6]$ was incubated at 37 °C for 1 h, while the concentration of mixed microorganisms (OD_{600}) varied from 0.5 to 10.0, and the anodic currents were recorded by chronoamperometry. As shown in Fig. 3B, the anodic current increased proportionally as the cell concentration increased. Cu^{2+} was selected as a standard toxicant to evaluate the biosensor's response to this toxicant. From Fig. 3A, we can clearly see that when 5 mg L^{-1} Cu^{2+} was added into the detection system, the inhibition ratio of the biosensor deteriorated as the concentration of cells increased. Thus, we can draw the conclusion that the sensitivity of the biosensor deteriorated with the increase in the concentration of mixed microorganisms. Therefore, OD_{600} = 0.5 was chosen as the optimal concentration in the following experiment.

Application for toxicity assessment of heavy metal ions

For the toxicity tests, the current–time curve was recorded to quantitatively measure the amount of the reduced mediator. The working potential of 450 mV is amply sufficient to reoxidize $K_4[Fe(CN)_6]$ produced by the reduction of $K_3[Fe(CN)_6]$ during cell respiration. To assess the feasibility and sensitivity of the as-prepared biosensor for the toxicity assessment of wastewater, the toxicities of three heavy metal ions (Cu^{2+}, Cd^{2+} and Zn^{2+}) were detected with both the single- and combined-mediator biosensors under the optimized conditions. The inhibition ratio of the toxicants at the designed concentration was calculated according to eqn (1). The plots of inhibition ratio against concentration were drawn and are shown in Fig. 4. The IC_{50} values deduced from these inhibition curves reflect the toxicity level of the heavy metal ions. Fig. 4A, B, C show the inhibition ratios obtained with the combined-mediator biosensor at different concentrations of heavy metal

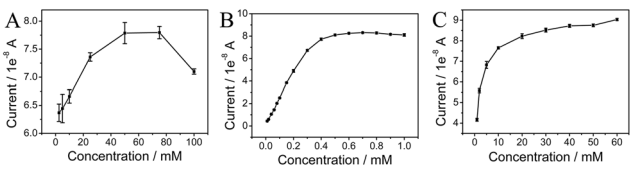

Fig. 2 Optimization of (A) PBS concentration at 10 μM BQ and 18 mM $K_3[Fe(CN)_6]$; (B) BQ concentration at 50 mM PBS and 20 mM $K_3[Fe(CN)_6]$; and (C) $K_3[Fe(CN)_6]$ concentration at 50 mM PBS and 0.4 mM BQ according to the anodic current intensity with the mixed microorganism suspension (OD_{600} = 3). The mean and standard deviation of all samples measured are in triplicate.

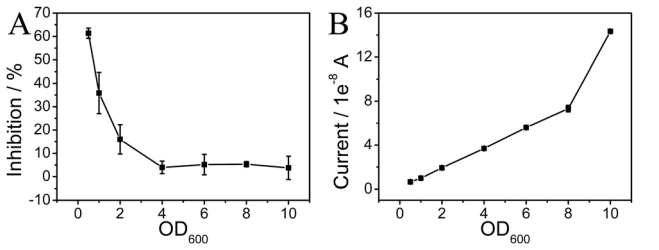

Fig. 3 Optimization of cell concentration at 0.4 mM BQ and 10 mM $K_3[Fe(CN)_6]$, (A) curve of inhibition ratio of different concentrations of mixed microorganisms in the presence of 5 mg L^{-1} Cu^{2+}; (B) optimization of concentration of mixed microorganisms according to the current intensity. The mean and standard deviation of all concentrations are from three replicated experiments.

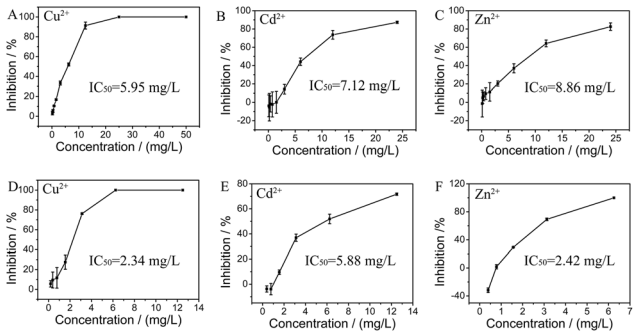

Fig. 4 The inhibition curves of three heavy metal ions, Cu^{2+}, Cd^{2+} and Zn^{2+}, with the combined-mediator sensor BQ–$K_3[Fe(CN)_6]$ (A, B and C) and the single-mediator sensor $K_3[Fe(CN)_6]$ (D, E and F). Error bars represent three replicated measurements.

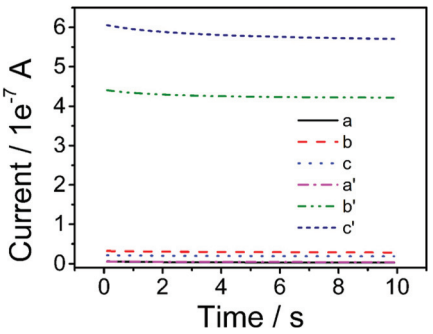

Fig. 5 *i–t* curves of samples with different mediators and incubation times. OD_{600} = 3, BQ concentration = 10 μM, $K_3[Fe(CN)_6]$ concentration = 18 mM (a, b and c are samples with mediators of BQ, BQ–$K_3[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$; a′, b′ and c′ are the corresponding samples after incubation for 1 h).

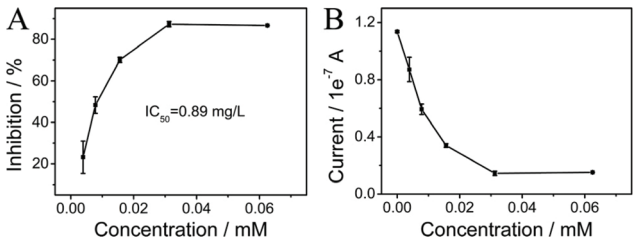

Fig. 6 (A) The inhibition curve of BQ; (B) the current intensity according to BQ concentration. 10 mM $K_3[Fe(CN)_6]$ was used as a single mediator. Data points represent the average of three replicates.

ions, and the IC_{50} values of Cu^{2+}, Cd^{2+} and Zn^{2+} were 5.95 mg L^{-1}, 7.12 mg L^{-1} and 8.86 mg L^{-1}, respectively. Fig. 4D, E, F show the inhibition ratios obtained with the single-mediator biosensor for the same samples, and the IC_{50} values of Cu^{2+}, Cd^{2+} and Zn^{2+} are 2.34 mg L^{-1}, 5.88 mg L^{-1} and 2.42 mg L^{-1}, respectively. The results demonstrated the promise of this bioassay in determination of the toxicities of heavy metal ions. Table S1† shows that the IC_{50} values of Cu^{2+}, Cd^{2+} and Zn^{2+} obtained by the proposed method were compared with those of the mediated electrochemical microbial biosensors reported in the literature. As can be seen from Table S1,† there are mainly two categories of test bacteria to prepare microbial electrochemical biosensors for toxicity detection. One is suspended bacteria, here, hydrophilic $K_3[Fe(CN)_6]$ is selected as the mediator to construct the biosensor for detection of water toxicity. The other type is fixed bacteria film, and BQ is generally selected as the mediator. The sensitivity of the biosensor with fixed bacteria is generally lower than that of the biosensor with suspended bacteria, which may be caused by the obstruction of mass transfer of fixed bacteria film. Single bacteria, *E. coli* in particular, have been widely used. Mixed bacteria have also been concerned and studied in recent years. However, the mixed bacteria mainly are active sludge with complex composition and artificially mixed bacteria with limited number. In this study, the mixed bacteria *in situ* cultured used as test would be more representative. The above results imply that the proposed biosensor with mixed microorganisms is a reliable tool for biotoxicity detection in aquatic ecosystems. The biosensor with a single mediator has higher sensitivity compared to that with a combined mediator. The above results imply that the proposed biosensor with mixed microorganisms is a reliable tool for biotoxicity detection in aquatic ecosystems. The biosensor with a single mediator has higher sensitivity compared to the biosensor with a combined mediator.

Effect of BQ on the current

It is obvious from Fig. 5 that BQ was used as the single electron mediator, and the anodic current of the sample remained very small after culturing for 1 h. In contrast, the anodic current markedly increased for 1 h cultivation when $K_3[Fe(CN)_6]$ was used as the single mediator. When the combined mediator of BQ–$K_3[Fe(CN)_6]$ was used with equal concentrations of BQ and $K_3[Fe(CN)_6]$, the anodic current after 1 h culture was larger than that of single BQ, but smaller than that of the single mediator $K_3[Fe(CN)_6]$. These experimental results may be attributed to the complex interaction between bacteria in the microorganism mixture, as well as the toxicity of BQ itself.

Toxicity assessment of BQ

The results can be clearly drawn as follows that neither detection current nor sensitivity could be improved when BQ was added to the $K_3[Fe(CN)_6]$ mediator. The reason may be related to the toxicity of BQ itself. To confirm this speculation, we used $K_3[Fe(CN)_6]$ as the mediator to test the toxicity of BQ. As shown in Fig. 6A, the IC_{50} value obtained, when BQ as the toxicant, was 0.89 mg L^{-1}. It can also be seen from Fig. 6B that the detection current gradually decreased with BQ concentration. It is suggested that BQ is highly toxic, which confirmed our above conjecture.

Conclusions

In this study, a mediated cell-based electrochemical biosensor was fabricated to investigate the single toxicity of heavy metal

ions by using as-prepared MEA as the working electrode and mixed microorganisms as the signal receptor. To amplify the current signal, combined BQ and $K_3[Fe(CN)_6]$ were used as mediators in the biosensor for measuring the toxicity of heavy metal ions. The results showed that the proposed biosensors based on mixed microorganisms are rapid and sensitive tools for toxicity assays. However, the sensitivity of the biosensors cannot be improved with combined BQ and $K_3[Fe(CN)_6]$ to replace the $K_3[Fe(CN)_6]$ mediator. We found that the addition of BQ could not improve the current intensity, and BQ itself had high toxicity through experimental verification. These experimental results explained the question of decreased sensitivity. Therefore, the self-toxicity of BQ used cannot be ignored in the detection of water toxicity. In conclusion, we found excitingly that BQ, a commonly used electron mediator for the electrochemical microbial biosensor, is toxic to bacterial cells and will inhibit the sensitivity of the biosensor, which has guiding significance for the selection of a mediator.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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