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A double-mediator based whole cell electrochemical biosensor for acute biotoxicity assessment of wastewater

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ABSTRACT

This work investigates the feasibility and sensitivity of a double-mediator based whole cell electrochemical biosensor to detect the acute biotoxicity of wastewater. The lipophilic mediator menadione was used to mediate the intracellular metabolic activities whereas hydrophilic potassium ferricyanide was employed as extracellular electron acceptor to transport the electron from the menadiol to anode. A chitosan hydrogel polymer film with boron-doped nanocrystalline diamond (BND) particles was electrodeposited onto a glassy carbon (GC) electrode to immobilize *Saccharomyces cerevisiae* cells and the mediators. The feasibility of the as-prepared biosensor was verified by determine the acute biotoxicity of four heavy metal ions(Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}), three phenol pollutants (3,5-dichlorophenol, 4-chlorophenol, phenol) and three real wastewater samples. The IC50 values for Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} are 10.12 mg/L,13.88 mg/L, 17.06 mg/L and 34.56 mg/L. And the IC50 value is 16.48 mg/L, 34.40 mg/L and 44.55 mg/L for 3,5-dichlorophenol, 4-chlorophenol and phenol, respectively. The results of this work indicate that the double-mediator based whole cell electrochemical biosensor could be applied into the acute toxicity assessment of real wastewater samples with excellent performance and highlight their merit as portable and sensitive, which may providing a reasonable and reliable way for wastewater toxicity online detection.

1. Introduction

With the booming of manufacturing industry and popularization of chemical products in the daily life, numerous chemicals and toxic compounds have permeated into the aquatic ecosystems, exerting potential harms to human health, threatening the safety of wild animals and food safety [1]. Besides, due to the powerless regulation and careless management of chemical plants and other manufacturing factories, the leakage accidents and illegal chemical dumping incidents happened frequently, causing not only tremendous harm to the environment but also giving rise to the latent possibility of contamination of downstream water system [2]. However, there are only limited methods for water toxicity and safe evaluation. The water safety assessment techniques are mainly depending on the detection of chemical oxygen demand (COD), biological oxygen demand (BOD) as well as extraction of sewage samples into subsequent laboratory analysis [3–5]. Although these methods can detect the toxicants qualitatively and quantitatively, it is difficult to measure the biotoxicity of every individual toxicant contained in water, since a wide variety of chemical species exist in natural water and a mixture of these may exhibit complex biotoxicity. The usage of above methods is also impeded by their technical limitations such as time-consuming, sophisticated detection procedure, needs of well-trained personnel as well as could not provide the real-time alert of the ambient ecosystem accidents [6].

On the other hand, bioassay tests as an excellent alternative technique in complementary to the above traditional water quality assessment methods, have been used to evaluate the biotoxicity levels of environmental and industrial wastewater. The biotoxicity assay provides a holistic approach that allows to evaluate the toxicity of the total effect of all constituent components, including toxicants and confounding variables in a given complex sample matrix. There have been various bioassays test reported to date for which the method of microbe-based biosensor was used. They can be divided into two main approaches, i.e., optical methods and electrochemical methods. Optical methods are based on the bioluminescence produced naturally by bioluminescent microbes, which using *Vibrio fischeri* [3],*Vibrio qinghaiensis* [5], *Photobacterium phosphoreum* [7] and other genetically engineered luminescent bacteria as signal receptor to determine the biotoxicity of water samples by the change of fluorescence intensity.

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Compare to luminescent bacteria methods, the electrochemical biosensor possesses the merits of detect online, prompt response, easy to operate, low-cost and not affected by the turbidity of water samples. Thus, fabricating electrochemical biosensors with high detection sensitivity and stability become an attractive research field in recent years. Many efforts have been made to develop an alternative method for acute biotoxicity estimation and consequently, a whole-cell based electrochemical biosensor assay has been proposed as a rapid and reliable method [8–10]. The sensing scheme of these methods normally rely on monitoring certain physiological changes of microbes under the stimulus of environment pollutants, typically, the respiration chain activity is commonly used to reflect the biotoxicity. However, the main drawback of this kind of microbial electrochemical biosensors is the slow electron transfer between the microbial cell wall and the electrode surface. In order to facilitate the electron transfer rate, many efforts have been devoted to effectively overcome the kinetic barriers. Various artificial electron mediators have been widely used to shuttle the electrons by replacing the oxygen to accept the electrons during the microbial respiration chain between the microbe and electrode, which called mediated electron transfer. The mediators that commonly used can be classified into two categories, i.e., hydrophilic mediators (such as potassium ferricyanide) and lipophilic mediators (such as benzoquinone, menadione, dichloroindophenol, 2,3,5,6-tetramethylphenylenediamine and neutral red, etc.). During the interaction with microbes, a hydrophilic mediator cannot cross the cell membrane and is restricted to reacting with the proteins located on the periplasm, but they possess the advantages of high water solubility and high diffusion coefficient in the aquatic system, which greatly promote their application in biosensor preparation and microbial fuel cells fabrication [11,12]. The lipophilic mediators like menadione, on the contrast, can permeate through cell membrane and interact with the intracellular redox centers in cytoplasm and mitochondria, be reduced by the intracellular enzymes and diffuse or transported out of the cell to transport electrons to the electrode surface. However, using lipophilic mediator as sole mediator in the aquatic system may not be a favorable choice, because their low aqueous solubility can greatly affect their concentration in the detection system and hence impact the magnitude of the current signal. To sort out the aforementioned problem, a double-mediator system comprising a hydrophilic and a lipophilic mediator can make up their drawbacks and enables the intracellular redox systems be accessed, providing a reflection of intracellular cell metabolism activities of target cells and high current signal intensity [13]. The potassium ferricyanide-menadione double-mediator system is commonly used to fundamentally investigate the redox activity of *S. cerevisiae* yeast cells and mammalian cells. For instance, potassium ferricyanide-menadione double-mediator system has been applied to analyze a specific enzyme activity or biochemical process in yeast cell [14,15], single cell imaging using Scanning Electrochemical Microscopy (SECM) [16] and the application in microbial fuel cells [17] etc. The combination of mediators can significantly increase the current magnitude and thus improve the detection sensitivity of the electrochemical biosensor.

However, in spite of the improved sensitivity of mediated biosensors, it seems there are still some drawbacks that hinder their practical application. The primary concern of these sensors is most mediators reported were usually added into the aqueous solution directly and their concentrations were kept at a relatively high level, which can be harmful to the cell [18]. Besides, the addition of the mediators may also cause second contamination of aquatic system. Thus, to fully display the merit of electrochemical biosensor, achieve real-time detection, an integrated and portable electrochemical biosensor is needed.

In present work, an integrated and miniaturized whole-cell based electrochemical biosensor was successfully fabricated. A yeast strain (S288C) was employed as the biological recognition element. The yeast cells were immobilized on a chitosan hydrogel polymer film with boron-doped nanocrystalline diamond (BND) particles which has electrodeposited onto a glassy carbon (GC) electrode. The conductivity of the as prepared electrode has greatly improved because of the uniform distribution of BND particles. The biotoxicity of four heavy metal ions, three phenol pollutants and three real wastewater samples were examined by the prepared biosensor. The results indicate that the double-mediator based whole-cell electrochemical biosensor prepared can be applied into the toxicity assessment of wastewater samples and display the advantages of integrated, miniaturized and sensitive, which imply a reasonable way for real wastewater online toxicity detection.

2. Materials and methods

2.1. Chemicals and reagents

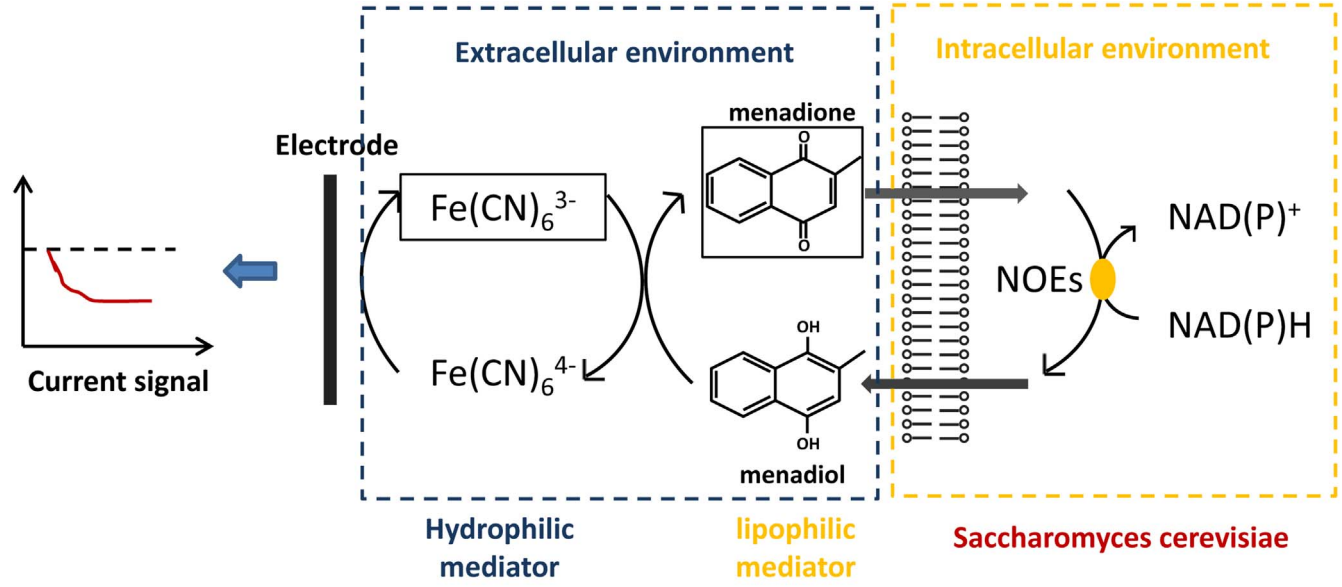
$Pb(NO_3)_3$, $CuSO_4{\cdot}5H_2O$, $Ni(NO_3)_2{\cdot}6H_2O$, $Cd(NO_3)_2$, 3,5-dichlorophenol, 4-chlorophenol and phenol were provided by Beijing Lanyi Chemical Products Co., Ltd., China. Yeast extract, beef extract and peptone were obtained from Beijing Aoboxing Bio-tech Co., Ltd., China. Potassium ferricyanide was purchased from Arcos USA. Menadione was provided by Adamas Reagent Co., Ltd. Glucose was purchased from Alfa Aesar. All reagents were of analytical grade and used as received without further purification. All solutions were prepared with deionized water (18.0 MΩ cm, Milli-Q Purification System, Millipore) freshly before use.

2.2. Cultivation of microorganism

Saccharomyces cerevisiae (S288C) and *Escherichia coli* (ATCC25922) were inoculated from China General Microbiological Culture Collection Center (CGMCC). A 300 mL flask containing 100 mL autoclaved Yeast Extract Peptone Dextrose medium (YEPD, 0.5% yeast extract, 1% peptone, 1% glucose) was inoculated with a colony of *S. cerevisiae* and grown aerobically at 30 °C for 16 h on a rotary shaker at 200 rpm to allow the *S. cerevisiae* grow into stationary phase. *E. coli* was cultured in 100 mL autoclaved Nutrient Broth solution (10g L^{-1} peptone, 3g L^{-1} beef extract, and 5g L^{-1} NaCl) for 16 h at 37 °C. The microorganism cells were harvested by centrifugation at 6000 rpm for 5 min at room temperature, then washed twice with PBS and resuspended in PBS. The final concentration of *S. cerevisiae* and *E. coli* cell suspension were adjusted by a Secoman Uvikon UV–Vis spectrophotometer at the wavelength of 600 nm (OD_{600}). The cell suspensions with desired concentration were stored at 4 °C until required. The cell suspension was used for the experiments on the day of harvesting.

2.3. Preparation of BND-chitosan hydrogel polymer film

A solid-state diffusion method has been applied to the preparation of boron-doped nanodiamond powder [19]. Detonation nanodiamond was treated in air at 425 °C for 5 h before boron doping to remove the sp^2 carbon. Then the heat-treated nanodiamond and boron powder was wet mixed with ethanol in the ratio of 1:2. The mixture was heated in 900 °C for 24 h under hydrogen gas flow. The boron-doped nanodiamond was finally collected after centrifuging, washing and drying. Chitosan solution was prepared by ultrasonically dissolve 1 wt% chitosan flakes in 1% acetic acid and 0.01 M KCl solution at 40 °C. 0.5 mg/mL boron-doped nanocrystalline diamond (BND) was added into the chitosan solution prepared above and ultrasonic agitate for 3 h until the BND nanoparticles were completely dispersed in the chitosan solution, forming BND-chitosan hydrogel. The 1 cm×1 cm glassy carbon electrodes were applied in present work and the electrodes were polished by 30–50 nm α-alumina powder each time before use to remove the oxidation layer. The polished glassy carbon electrodes were successively ultrasonic washed by nitrate, ethanol and distilled water. A conventional three-electrode system was applied to electrodeposit the BND-chitosan hydrogel onto the glassy carbon electrodes. The polished



Scheme 1. The mechanism of menadione-$K_3Fe(CN)_6$ double-mediator system interact with *S. cerevisiae* cell.

glassy carbon electrode was fixed on the vial containing BND-chitosan hydrogel with an O-ring and a platinum wire was used as auxiliary electrode, Ag/AgCl (saturated KCl) electrode was used as reference electrode. The electrochemical deposition experiment was performed with a potentiostat/galvanostat (Model 263A, Princeton, USA) at −3V vs Ag/AgCl (sat' KCl) for 5 min. The electrode was then removed from the vial and rinsed with deionized water to obtain a BND-chitosan polymer film modified electrode. The as prepared electrodes were stored at room temperature in deionized water before use.

2.4. The fabrication of integrated biosensor

The *S. cerevisiae* cell suspension with desired concentration was incubated with mediators and glucose firstly. A total volume of 10 mL incubation suspension was prepared for each test. The standard incubation suspension comprises: 9.0 mL cell suspension with desired concentration, 100 μL glucose solution with the final concentration of 7.5 mM, 800 μL potassium ferricyanide solution and 100 μL menadione solution in optimized concentration. The above suspension was mixed intensively and incubated at 30 °C for 1 h. The incubated cell suspension was centrifuged at 6000 rpm for 8 min at room temperature, and then resuspended in PBS with the final volume of 10 mL. The BND-chitosan hydrogel modified electrodes prepared above were dipped into the cell suspension for 30 min at room temperature to allow the *S. cerevisiae* and mediators adsorbing on the polymer film. The obtained polymer biofilm modified glassy carbon electrode was rinsed with deionized water repeatedly. After above procedure, an electrode with both yeast cells and mediators immobilized on the surface was fabricated, and then stored at 4 °C refrigerator for further biotoxicity test.

2.5. Acute biotoxicity tests

The biotoxicity test of pollutants in water was carried out in a stirring vial with a potentiostat/galvanostat (Model 263A, Princeton, USA). The modified electrode fabricated above was set as working electrode and fixed on the bottom of the cell by an O-ring, a platinum wire was used as auxiliary electrode and Ag/AgCl (sat' KCl) electrode as reference electrode. The chronoamperometry was applied to monitoring the real-time anode current change in the biotoxicity detection procedure. The potential was set as 400 mV vs Ag/AgCl (sat' KCl) and the data was displayed in real-time as current against time plot. After a stabilization period about 5 min, the target toxicant was added into the cell, and the current was dropped immediately due to the lethal effect of the detected toxicant. For each toxicant concentration, the anodic currents were converted to equivalent inhibitory percentage values according to Eq. (1):

$$\text{Inhibition } \% = (1 - I_2/I_1) \times 100\% \quad (1)$$

Where I_1 is the steady-state current before adding the toxicant, I_2 is the steady-state current after adding the toxicant.

2.6. The biotoxicity assay of real wastewater samples

The biotoxicity of three real wastewater samples (landfill wastewater, electroplating wastewater and laboratory wastewater) were estimated by chronoamperometry, the *i*-t curves were recorded to observe the real-time signal changes in the toxicity detection procedure. The three kinds of real wastewater samples were taken from garbage-treatment plant, electroplating factory and our laboratory, respectively. The biotoxicity of above three real wastewater samples were tested without further treatment. The inhibition rate was calculated according to Eq. (1), and the biotoxicity level of three real water samples was determined accordingly. The composition analysis of real water samples was performed by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Varian 710-ES ICP Optical Emission Spectrometer). The chemical oxygen demand (COD) values of these three samples were measured by the closed reflux colorimetric method with spectrophotometer (HACH DRB 200).

3. Results and discussion

3.1. Specificity verification of double-mediator system

As reported previously, NAD(P)H-oxidizing enzymes (NOEs) are a group of intracellular enzymes specific existing in the cytosolic and mitochondrial of *S. cerevisiae* (S288C) cells, which can catalyze electron transfer from intracellular NAD(P)H to quinone substrates and keep the balance of NAD(P)H/$NAD(P)^+$ [20]. In our double-mediator assisted system, due to the hydrophobic nature of menadione, it can permeate through the cell membrane and enter into the yeast cell without the assistance of pumps or transport proteins. As shown in Scheme 1, menadione is reduced by NAD(P)H to menadiol on the catalysis of NOEs and menadiol further diffuse out of the yeast cell

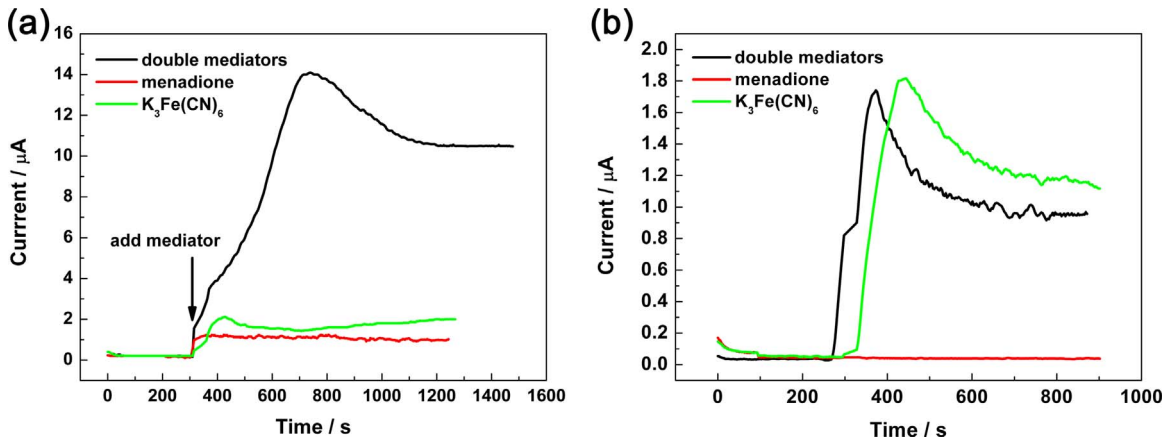


Fig. 1. The chronoamperometry curves of *S. cerevisiae* cell suspension and *E. coli* cell suspension after addition of menadione and $K_3Fe(CN)_6$ mediators individually and in combination. (a) The *i*-t curves of *S. cerevisiae* cell suspension. OD_{600}=2.5, menadione concentration=100 μM, $K_3Fe(CN)_6$ concentration=20 mM; (b) The *i*-t curves of *E. coli* cell suspension. OD_{600}=2.5, menadione concentration=100 μM, $K_3Fe(CN)_6$ concentration=20 mM;.

membrane into the extracellular environment. Outside the yeast cells, the reduced form of menadione reacts with the hydrophilic $K_3Fe(CN)_6$, $K_3Fe(CN)_6$ accepts one electron and reduced to $K_4Fe(CN)_6$. With the excellent solubility of $K_4Fe(CN)_6$ it can diffuse to the surface of electrode and the current signal can be observed immediately.

To verify the specific existence of NOEs in the *S. cerevisiae* (S288C) cells, we compared the changes of current intensity after the addition of single mediators and double-mediator into *S. cerevisiae* cell suspension and *E. coli* cell suspension, respectively. As depicted in Fig. 1, (a) is the *i*-t curves of *S. cerevisiae* cell suspension after the addition of menadione and $K_3Fe(CN)_6$ individually and in combination. When menadione and $K_3Fe(CN)_6$ were added separately, their steady current intensity is around 1 μA and 2 μA respectively. Whereas, the final signal magnitude of double-mediator system reached above 11 μA in the same concentrations. On contrast, after the addition of mediators, the current of *E. coli* suspension appear no obvious change when menadione was added. Moreover, there is no remarkable current increase after the injection of double mediators compared single $K_3Fe(CN)_6$ mediator when the detected system reached steady. By above discussion, we can reach the conclusion that NOEs are specific exist in yeast cell such as *S. cerevisiae* (S288C) and thus can shuttle the electrons from quinone to NAD(P)H.

3.2. Optimization of experimental conditions

3.2.1. Optimization of menadione concentration

Menadione is a favorable electron transporter between the cell respiratory chain and ferricyanide. Menadione can generate the reactive oxygen species (ROS) through redox cycling. Low levels of ROS can function as redox-active signaling messengers, whereas high level of ROS will induce cellular damages and intrigue cell death [21]. Thus, to improve the sensitivity of biosensor, the menadione concentration was optimized. The current of cell suspension with different menadione concentration after incubation of 1 h with 5 mM $K_3Fe(CN)_6$ was detected by chronoamperometry, the suspension concentration of the yeast used is OD_{600}=1.5. As shown in Fig. 2(a), the detected current increased as menadione concentration increased, when come to 0.05 mM, the current value reached the peak and dropped in the higher concentration. Thus, when the menadione concentration is at 0.05 mM, the highest electron transfer efficiency is observed while no harm to the yeast cell viability. As a result, the menadione concentration of 0.05 mM was used as the optimal condition in the following experiments.

3.2.2. Optimization of potassium ferricyanide concentration

It has been reported that a low potassium ferricyanide concentration can stimulate the cell growth whereas a high potassium ferricyanide concentration will injure cells [18]. Therefore, the optimal concentration of $K_3Fe(CN)_6$ mediator was determined in the same way as menadione. The yeast cell suspension with a concentration of OD_{600}=1.5 was incubated with 0.05 mM menadione at 30 °C for 1 h while the concentration of $K_3Fe(CN)_6$ vary from 5 to 50 mM. The anodic currents of the resulted cell suspension were recorded by means of chronoamperometry. As shown in Fig. 2(b), the anodic current went up with the increase of $K_3Fe(CN)_6$ concentration first until it reached 20 mM, and then the currents decreased slightly as the amount of $K_3Fe(CN)_6$ increase. Thus, 20 mM $K_3Fe(CN)_6$ was applied as the optimal concentration for the following experiments.

3.2.3. Optimization of yeast cell concentration

To improve the detection sensitivity, the parameter of cell concentration was also optimized. The yeast cell suspension was incubated with 0.05 mM menadione and 20 mM $K_3Fe(CN)_6$ at 30 °C for 1 h while the concentration of yeast cell (OD_{600}) vary from 0.5 to 3.0, the anodic currents were recorded by means of chronoamperometry. As shown in Fig. 2(c), the anodic current increase proportional with elevation of cell concentration. Moreover, the sensitivity of as prepared biosensor to the detected toxicants is a crucial factor which should be taken into consideration. To test the sensitivity of the as prepared biosensor when the yeast cell concentration varies, 3,5-dichlorophenol (DCP) was selected as a standard toxicant to evaluate the biosensor's response. From Fig. 2(d), we can clearly see that, when 25 mg/L DCP was added into the detection system, the inhibition rate of the biosensor improved as the cell amount increase. Thus, we can draw the conclusion that with the increase of the yeast cell concentration, the sensitivity of the as prepared biosensor increases as well. Considering the cell culture condition, the OD_{600}=3.0 was used as optimal concentration in the following experiment.

3.3. The Fabrication and characterization of the integrated biosensor

3.3.1. The fabrication and surface morphology of BND-chitosan polymer biofilm modified electrode

Various nanomaterials have emerged as a powerful tool to modified electrodes so as to improve the sensitivity and the electron transfer efficiency of electrodes [22–24]. Among all of promising nanomaterials, boron-doped nanodiamond (BND) nanoparticles were considered as an excellent additive to electrode modification because of their superior conductivity, chemical stability, especially its good biocompatibility can serve as an enhanced and versatile material for biological applications. On the other hand, chitosan is an amino-polysaccharide derivative from chitin, existed in the shells of crustaceans. It is widely

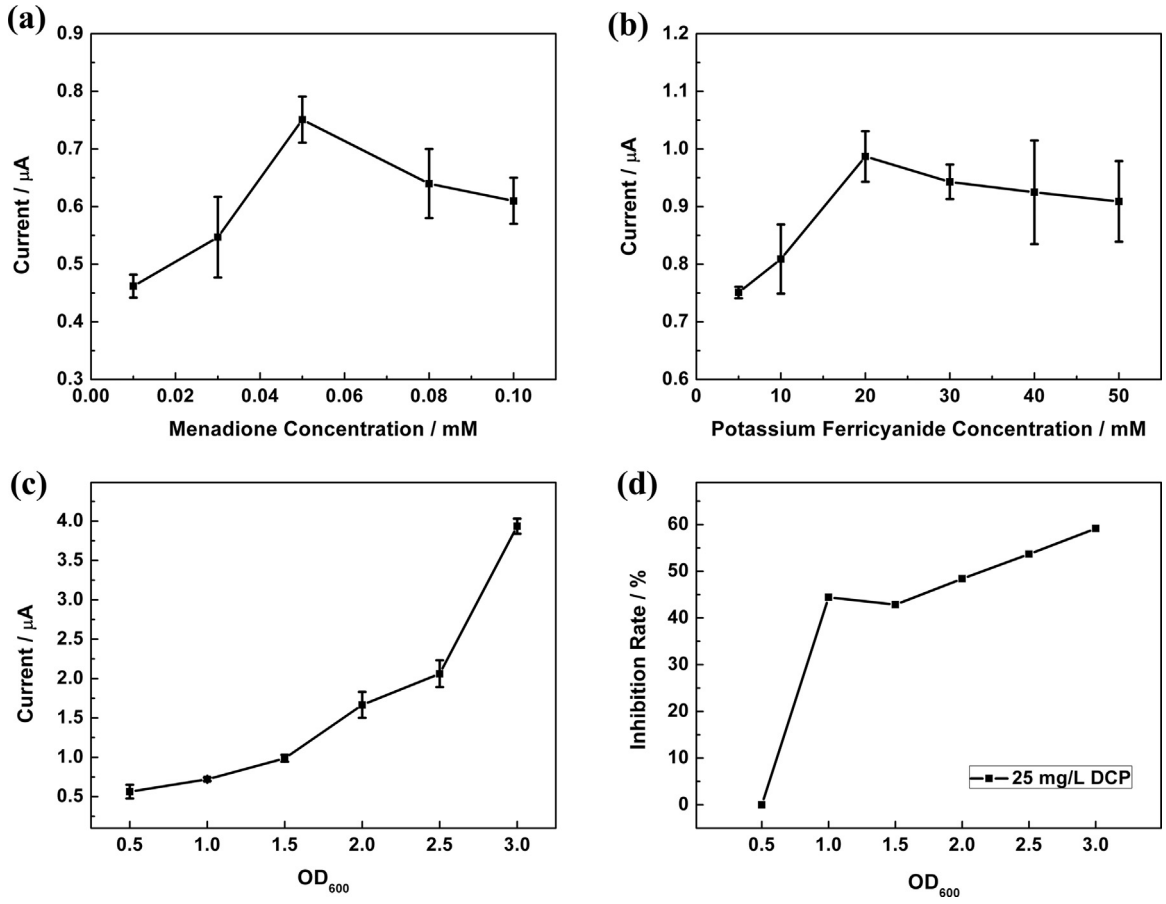
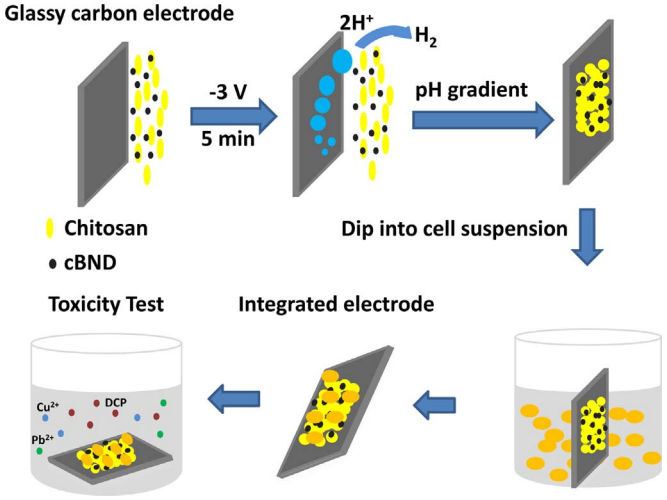


Fig. 2. Optimization of experimental conditions. (a) Optimization of menadione concentration according to the anodic current intensity; (b) Optimization of $K_3Fe(CN)_6$ concentration; (c) Optimization of yeast cell concentration according to the current intensity; (d) The inhibition rate curve of different yeast cell concentration to 25 mg/L DCP.

fabricated and casted on the metal/glassy carbon electrodes or carbon paper to prepared different functionalized electrochemical biosensors. The merit of chitosan polymer film is its electrochemically inactive property, but the film charge is positive in acidic solution which lead to the negative charged mediator such as $Fe(CN)6^{3-}$ can trapped into the film, endowing the above polymer electroactive property [25].

In present work, the BND nanoparticles were mixed into the chitosan hydrogel and co-electrodeposited on the GC electrode to fabricate a BND-chitosan biofilm modified electrode. The procedure of fabricating the BND-chitosan hydrogel polymer biofilm on GC electrode and preparing the integrated biosensor is illustrated in Scheme 2. When a potential of −3V vs Ag/AgCl (sat' KCl) was applied, H^+ in the acidic BND-chitosan hydrogel could be reduced to hydrogen and electrodeposition process occurred. Due to chitosan is only soluble on the condition that environment pH below 6.3, the production of hydrogen caused a local increase of pH on the electrode surface which induced a sol to gel transition of the pH-sensitive chitosan. Besides, the releasing of hydrogen bubbles during the sol to gel transition process of chitosan hydrogel can also contribute to the forming of porous scaffold of BND-chitosan hydrogel modified electrode. The porous structure of the prepared polymer film can be a favorable template for the further absorption of *S. cerevisiae* cells. Moreover, the as prepared polymer film is positive charged because of the amino groups of chitosan are protonated in the acidic solution [25], which lead the negative charged mediator $Fe(CN)_6^{3-}$ be trapped into the film, endowing the above polymer electroactive property. After the co-immobilization of yeast cells and mediators, an integrated whole-cell based electrochemical biosensor is successfully fabricated and can be applied in the water biotoxicity assessment.

The field-emission environmental scanning electron microscopy



Scheme 2. The diagram of the fabrication process of integrated electrode and toxicity test.

(FESEM) was used to characterize the surface topography of the modified electrode prepared above. The images in Fig. 3 show the surface morphology of BND nanoparticles and BND-chitosan polymer biofilm modified electrode. In Fig. 3(a), the morphology of boron-doped nanodiamond particles are clearly shown, the shape of nano-diamond particles are irregular with sizes varies from 300 to 800 nm. The surface morphology of BND-chitosan polymer biofilm is shown in Fig. 3(b), the high brightness spots represent the BND existence in the biofilm due to its high conductivity. From the FESEM image, we can

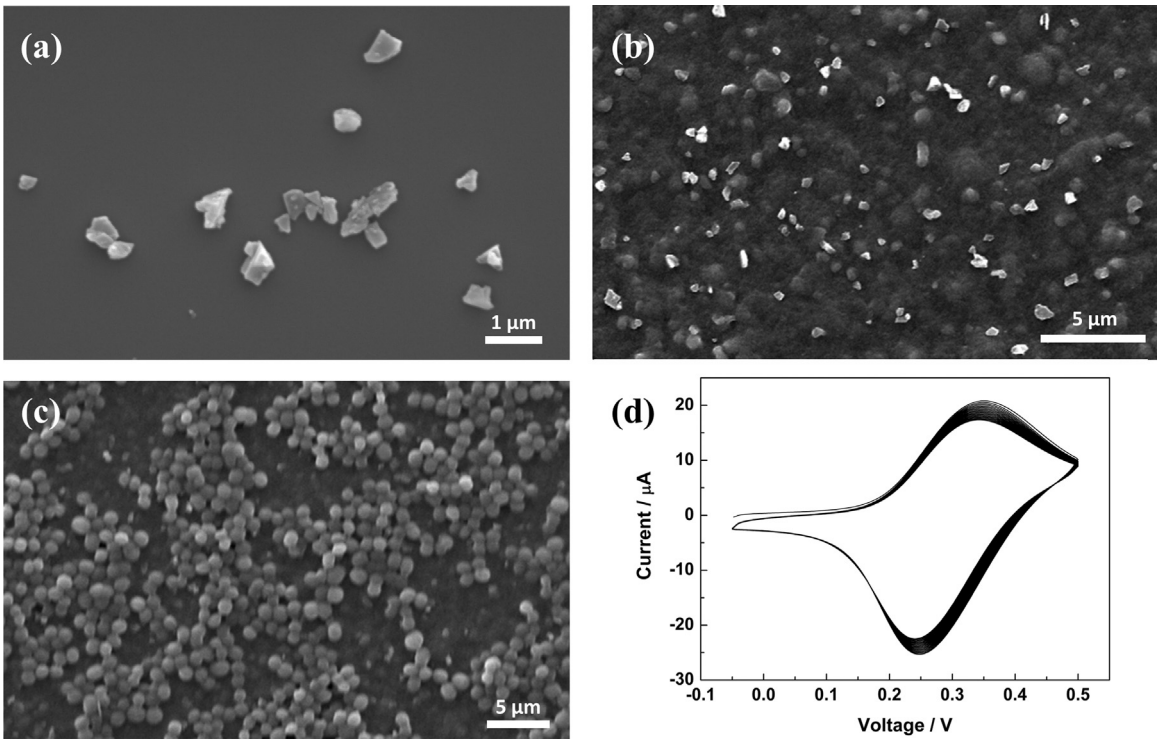


Fig. 3. The FESEM images and cyclic voltammetry curve of BND-chitosan polymer biofilm modified electrode. (a) The FESEM image of BND nanoparticles; (b) The FESEM images of BND-chitosan polymer film modified electrode; (c) The FESEM image of BND-chitosan polymer biofilm modified with S. cerevisiae cells; (d)The cyclic voltammetry curves of BND-chitosan polymer biofilm modified electrode.

observe that the BND is uniform distributed in the film without aggregate, suggesting that the chitosan polymer film with BND is formed through one-step electrodeposition process. Furthermore, the distribution of *S. cerevisiae* cells in the integrated biosensor was also observed. As shown in Fig. 3(c), the *S. cerevisiae* cells were successfully adsorbed on the surface of biofilm, indicating the biosensor was successfully fabricated. The cyclic voltammetry was applied to further prove the mediator $K_3Fe(CN)_6$ was absorbed onto the biofilm. In Fig. 3(d), the redox peak of $K_3Fe(CN)_6$ mediator was clearly seen, indicating that the $K_3Fe(CN)_6$ mediator was successfully absorbed in the biofilm. In addition, we further measure the cyclic voltammetry for 20 cycles, the current was slightly decreased as the scan cycle increased and finally went steady at 20 cycles. This result indicates that there is a small amount of mediator leakage of the modified electrode, but the current signal went stable after a short period of time.

3.3.2. The EIS experiment

The electrochemical impendence spectroscopy (EIS) experiment was conducted to characterize the interfacial charge transfer resistance of the above electrodes. The EIS experiments were carried out in a 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture solution with 0.1 M KCl as supporting electrolyte, the frequency range from 0.1 to 100 kHz with a scan rate of 5 mV/s. The electron transfer kinetics of the redox probe $Fe(CN)_6^{3-/4-}$ at the different modified electrodes were explored. From the EIS spectra in Fig. 4, we can observe a well-defined single semicircle over the high frequency range, followed by a long straight line in the low frequency range for the all four electrodes. The diameter of the semicircle corresponds to the interfacial charge transfer resistance (R_{ct}), which represents the resistance of the electrochemical reactions on the electrode. A smaller R_{ct} indicates a faster electron transfer rate. Fig. 4 shows that R_{ct} is remarkably reduced by electrodeposit BND-chitosan hydrogel onto the GC electrode. This may contribute to the excellent electrical conductivity of the BND nanoparticles. To further verify the conductive effect of BND nanoparticles, the properties of the electrodeposit polymer film without BND nanoparticles were also investigated. As a result, the pure chitosan hydrogel without BND nanoparticles mixed can be hardly electrodeposited on the electrode, and the obtained polymer biofilm is fragmentary, indicating that the conductivity of the hydrogel without BND is rather poor. Moreover, the Fig. 4 also shows that the electrode which is adsorbed both *S. cerevisiae* and mediators on the film exhibit an even lower R_{ct} compare to the electrode that only trap yeast cell in the polymer film, which indicates that the adsorbent of mediators can also facilitate the charge transfer between solution and electrode.

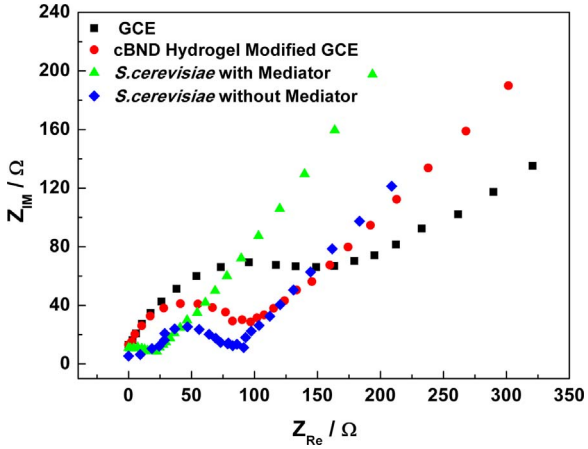


Fig. 4. The EIS spectra of four different modified electrodes.

3.4. The acute biotoxicity test in aquatic system

To prove the feasibility of as-prepared biosensor to the acute biotoxicity assessment of wastewater system, the biotoxicity of three phenols (3,5-dichlorophenol, 4-chlorophenol and phenol) and four heavy metal ions (Cu^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+}) were detected to verify the sensitivity and broad spectra detection property of the sensor. The

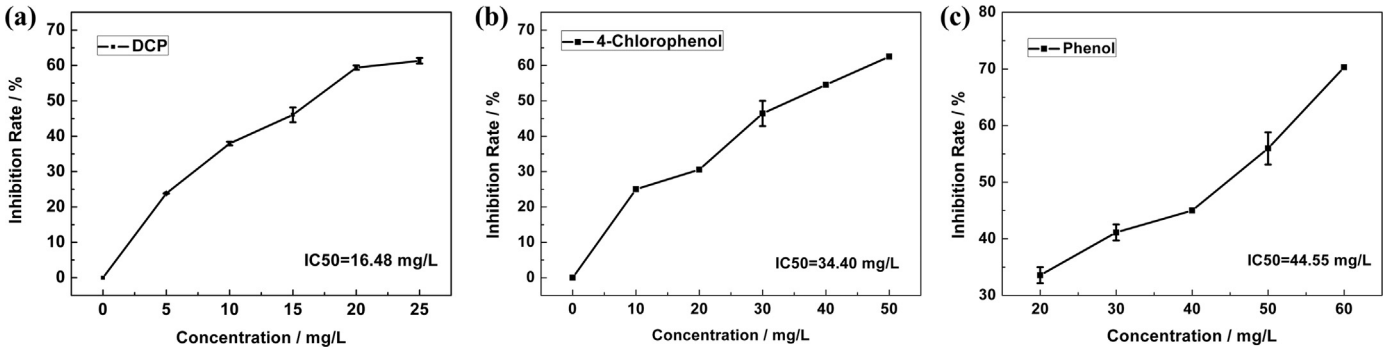


Fig. 5. The inhibition curves of three phenol pollutants, 3,5-dichlorophenol, 4-chlorophenol and phenol, respectively.

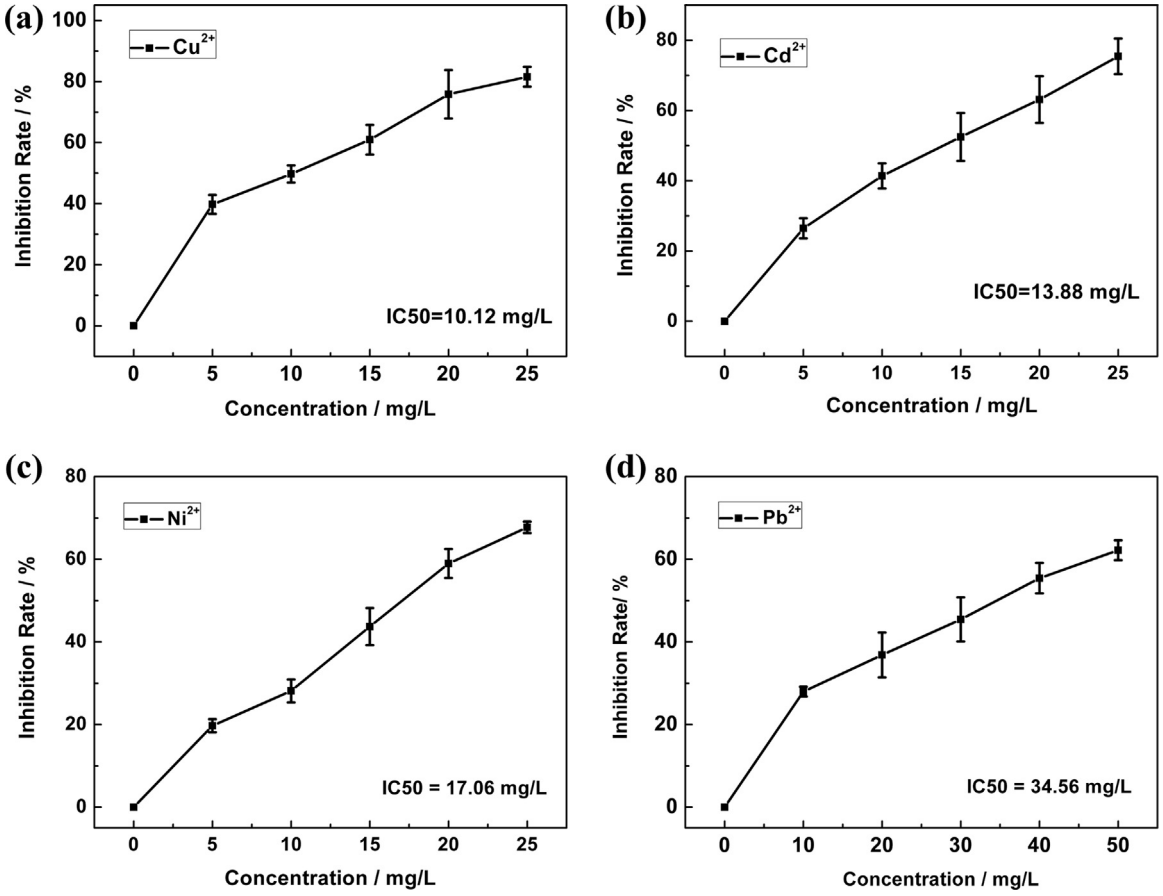


Fig. 6. The inhibition curves of four heavy metal ions, Cu^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+}.

Table 1
Comparison of IC50 values of different methods.

Toxicity assays	Toxicants, IC50 (mg/L)							References
	Cu^{2+}	Cd^{2+}	Ni^{2+}	Pb^{2+}	Phenol	4-chlorophenol	DCP	
Amperometry, *S. cerevisiae* $K_3Fe(CN)_6$-menadione	10.12	13.88	17.06	34.56	44.55	34.40	16.48	Present study
Amperometry, *Psychrobacter* sp. p-benzoquinone	2.6	47.3	–	110.1	–	–	–	[26]
Amperometry, *E. coli* p-benzoquinone	44	79	–	–	–	–	–	[27]
Amperometry, *E. coli* $K_3Fe(CN)_6$	–	93.35	–	–	–	–	35	[28]

chronoamperometry was applied to monitoring the online current changes in the detection process. At the beginning, the biosensor was stabilized for 5 min, and the toxicant with certain concentration was added into the reaction cell, and a dramatic current drop can be detected in the *i*-t curve, reflecting the detrimental effect of the toxicant to the yeast cells and the inhibition to their overall metabolism activity,

Table 2
The composition of three wastewater samples.

Real wastewater samples	Heavy metal ions (mg L^{-1})				COD (mg L^{-1})	pH
	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cr^{2+}		
Landfill wastewater	48	–	–	0.2	19,203	6.88
Electroplating wastewater	30–80	30–60	50	–	5760	7.24
Laboratory wastewater	79.09	42.65	6.43	0.03	8442	6.62

as is shown in Fig. S1. The inhibition rate of toxicant in designed concentration was calculated according to Eq. (1). The line charts of inhibition rate against concentration were draw and shown in Figs. 5 and 6. The half maximal inhibitory concentration (IC50) was deduced from the inhibition curve, which reflects the toxicity level of detected pollutant. For the three detected phenol toxicants, the IC50 value is 16.48 mg/L, 34.40 mg/L and 44.55 mg/L for 3,5-dichlorophenol, 4-chlorophenol and phenol, respectively. The toxic order of the three phenol toxicants ranks as 3,5-dichlorophenol > 4-chlorophenol > phenol, accordingly. This toxic order is in consistent with the discipline that more chloric substituent in the phenol derivatives more toxic it will be. The IC50 values of four heavy metal ions Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} are 10.12 mg/L,13.88 mg/L, 17.06 mg/L and 34.56 mg/L, respectively. The toxic order deduced from the IC50 values of the heavy metal ions rank as $Cu^{2+} > Cd^{2+} > Ni^{2+} > Pb^{2+}$, this toxic order is also in coincidence with the results reported by other methods. From the above results, we can draw the conclusion that the prepared biosensor has a broad spectra detection range, which can not only determine the biotoxicity of organic pollutants, but can also reflect the detrimental effect of heavy metal ions to the microorganism. Moreover, the biosensor we prepared also shown consistent high sensitivity to all the organic pollutants and heavy metal ions compare to biosensors prepared by others. As shown in Table 1, we can clearly see that the detection sensitivity of this double-mediator based biosensor has greatly improved with contrast to the biosensor using single mediator. The above results imply that above biosensor is a reliable tool for the biotoxicity detection in real water ecosystem.

3.5. The toxicity detection of real wastewater samples

The ultimate goal of the biosensor we developed is to detect the biotoxicity of real wastewater samples and achieve the goal of early risk warning of water quality change. To achieve that purpose, the ability of quantify the biotoxicity level of real wastewater samples need to be verified. In this work, three real wastewater samples: landfill wastewater, electroplating wastewater and laboratory wastewater were tested and their biotoxicity were determined according to the inhibition rate. By the calculation according to Eq. (1), the inhibition rates were 38.74%, 28.6% and 35.66% for landfill wastewater, electroplating wastewater and laboratory wastewater respectively, which reflect the toxic order of these water samples were ranked as landfill wastewater > laboratory wastewater > electroplating wastewater. The biotoxicity of these three real water samples may contribute to their composition. As listed in Table 2, the ingredients of heavy metal ions in water are measured by ICP-AES and organic chemicals amount are deduced from the COD values. As the pH values of the samples are all near neutral, their toxicity difference may mainly depend on their composition of heavy metal ions and organic toxicants. From the results, we can clearly observe that, even though the content of heavy metal ions in landfill wastewater is relatively low, there are massive organic chemicals which is the main reason result in the toxic effect on the microbes. On the contrary, the electroplating wastewater sample is rich in heavy metal ions, but there are few organic toxicants existing in the sample which lead to toxicity difference between landfill wastewater sample. To be concluded, the results detected by the as-prepared biosensor is consistent with the predicted toxic order referring from their compositions, which implying that this double-mediator biosensor can be effectively used to evaluate the overall biotoxicity of water samples and there is a promising potential for the as-prepared biosensor to be applied into the on-site detection of water quality change.

4. Conclusions

An integrated electrochemical biosensor was prepared by using *S. cerevisiae* as signal receptor and potassium ferricyanide-menadione as signal amplified mediator. Both yeast cells and mediators were immobilized on the electrode by electrodeposit BND-chitosan polymer film on the GC electrode. The double mediators can greatly elevate the current intensity thus improve the sensitivity of the biosensors compared to single mediator biosensors. Furthermore, the BND-chitosan polymer biofilms fabricated by electrodeposition have the advantages of can adsorb various kinds of microbes, which is possible to fabricate many kinds of biosensors with different signal reception elements. The as-prepared biosensor was used to detect the biotoxicity of three real wastewater samples, which prove the feasibility of biotoxicity assessment and early risk warning of instant water quality change.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.01.081.

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