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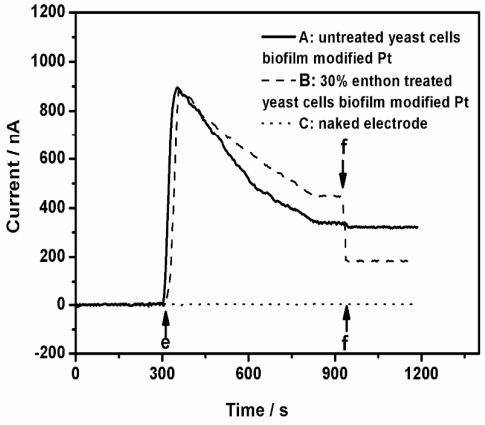
Graphical abstract



A disposable biofilm modified amperometric microbial sensor was developed and employed as an analytical tool for evaluating biotoxicity of pesticides and the real wastewater. The microbial biosensor is a sensitive, rapid, convenient and cheap alternative to toxicity screening of chemicals and real wastewater.

A disposable biofilm modified amperometric biosensor for sensitive determination of pesticide biotoxicity in water

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Abstract

In the present study, a disposable biofilm modified amperometric microbial sensor was developed and employed as an analytical tool for evaluating biotoxicity of pesticides and the real wastewater. The proposal biotoxicity biosensor is fabricated by using a polymeric disposable biofilm, which was prepared by immobilizing the pretreated *S. cerevisiae* cells (as a highly active biocatalyst), a combination of polyvinyl alcohol (PVA) hydrogel and sodium alginate crosslinked by $CaCl_2$ as a matrix at the electrode surface. It was found that the *S. cerevisiae* pretreated with alcohols is needed for increasing response sensitivity of the microbial sensor, therefore, the effects of treated reagents, concentration and treating time, etc., on the performance of biosensor were optimized. Atomic force microscopy (AFM), flow cytometry (FCM), as well as the electrochemical response was employed to investigate the morphology characteristics, cells viability and electrochemical characteristics respectively. 3, 5-Dichlorophenol (DCP) was taken as the reference toxicant. The pesticides solutions, including Acephate, Ametryn and Thiram were taken as the model toxicants. The biotoxicity of three kinds of real wastewater was also determined by this amperometric microbial sensor. The traditional parameter of 50% concentration (IC_{50}) was measured within 30 min and results obtained were better than those of other toxicity bioassays reported. The microbial biosensor prepared is a sensitive, rapid, convenient and cheap alternative to toxicity screening of chemicals and real wastewater.

1. Introduction

Pesticides are widely used for control of pests in the environment, and may exert a significant effect, especially on aquatic ecosystems.[1] Conventional physical/chemical analysis methods can only quantify concentrations of the individual pollutants. In recent years, a large amount of laboratory models of biotoxicity analyzers have been described by using plants, invertebrates, daphnids and luminescence bacteria as test organisms,[2-5] but most of these bio-based assays are time-consuming, complex and must be employed off-line or expensive instruments. Electrochemical biosensors detect the analytes more rapidly, sensitively in comparison to conventional techniques, but the more typical problems in the development of electrochemical biotoxicity bioassay are how to increase sensitivity and prolong lifetime of biomaterials, and also broad spectrum assay for toxic substances. Efforts have been focused on the development of novel biosensor that would provide a stable, simple, rapid and on-line response for monitoring and detecting water pollution or potential risk to human health.

For the electrochemical bioassay, whole cells are excellent indicators for variety of parameters, in particular for toxic compounds. They can respond unspecifically and thus represent an excellent means for determining the overall toxicity of pollutants. In recent studies, most whole cell biosensors that have been reported are based on bacterial cells such as *E. coli*, *Bacillus subtilis* and *Pseudomonas putida*.[6-8] However, there are also some disadvantages in their use. For example, protokaryotic bacterial cells may be relatively weak in sensor environments, leading to short lifetime, limited pH, osmotic and temperature tolerances of individual species, which means that the operating parameters of the sensor could also be limited.

While eukaryotes occupy a wide range of environmental niches, so the determination results are more reliable and good models for assessing toxicity compared to protokaryotic bacterial cells. Two reviews have outlined the advantages of using yeasts in sensing applications.[9,10] Parry noted that the physical robustness of these yeasts in comparison to bacteria, and superior pH, temperature and osmolarity/ionic strength tolerances.[9] Walmsley et al. in their review gave two further reasons to use yeast in whole cell sensors.[10] The first is an advantage shared with the bacteria: rapid growth, ease of manipulation and growth on a broad range of substrates; the second advantage, also noted by Parry,[9] is that these organisms are eukaryotes and sometimes can provide information of direct relevance to other eukaryotes that prokaryote cells can not.

Despite these advantages, the number of yeast species used in toxicity application to date is little compared to the number of bacterial cells that have been exploited.[11-12] This may be due to the permeability barrier of cell envelope for substrates and products which often causes very low reaction rate of whole cells.[13] Therefore, it is important to develop an effective method to reduce the permeability barrier and to prepare whole cell biocatalysts with high activities. Garjonyte et al. reported that the permeabilization of yeast cells could achieve significantly higher electrode responses compared to those intact yeast cells.[14] Common permeabilization methods include cell treatment with solvents, detergents, salts, cell freezing and thawing or electro-permeabilization, etc.[15-16] To the best of our knowledge, a mild cell walls permeabilization technology aimed at *S. cerevisiae* by alcohols using for biotoxicity assay has not been reported.

Moreover, during the fabrication process of the electrochemical whole cells biosensor, coating the microorganism cells solutions on the electrodes surface is typical used,[17-18] but its shortcoming is that the surface of electrodes could be easily contaminated during electrochemical biotoxicity assay. While the electrode with a disposable microbial film can avoid inaccurate data caused by film secondary contaminated in the repeat detection biotoxicity process, and it is also convenient for a next

biotoxicity determination by change a new film. Especially, such single–used probes are advantageous in polluted water and complex media, therefore, the fabrication of the low-cost and disposable electrode for biosensor is of vital importance.

Therefore, we report for the first time the development of a single-use biofilm modified amperometric microbial sensor for on-line sensitively determining the biotoxicity of pesticides in water. Remarkably, the *S. cerevisiae* cells were pretreated with alcohols to increase the permeability of its cell walls, which could improve the determination sensitivity for organic pesticides pollutants effectively. The pretreatment effects on *S. cerevisiae* cells were evaluated by the combination of AFM images, FCM as well as the electrochemical responses. Furthermore, the amperometric biosensor was fabricated by a single-use biofilm modified Pt electrode which was prepared by immobilized pretreated *S. cerevisiae* by using a complex of PVA solution and sodium alginate solution crosslinked with $CaCl_2$, which is porous enough and to allow the diffusion of substrates to the cells. The feasibility of the proposed bioassays in determination of pesticides toxicity is further successfully demonstrated using three different kinds of pesticide, Ametryn, Acephate and Thiram, and the IC_{50} values are determined to be 22.0 mg/L, 29. 0 mg/L and 47.5 mg/L respectively. According to the IC_{50} values, the toxicity, in descending order, is Ametryn > Acephate > Thiram. 3, 5-dichlorophenol (DCP) was taken as a reference toxicant and its IC_{50} value is 9.83 mg/L. Finally, the biotoxicity of three kinds of real wastewaters were also determined respectively. All above determination results show that this prepared disposable *S.cerevisiae* biofilm modified amperometric microbial sensor can effectively determine the biotoxicity of chemical toxicants .

2. Experimental

2.1. Reagents

A cocktail of respiratory substrates (sodium lactate, sodium succinate and glucose each at 10 mM) in 0.85% saline was used for resuscitating and monitoring the biosensors. p-benzoquinone (BQ) was used as mediator. All chemicals were of analytical grade (Beijing Lanyi Chemical Products Co., Ltd., China). All toxicant (3, 5-DCP, Acephate, Ametryn, Thiram) solutions with desired concentrations were prepared by diluting their stock solutions with deionized water. Stock solutions were prepared freshly or kept at 4 °C. Low solubility compound was dissolved through the assistance of 0.5% (v/v) of dimethyl sulfoxide (DMSO).[19] The selected toxicants possess different chemical structures, for example, Ametryn, Acephate and Thiram are triazine weedkiller, organophosphorus insecticide and sulfur fungicide respectively, which were widely used and chosen as target toxicants. The three kinds of real wastewaters were respectively taken from garbage-treatment plant, electroplating factory and laboratory without any treatment. The usedl real wastewaters contained organics, inorganics or both are our selecting basis for wastewater, in order to reflect the broader spectrum responses to water samples by our prepared sensor.

2.2. Microorganism preparation

S.cerevisiae S288C was obtained from China General Microbiological Culture Collection Center (CGMCC). Rich medium (YEPD) contained 2% glucose, 1% yeast extract. A 100mL solution of autoclaved YEPD medium was inoculated with a colony of *S.cerevisiae* and grown aerobically at 30 °C for 24 h on a rotary shaker (200rpm). Then *S.cerevisiae* cells were harvested by centrifugation at 10000 rpm for 10 min at room temperature, and washed twice with PBS and resuspended in PBS. The ultimate concentration of cells was adjusted to an absorbance value of 24.0, measured at 600 nm using a Secoman Uvikon UV–Vis Spectrophotometer. The *S.cerevisiae* suspension was stored at 4°C until required.

The procedures of pretreat *S.cerevisiae* were firstly incubated the untreated *S.cerevisiae* cells to different concentrations (20, 30, 40%, v/v) of methanol or ethanol solutions in phosphate buffer. Then this pretreated cells suspensions were respectively centrifuged and washed with sterile deionized water for three times. To find the optimized pretreatment effect, electrode responses to acute biotoxicity of 10 mg/L DCP were tested and repeated for three times.

2.3. Morphology studies by AFM

The AFM samples were prepared by spreading 100 μL of the pretreated yeast cell suspensions onto the surfaces of glass slides, and then left to dry 30 min at room temperature. All images were obtained from a ScanAsyst-AIR mode atomic force microscope (AFM, BRUKER MultiMode® 8, GER). A rotated (symmetric) silicon tip/ a triangular silicon nitride cantilever with a spring constant of 0.4 N/m and a resonance frequency of ~ 70 kHz were used. The scan speed was set at 0.7 Hz and final resolution was 256 by 256 pixels. Each scan resulted in a topography image and a phase image simultaneously. The height scale of cell was depicted as shades of gray, with bright area being nearer to the tip in topography images. All the AFM experiments were performed at room temperature.

2.4. Yeast cells viability by FCM

S. cerevisiae cell suspensions (pretreated or untreated) were incubated in 50 μg/L propridium iodide (PI) at room temperature for 5 min. The viabilities of the yeast were analyzed on a FACScan flow cytometer (FCM, BD Biosciences, San Jose, CA) equipped with a 15mW air-cooled 488 nm argon ion laser for excitation of PI. At least 10,000 cells were analyzed for each sample.

2.5. Preparation of disposable Biofilm and Characterization by SEM

A 100 μL of 10% (w/v) PVA (degree of saponification 98%, degree of polymerization 2400) aqueous solution was mixed with 10 μL of 1.0 M sodium sulfate aqueous solution and 40 μL of 0.8% (w/v) sodium alginate aqueous solution adequately (Supporting Information Figure S1). The treated yeast cells suspension was centrifugated at 5000 rpm for 10 min at room temperature, and then washed twice with pH 7.0 PBS before use. Finally, the 50 μL washed cells suspension was added to the above mixture solution. The resulting mixture was spread over the surface of a Polyethylene terephthalate (PET) plastic sheet by spin coating to distribute well. The PET sheet coated with yeast cells was further dripped in 2% (w/v) calcium chloride solution and crosslinked for 15-20 min, forming the PVA-alginate microbial biofilm with 0.05-0.10 mm in thickness. The prepared biofilms were peeled off from the PET sheet and was washed thoroughly with PBS and stored at 4 ℃ until use. The surface morphology of the prepared microbial films was investigated by SEM. (Hitachi Ultra-High-Resolution S-4300).

2.6. Electrochemical measurements and toxicity assay

The electrochemical experiments were performed with a potentiostat/galvanostat (Model 263 A, Princeton, USA). Electrochemical impedance spectroscopy (EIS) measurements were carried out with a 263A Potentiostat/Galvanostat and a FRD 100 frequency response detector (Princeton, USA). The electrochemical measurements were carried out in a biofilm reactor (BFR), including with the microbial working electrode, platinum wire auxiliary electrode, and Ag/AgCl (saturated KCl) reference electrode, respectively. The working electrode was obtained by attaching and fixing an appropriate size of biofilm described above on the surface of a platinum disk through a rubber O-ring (Scheme 1a).

The biosensors were monitored in a stirred vial (700 rpm) containing 10 mL pH 7.0 respiratory substrates solution by applying a potential of 0.3 V (vs. Ag/AgCl) at room temperature, and the current was monitored and data were displayed in real-time as current against time plots. After a stabilization period about 5 min, 100 μL of BQ solution as a redox mediator was added to the respiratory substrates to get 0.4 mM final concentration. Target toxicants were added in the same way behind the second period of stabilization. After adding a toxicant sample, the current was decreased because of their detrimental effect to metabolic activity of microorganism. The toxicity can be determined by measuring changes of the BQ-mediated respiration chain activity. As shown in Scheme 1b, BQ can be reduced to hydroquinone (HQ) during cell respiration, and the resultant HQ can be reoxidized to BQ at the electrode surfaces. 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.

2.7. EIS characterization of the disposal biofilm on the Pt electrode

We are aiming to develop a single–used, disposal biofilm to modify the Pt electrode, so the electron transfer properties of the interface between Pt electrode and biofilm become important. EIS characteristics were performed when the biofilm was attached and fixed on the Pt surface because it is capable of monitoring the adhesion of biofilms with Pt electrode and measuring the resulting change in the electron-transfer resistance. As a control, duplicating an electrochemical sensor which constructed by no biofilm modified bare Pt electrode, as well as a conventional nondisposable biofilm modified Pt electrode which obtained by directly coating the PVA/pretreated S. cerevisiae gel onto surfaces of Pt electrode, were also used to compare their difference in electron transfer processes.

3. Results and discussion

3.1. Optimization of the pretreatment conditions of *S. cerevisiae* cells.

S. cerevisiae cells, as a micro-organism used in the present biosensor, have thick and strong cell walls which cause very low biocatalytic activity of whole cell biosensors. It is important to develop an effective method to reduce the permeability barrier of the cell wall. During the pretreatment, particular care has to be taken in order to prevent loss of cell viability, considering efficiency and cost factors, alcohols solvents are considered the more adequate treated agents for *S. cerevisiae* cell permeabilization.[14] In the present study, methanol and ethanol as permeabilizing agents of *S. cerevisiae* cells were selected. To optimize the pretreatment conditions, the alcohol species, concentration and treating time were investigated by AFM, FCM.

3.1.1 The AFM morphology images of pre-treated *S. cerevisiae* cells

Firstly, we used the ScanAsyst mode in air, which is the imaging mode with automatic image optimization technology for AFM, to study the effects of methanol or ethanol as the permeabilizing agents by morphological characteristics of *S. cerevisiae* cells. As

shown in Fig. 1 the surface topographies were different depending on concentrations and kinds of alcohols used. Normal cells image reveals that *S. cerevisiae* cells were round as an ovoid, with a smooth and homogeneous surface, and their surfaces showed a bud scar in their native state (Supporting Information Figure S2a). When the cells were incubated in solution containing 20% (v/v) ethanol for 16 h, no obvious changes were observed (Supporting Information Figure S2b). While increasing ethanol concentration up to 30% (v/v), it can be observed that the cells surface became more rough and some obvious wrinkle and gaps occurred, but the cytoplasmic membrane remained intact (Fig. 1a and Supporting Information Figure S2c). Furthermore, when the ethanol concentration reached 40% (v/v) for 16 h, the cells wall peeled half and some cells were shrinkage (Fig. 1b).

*S. cerevisia*e cells grown in different methanol concentrations were also detected. The effect of the permeabilization at lower concentrations (20%) 16h was similar to those at 20% ethanol, i.e., no obvious changes were observed (Supporting Information Figure S2d); when cells exposure to 30% and 40% (v/v) methanol for up to 16 h, respectively, it can be observed that, most of the cell walls peeled off and nearly become protoplast (Supporting Information Figure S2e and f). Moreover, some cell shapes became irregular compared to those of the control cells.

3.1.2 The viability of pretreated *S. cerevisiae* cells

Flow cytometry (FCM) has been extensively used to measure the total, viable and/or metabolically active microorganisms.[20] The significant advantage of FCM is its rapid automatic counting total cell concentration and viability. Therefore, in the present study, FCM combining with fluorescent dye (PI) was employed to evaluate the viability of yeast cells before and after pre-treatment by alcohols. Results were shown in Fig. 2. It can be observed that the cells treated by ethanol or methanol for 6 h to 16 h, showed maximal about 15% lower viabilities than the untreated cells. Compared to the high viability (97%, 94.5%) viability of the untreated yeast cells over 6 h and 16 h periods, the viabilities of *S. cerevisiae* cells which were subjected to 20% (v/v) methanol and ethanol after 16 h exposure decreased to about 89% and 90%, respectively. When the concentrations of the ethanol reach 30% and 40% (v/v) ethanol, the viability of the yeast further decreased to 87% and 85%, respectively. Based on this results, we can concluded that, although some cells dead during permeabilization, most of the permeabilized cells retained their viability, and therefore, an optimized permeabilization conditions may be effective for obtaining whole cell biocatalysts with high activity.

So combining to the results of the AFM imaging (Fig. 1) and the viability experiments of the cells, 30% (v/v) ethanol and treating time 16 h was set as the optimal condition for the furthermore applications. This was in accordance with that obtained by Garjonyte.[14] They thought that the high activity of *S. cerevisiae* treated by ethanol is probably attributable to increase water permeability of *S. cerevisiae* membrane to a great extent.

3.2. Characterization of PVA/Ca alginate /yeast cells disposable biofilm

3.2.1 SEM characterization

A promising immobilization technique for whole cells is the entrapment in some gels, such as PVA.[21] In the present study, a PVA-Ca alginate complex gel prepared by mixing PVA and sodium alginate solution, then cross-inking with sodium sulfate was used to immobilize *S. cerevisiae* cells. PVA is a kind of chemically stable, nontoxic and biocompatibility material for immo-bilization of microorganisms and is capable of maintaining the microbial activity,[22] while composite PVA hydrogels with a low content of Ca alginate are soft and form a more porous hydrogel.[23] To better understand how the pretreated *S. cerevisiae* cells are entrapped into the gel matrices, we have performed SEM images. As can be observed in the Fig. 3, a PVA-Ca alginate biofilm

prepared in the present study is a kind of macroporous network structure (Fig. 3a), and in which the living *S.cerevisiae* cells are enclosed in this polymeric matrix which is porous enough. The PVA biofilm does not have porous structure (see supporting information). The two biofilm were also used to determine the toxicity of DCP, and the results showed (see supporting information) that the biosensor based on PVA-Ca alginate biofilm is more sensitive to DCP than that based on the PVA biofilm, which might result from that the macroporous network structure may be beneficial to allow the diffusion of substrates to the cells. This micropore frame provides an excellent interspace and condition for the diffusion of substances and for the growth of cells. Schematic diagram of reaction involving PVA-alginate/ Na_2SO_4 was shown in Supporting Information Figure S4.

Additionally, it is important to mention that sodium sulfate induced crystallite formation in PVA as sulfate ions possess the ability to form linkages among PVA.[24] In the PVA-sodium sulfate hydrogel preparation, the sulfate ions penetrated into the PVA hydrogel solution soon and destabilized hydrogen bonding between hydroxyl groups of PVA and H_2O by polarizing the water molecules.[25] As a result, the formation of hydrogen bonds between the hydroxyl groups of PVA was facilitated, resulting in PVA crystallite formation.[22] XRD patterns of PVA-sodium sulfate hydrogel, dried PVA powder, dried sulfate hydrogel powder and PVA hydrogel was shown in Supporting Information Figure S3. Finally, we found that this crystallite formation can effectively overcome the swelling of the hydrogel matrix.

3.2.2 Electrochemical characterization of the disposable biofilm biosensor

In order to ascertain whether the pretreatment effect of 30% (v/v) ethanol on *S. cerevisiae* cells wall was capable of producing a higher sensitive biotoxicity response, the electrochemical responses of the Pt electrode modified by a biofilm which entrapped the pretreated *S. cerevisiae* cells were compared with that of a biofilm entrapped the untreated *S. cerevisiae* cells in the PVA gels. Biofilms are attached and fixed on Pt electrode surface as described in the experimental section, and generated electrochemical signals were captured by a potentiostat system. The control test was prepared as the same way but without biofilm on Pt electrode surface. 3, 5-dichlorophenol (DCP) was chosen as the reference toxicant as its toxicity has been widely studied using different approaches.[26]

As shown in Fig. 4, the analysis process was as follows: (1) The biosensors were carried out in a stirred vial containing 10 mL pH 7.0 respiratory substrates solution by applying a potential of 0.3 V (vs. Ag/AgCl) at room temperature; (2) Following a biosensor stabilization period I about 5 min, 100 µL of p-benzoquinone solution as a redox mediator (as shown in Fig. 4, position e) was injection to a final concentration of 0.4 mM. Then the current began to increase significantly firstly, followed by a decrease and finally reached a second period stabilization II about 5–10 min and noted as I_1 (baseline current); (3) When the toxicant samples were performed in the same way at the second stabilization period II (Fig. 4, position f), the current was monitored and noted as I_2; (4) The current data were displayed in real-time as current against time plots; (5) Then, the anodic currents were converted to equivalent inhibitory percentage values according to Eq. (1): $\text{Inhibition}\% = (1 - I_2 / I_1) \times 100\%$.

It was found that there were both no obvious changes of the current when injected 100 µL p-benzoquinone (position e, dotted line, Fig.4) and followed 100 µL 10 mg/L DCP (position f, dotted line, Fig.4) on naked Pt electrode respectively. It is well known that mediator fundamentally work by interacting with the metabolic pathways of the biocatalyst. The reduction activity of mediator is a sensitive indicator of xenobiotic toxicity to microorganisms because it is directly coupled to respiration via the electron transport chain, and decreases when noxious substances are present.[27] BQ can accept electrons from cells easily, which was proved evidently by the immediate increase of anodic current when injected BQ (position e, Fig.4) on electrode which modified by 30% ethanol pretreated *S. cerevisiae* cells 16 h disposable biofilm. Compared to the untreated *S. cerevisiae* cells

disposable biofilm modified Pt electrode (solid line), when injected 10 mg/L DCP, the pretreated cells biofilm modified Pt electrode response curves, as expected, showed a big current decline (position f, dash line, Fig.4). For biosensor A and biosensor B, the inhibition values of DCP to *S. cerevisiae* are 4.82% and 57.78% respectively according to Eq (1). These results showed that respirator activity of *S. cerevisiae* can be inhibited effectively by DCP, and the permeabilized *S. cerevisiae* cells are more sensitive to DCP than the untreated *S. cerevisiae* cells. It further suggests that the permeabilized *S. cerevisiae* cells should be useful as a sensitive bio-component for electrochemical biotoxicity assay.

Fig. 5 shows the inhibition curves of the pretreated yeast cells with different concentrations of methanol or ethanol for different time to 10 mg/L DCP. Whether treated with methanol or ethanol, the sensitivities of electrodes increased when their concentrations of alcohol were lower than 30%. However, the maximum responses values of the electrodes that contained cells treated with 30% ethanol are much higher than that of 30% methanol treated. When the concentration reached 40% and the treatment time is about more than 16h, the responses sensitivities decreased. Therefore, when yeast pretreatment with 30% ethanol for 16 h, the maximum sensitive and stable responses were obtained. This was consistent with the results of AFM. We speculated that the reason of high response sensitivity is that mediator could reach the enzyme in cells and membrane permeabilization was also favorable for DCP penetrating into yeast cells.

3.3 EIS characterization of the disposal biofilm on the Pt electrode

A Nyquist plot commonly includes a semicircle region lying on the axis followed by a straight line. The semicircular part seen at higher frequencies corresponds to the electron transfer-limited process and its diameter is equal to the charge transfer resistance (R_{ct}), which controls electron transfer kinetics of the redox probe at the electrode interface. Fig. 6 displays EIS observed for bared Pt, nondisposable biofilm modified Pt, and a disposal biofilm/Pt in 10 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl from 100 KHz to 10 Hz, respectively. The inset in Fig. 6 shows the most frequently used equivalent circuit for modeling the EIS experiments, Randles equivalence circuit model, which contains the electrolyte resistance (R_S) of the bulk solution in series with a double-layer capacitance (*Cdl*), charge transfer resistance (R_{ct}), and Warburg impedance (Z_W). Compared to the bare Pt electrode (curve a), the Nyquist plots of the both electrodes modified by biofilms are all exhibited the characteristics semicircles at high frequencies and a straight line at low frequencies, corresponding to kinetic and diffusion processes, respectively. The R_{ct} of the nondisposable biofilm/Pt electrode (curve c) was estimated to be 206 Ω, however, the R_{ct} of disposable biofilm/Pt electrode (curve b) displayed a lower value 185 Ω. These results imply that, firstly, as expected, the attachment of the biofilms on the metal surface retards the interfacial electron-transfer kinetics and increase the electron-transfer barrier between the redox analytes (p-benzoquione in the present study) and electrode surface; and moreover, the similar EIS behaviors of the two biofilms modified Pt electrodes suggest that attaching the disposal biofilm onto the Pt surface make no difference with the conventional biofilm modified Pt in electrochemical characteristic despite a little increasing in R_{ct}. Therefore, the present result is encouraging as disposable biofilm was shown to compare well with the conventional nondisposable biofilm electrode and demonstrates not bad response over the date presented.

3.4. Toxicity assessment

Mediated, amperometric, microbial biosensors have been developed for toxicity assessment.[5] In the present study, AFM observations, electrochemical responses and FCM results showed that the optimized conditions of pretreatment were 30% (v/v) ethanol for 16 h. Therefore, we selected 30% (v/v) ethanol for yeast pretreatment 16 h in following biotoxicity tests. BQ was used

as an electron transfer mediator and the pretreated *S. cerevisiae* cells loaded into the PVA gel as biological component. After being activated, the biofilm, fabricated by the PVA gel/pre-treatment *S. cerevisiae*, was attached and fixed onto the Pt electrode to construct a disposable amperometric biosensor for the assay of biotoxicity. This Biosensor with a disposable microbial film can avoid inaccurate data caused by film pollution in repeat detection biotoxicity process.

The responses of biosensor operated under optimal conditions (i.e., 0.4 mM p-benzoquinone; pH 7.0 respiratory substrates solution) were measured, and 3, 5-dichlorophenol (DCP), Ametryn, Acephate and Thiram were chosen as target toxicants. The selecting basis for several pesticides is that we chose the typic pesticides of different chemical structures. Ametryn, Acephate and Thiram are triazine weedkiller, organophosphorus insecticide and sulfur fungicide respectively, which were widely used and chosen as target toxicants. Inhibition percentage values are calculated according to Eq. (1). Fig.7 shows the respiration rates obtained at different concentrations of DCP and three pesticides, and the IC_{50} values of 9.83 mg/L for DCP, 22 mg/L for Ametryn, 29 mg/L for Acephate and 47.5 mg/L for Thiram, respectively, were determined within 30 min. The lowest IC_{50} value corresponded to DCP, followed by Acephate, Ametryn and Thiram, indicating its high toxicity. Table 1 compares IC_{50} results of DCP by using the present biosensor and others under comparable conditions. It can be seen that this proposed bioassay is more sensitive than biosensor (eukaryote *T. Cutaneum*),[28] Biolog (*activated sludge*)[29], CellSense (Genetically Engineered Bacterium *E.coli* PMP 101)[30] and Bioluminescence (*Vibrio qinghaiensis sp.* Q67 and BF-2/luc1 cell)[30-31] biotoxicity assay, further demonstrating the potential applications of this novel and disposable *S. cerevisiae*–based sensor bioassay in determination of pesticides toxicity.

Furthermore, In order to demonstrate IC_{50} values determined using the electrochemical method is reliable, three pesticides were investigated and comparing the results with that of published data by other methods. Table 2 reports the IC_{50} values to Ametryn, Acephate and Thiram as 22 mg/L, 29 mg/L and 47.5 mg/L, respectively. Obviously, Ametryn was the most toxic, Acephate in the middle and Thiram was the lowest of the three pesticides. Although Toxalert assay using *V. fischeri* shows more sensitive response to Ametryn, it is not suitable for measurement of turbid solutions due to the decrease of light intensity. In contrast, measurements by the electrochemical method are not disrupted by turbidity, even when measuring suspensions, which is an advantage, especially for wastewater samples. Thus, the improvement of the biosensor's system is needed for further investigation. Nevertheless, the prepared disposable *S.cerevisiae* biofilm modified amperometric microbial sensor can effectively determine the biotoxicity of organic toxicants especially for DCP, and another advantage is that it can provide a constant monitoring of the microbial activity, which makes it possible for the real-time monitoring of water quality and early warning of emergent pollution.

3.5. Reproducibility and stability of the disposable microbial biosensor

The reproducibility of the prepared microbial biosensors was evaluated by five replicate assays for 10 mg/L DCP, Ametryn, Acephate and Thiram inhibition respectively on *S. cerevisiae*. As shown in Fig. S4(a), the relative standard deviations (RSD) obtained were 2.15%, 3.33%, 3.61% and 3.53% respectively. Thus, the prepared biosensors showed a good and reproducible performance.

When the stability was concerned, the integrated microbial biosensors were kept at 4 °C. As shown in Fig. S5(b), the responses to 10 mg/L DCP, Ametryn, Acephate and Thiram respectively were measured every two days and the results showed a relative standard deviation (RSD) of 5.55%, 6.52%, 7.0%, 7.3% respectively for one week. The results indicated that the biocompatibility of hybrid material was good and the pretreated *S. cerevisiae* cells could be applied for biosensor fabrication.

3.6. Analysis of four real wastewater samples

In real environmental pollution, a wide variety of chemicals simultaneously exist in water. This disposable biofilm modified amperometric microbial sensor prepared can provide a sensitive, rapid, convenient assessment of overall composite toxicity and a promising approach for early risk warning of acute water toxicity. The usual real wastewaters contained organics, inorganics or both are our selecting basis for wastewater, in order to reflect the broader spectrum responses to water samples by our prepared sensor. The wastewaters which were respectively taken from a river around suburban farms of Beijing, garbage-treatment plant, electroplating factory and laboratory were measured as the real samples. 10 μL real sample was added to the 10 mL pH 7.0 respiratory substrates to get the volume ratio 1% (v/v) and its biotoxicity was measured with the prepared biosensor. As shown in Fig. S6, the integrated biosensor was sensitive to the four kinds of real wastewater samples and the inhibitory percentage values were 30.75% for river around suburban farms, 44.81% for garbage wastewater, 59.07% for laboratory wastewater and 64.57% for electroplating wastewater respectively. Therefore, the toxicity can be ranked in a descending order: electroplating wastewater > laboratory wastewater > garbage-treatment wastewater > river water.

4. Conclusions

A novel, disposable and sensitive whole cells amperometric biosensor based on permeabilized *S. cerevisiae* has been fabricated successfully for the biotoxicity assessment of pesticides in water and three kinds of real wastewater samples. Amperometric measurement suggested that the *S. cerevisiae* cells treated with ethanol caused similar "mild" permeabilization of *S. cerevisiae* cells membrane, which resulted in electrode more sensitivity to DCP than non-treatment one. In optimized assays, IC_{50} values of 9.83 mg/L for DCP, 22 mg/L for Ametryn, 29 mg/L for Acephate and 47.5 mg/L for Thiram were determined, respectively. These results obtained were better than those of other toxicity bioassays. Moreover, this prepared disposable biofilm modified amperometric microbial sensor can effectively determine the biotoxicity of the real wastewater samples, showing that the prepared biosensor can be easily applied to practical mobile system for biotoxicity assay of chemicals and environmental water monitoring. Therefore, the present disposal biofilm electrochemical biosensors not only provide the advantages which avoided complicated operation, expensive electrode material and increasing sensitivity of response, but also the potential for expanding the technique to utilized *S. cerevisiae* with complementary toxicity responses, thereby allowing use of the biotoxicity in a wide range of application.

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† Electronic Supplementary Information (ESI) available: See DOI: .

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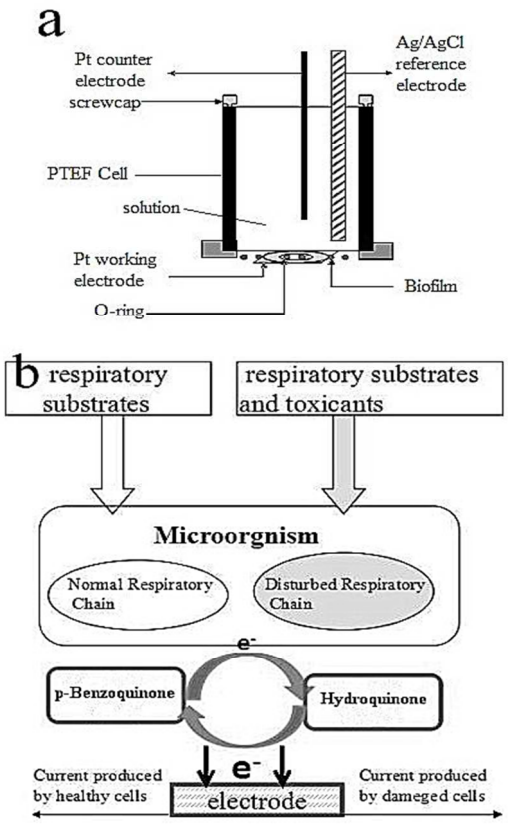

Scheme 1. (a) Schematic diagram of the disposable microbial film sensor. (b) Principle of the BQ-mediator biotoxicity assay based on sensor.

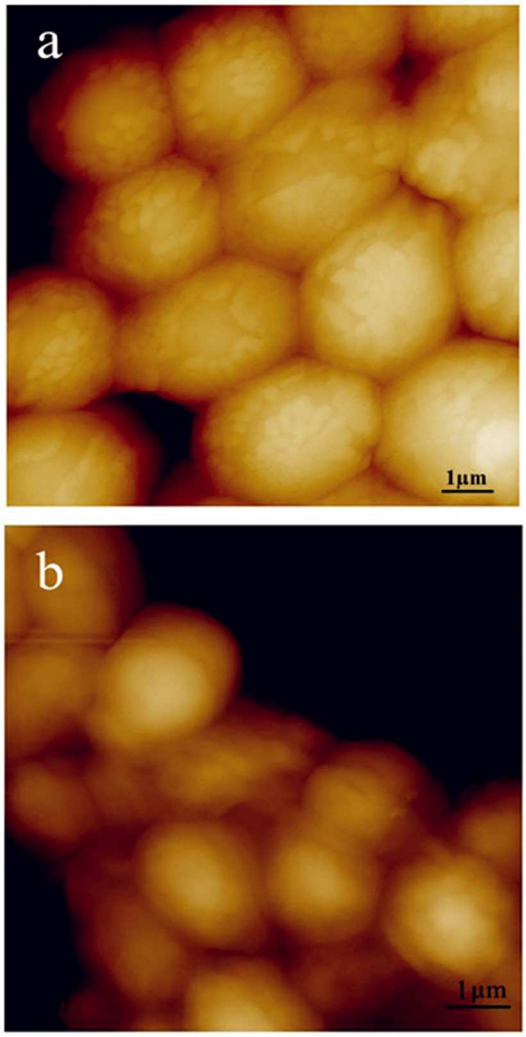

Fig.1. AFM images of *S. cerevisiae* cells pretreated by ethanol with different concentrations. (a) 30% (v/v) ethanol, 16 h; (b) 40% (v/v) ethanol, 16 h; respectively.

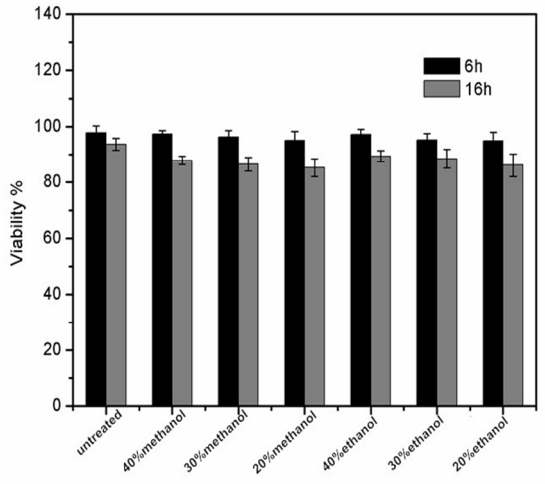

Fig. 2. Viability of *S. cerevisiae* cells in methanol and ethanol (20%, 30%, 40%(V/V)) after 6 h(black) and16 h (gray) treatment. Bar: mean ±SD (n=3).

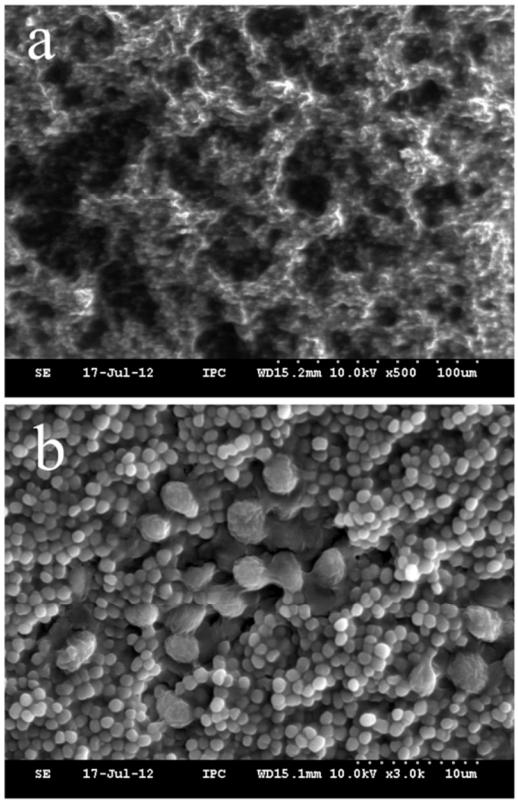

Fig. 3. (a) The low-magnification SEM image of the PVA-Ca alginate biofilm enclosed with the pretreated *S. cerevisiae*; (b) The magnified image of (a).

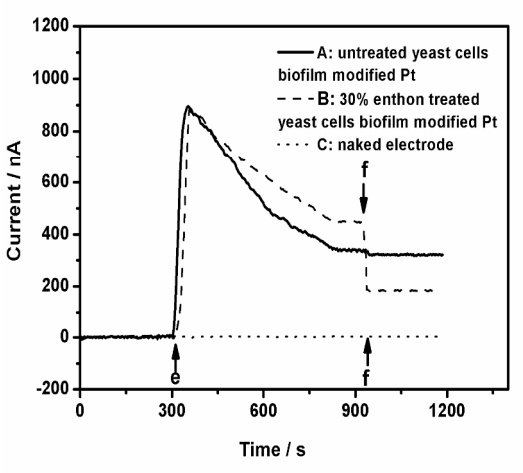

Fig. 4. Toxicity analysis current curves of different biosensors: A: electrodes modified with untreated *S. cerevisiae* (solid line); B: electrodes modified with pretreated *S. cerevisiae* by 30% ethanol 16h (dash line); C: naked Pt electrode (dotted line). a, b are operation starting points of p-benzoquinone and toxicant samples respectively. Operating potential is 0.3 V (vs. Ag/AgCl).

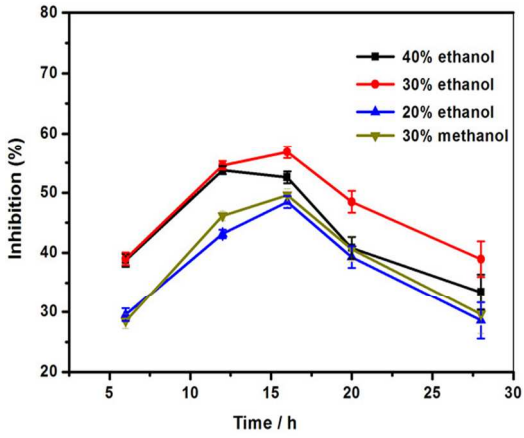

Fig. 5. Inhibition curves of electrodes modified with yeast by different pretreated concentration and time methanol or ethanol to 10 mg/L DCP in pH 7.0 respiratory substrates solution using p-benzoquinone as a mediator. Operating potential 0.3 V (vs. Ag/AgCl).

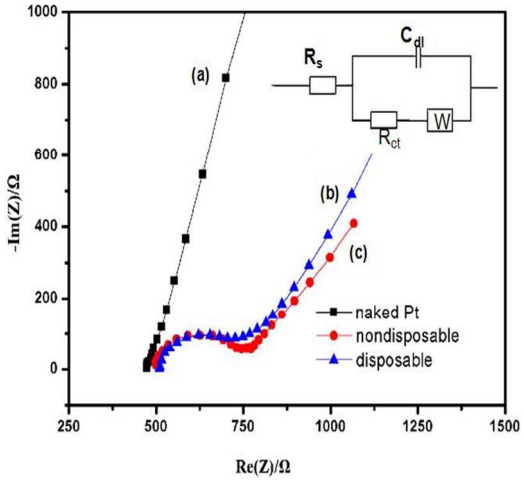

Fig. 6. Nyquist plots for of (a) naked Pt electrode; (b) disposable biofilm modified electrode; (c) nondisposable biofilm electrode electrode; respectively, obtained in 10 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl. Inset: the Randles equivalent circuit model for modified electrodes.

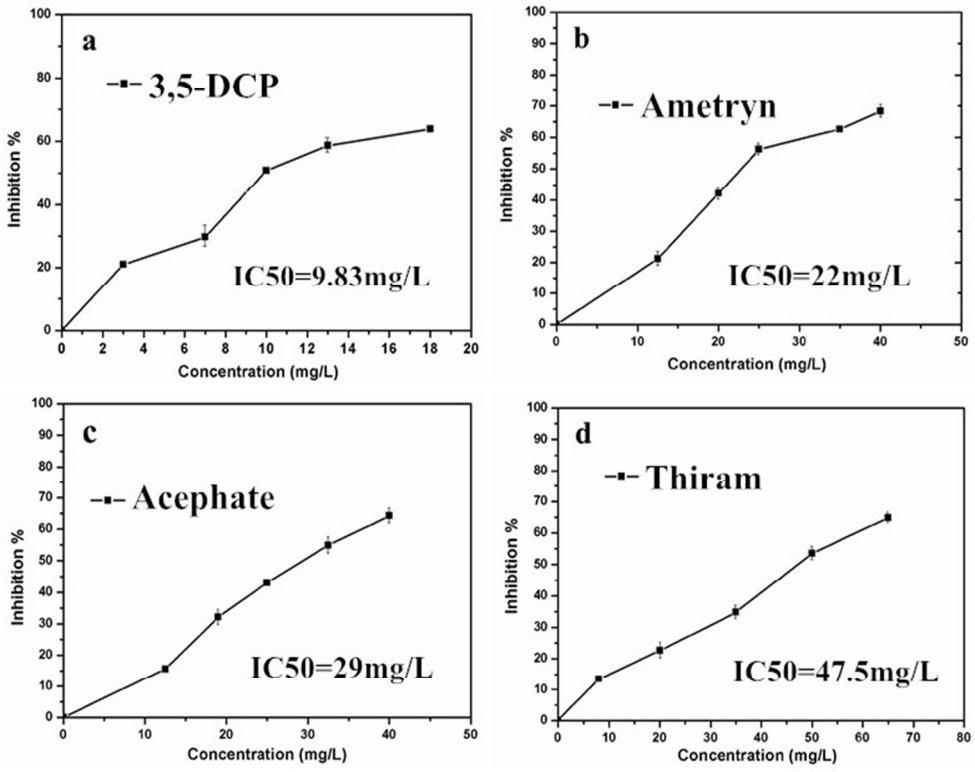

Fig.7. Inhibitory curves of pretreated *S.cerevisiae* at different concentrations of (a) 3, 5-dichlorophenol, (b) Ametryn, (c) Acephate and (d) Thiram respectively under the optimized conditions. Date points represent the average of three replicates.

Table 1. Comparison of IC_{50} for DCP values by different methods

Toxicants, IC_{50} (mg/L)			
3,5-DCP	Biotoxicity assays	Microorganism	Reference
9.83	Biosensor, Amperometry	*S.cerevisiae* (pretreated)	This work
32.1	whole cell biosensor, Amperometry	*T. Cutaneum* (eukaryote)	(ref. 28)
14.4	Biolog MT2 microplates procedure, incubation 24 h	*activated sludge*	(ref. 29)
15.10	CellSense, Amperometry	Genetically engineered bacterium *E.coli* PMP 101	(ref. 30)
25.19	Bioluminescence	*Vibrio qinghaiensis sp*. Q67	(ref. 31)
52.0	Bioluminescence	BF-2/luc1 cell	(ref. 30)

Table 2. Comparison of IC_{50} for Ametryn, Acephate and Thiram values by different methods

Pesticides, IC_{50}			Biotoxicity assays	Reference
Ametryn	Acephate	Thiram		
22	29	47.5	*S.cerevisiae* (pretreated); Biosensor	This work
18.6 mg/L	-	-	*V. fischeri;* Toxalert, 30 min exposure	(ref. 32)
-	141.25 ±10.49 mM	-	*Crassostrea hongkongensis*; AChE activity	(ref. 33)
-	-	> 40 mg/L (not give the specific values)	*M. phaseolina* (eukaryote); in vitro	(ref. 34)