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A novel colorimetric biosensor for monitoring and detecting acute toxicity in water†

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This work presents a new colorimetric microorganism biosensor for monitoring and detecting acute toxicity in water, where prussian blue (PB) is used as the colorimetric indicator and *E. coli* as the model bacterial. In this biosensor, the electron mediator, ferricyanide, accepts electrons from *E. coli* during respiration to produce ferrocyanide, which subsequently reacts with ferric ions to yield PB, a famous material with a blue color. Since toxicants can inhibit the respiratory activity of *E. coli* and then reduce the ferrocyanide and consequent PB production, toxicity can be easily detected by measuring the decrease in the production of PB induced by toxicants. Three important toxicants, 3,5-dichlorophenol (DCP), As^{3+}, Cr^{6+} are tested and the detection limits are 3.2, 25, and 3.2 ppm, respectively. Moreover, we could identify the yellow green to dark green color change by naked eye even at concentrations as low as 12.5 ppm for both DCP and Cr^{6+}. Subsequently, the acute toxicities of groundwater and south lake water are successfully determined by this sensor. This biosensor is rapid, sensitive and cost-effective, and can thus be regarded as a promising biosensor for giving an early warning of acute water toxicity.

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Introduction

There is a growing interest to design and develop simple, reliable, rapid and inexpensive biosensors for monitoring and detecting toxicity in water due to the increased pollution from the rapid development of industrial activities.[1–3] Microorganism biosensors meet these criteria because microorganisms have a short life cycle and sharp response to toxin, and rapidly adapt to toxicological studies for assessing organisms or ecosystem health.[4,5] Colorimetric sensors have been extensively studied in sensing biomolecules, metal ions, and so on.[6,7] Because their response signals are visible, that is, the results can be directly observed by naked eye; the special equipment for readout results are not indispensable in colorimetric sensors and thus the cost of sensors and hence of tests can be significantly reduced.[8–10] Conventional colorimetric bioassays such as MTT assay and neutral red uptake assay screen for toxicity by using dyes as indicators to distinguish viable and dead cells.[11–13] Although huge success has been obtained, the long incubation times (more than 24 h) of cell seeds with toxicants make these assays unsuitable for giving an early warning of acute water toxicity. Till now, no colorimetric toxicity biosensor capable of giving an early warning of acute water toxicity (detecting toxicity rapidly) has been developed.

Recently, the ferricyanide-mediated bioreaction, where ferricyanide acts as a terminal electron acceptor during respiration by bacteria instead of oxygen and is reduced to ferrocyanide,[14–16] has been successfully used to develop microorganism biosensors for rapid toxicity assessment. In the presence of toxicants, the production of ferrocyanide by this bioreaction is reduced because the respiration of cells is inhibited by toxicants. Then the toxicity could be determined by measuring the decrease in ferrocyanide production.[14,17–19] In previous works the methods for ferrocyanide measurement are limited to electrochemical methods,[15,20] because ferrocyanide is a well known electron mediator. However, these biosensors combined with an electrochemical test are not suitable for samples containing redox active molecules, which can cause over- or underestimated toxicity. On the other hand, prussian blue (PB), a famous mixed-valence compound with a blue color,[21,22] can be simply prepared by mixing the solutions of ferric ion and ferrocyanide. In particular, this PB formation reaction can be used to quantify ferrocyanide and ferric ions, and a successful example is the PB staining assay for intracellular iron content.[23,24] Therefore, the ferrocyanide produced by the bioreaction can be detected by this PB formation reaction, and sample solution colors would be changed to blue (or green) when PB is formed. So PB can serve as the colorimetric indicator for microbially produced ferrocyanide and hence the toxicity, as shown in Scheme 1, however, no attention has so far been paid to it.

In this work, by coupling the PB formation reaction and the ferricyanide-mediated bioreaction, we have designed a new colorimetric microorganism biosensor capable of rapidly

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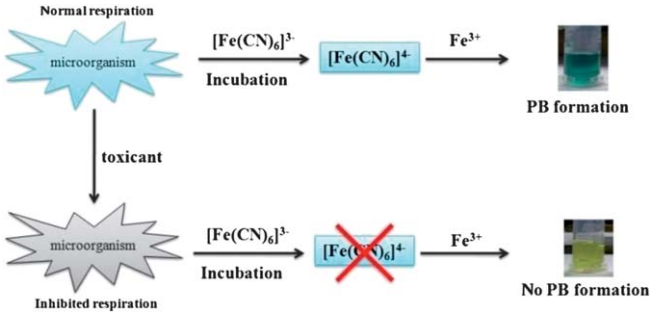

Scheme 1 Schematic illustration of the principle of toxicity detection.

detecting acute toxicity. The formed PB is used as the colorimetric indicator for toxicity and *E. coli* as the model microorganism. The principle of toxicity detection is shown in Scheme 1 and the feasibility of this biosensor is demonstrated by the toxicant-dependent color changes of the samples. The toxicities of 3,5-dichlorophenol (DCP), As^{3+} and Cr^{6+} are detected and the detection limits are 3.2, 25, and 3.2 ppm, respectively. Moreover, the unique feature of this biosensor compared with other reported biosensors such as Microtox,[25] Micredox[20] and Baroxymeter,[26] is the that the results (or toxicity) can be directly observed by naked eye. For example, the yellow green to dark green color change can be identified by naked eye even at concentrations as low as 12.5 and 12.5 ppm for DCP and Cr^{6+}, respectively. Importantly, the acute toxicities of polluted groundwater and south lake water are successfully determined by this biosensor, demonstrating its potential in giving an early warning of acute water toxicity.

Experimental details

Chemicals

DCP was purchased from Sigma-Aldrich. Standard Stock solutions of Cr^{6+}, Hg^{2+} and As^{3+} were obtained from the National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials, China. Peptone and yeast extract were from OXOID Ltd. All other chemicals were of analytical grade and obtained from Beijing Chemical Corporation. 0.2 M phosphate buffer solutions (PBS) were prepared by adding 10.88 g Na_2HPO_4 and 42.98 g $KH_2PO_4 \cdot 12H_2O$ into 1000 mL of deionized water. The pH value was adjusted to 7.2 by adding 1 M HCl or 1 M NaOH. 5 mM ferric solutions with pH values of 0.08, 0.80 and 2.20 were prepared with HNO_3.

Microbial cultures

E. coli (DH 5α) was obtained from the China General Microbiological Culture Collection Center (CGMCC) and maintained on nutrient agar plates at 4 °C. The suitable medium and incubation temperature were confirmed by CGMCC and Cole-Parmer Instrument Company guides. The microorganism was cultivated in a 220 rpm shaker bath unless otherwise stated. Cells were harvested by centrifugation at 6500 rpm for 5 min at room temperature, and washed twice with PBS. And then the cells were resuspended in PBS for obtaining microbe cell slurries and kept at 4 °C. All cell suspensions should be used for experiment on the day of harvesting.

Sensing toxicity

A 450 mM stock solution of ferricyanide was prepared and stored at 4 °C for experiments. The cell suspensions were diluted to desired OD_{600} values with PBS. All toxic chemical solutions with desired concentrations were prepared by diluting their stock solutions with PBS. Samples were prepared by adding 400 μL of cell suspension, 400 μL of PBS, 100 μL of toxic chemical solution and 100 μL of 450 mM ferricyanide solution. Control samples contained PBS in place of toxic chemical solution. After incubation at 37 °C for 30 min, the samples were centrifugated at 11 000 rpm for 10 min and the supernatants were decanted into new sample containers. 200 μL of the resultant supernatants of samples were mixed with 200 μL of ferric solutions (pH = 0.8). The resultant 400 μL solutions were diluted to 1 mL with deionized water, and then the absorption of PB was measured by UV-vis absorption spectroscopy. The OD values of cell suspensions were determined by measuring their diluents with OD_{600} in the range of 0.3–0.9. The CFU (colony forming unit) concentration was determined to be 5×10^7 mL^{-1} for the cell suspension with the $OD_{600} = 0.5$.

For real samples, 1.08 g Na_2HPO_4, 4.30 g $KH_2PO_4 \cdot 12H_2O$ and 1.48 g potassium ferricyanide were firstly added into 100 mL of groundwater or south lake water, and dissolved by sonication. Polluted groundwater or south lake water were prepared by diluting stock solutions of toxicants with the above-mentioned groundwater and south lake water. Samples were prepared by adding 200 μL of cell suspension, 800 μL of the polluted groundwater or south lake water. The PBS containing ferricyanide was prepared by dissolving 1.08 g Na_2HPO_4, 4.30 g $KH_2PO_4 \cdot 12H_2O$ and 1.48 g potassium ferricyanide in 100 mL of deionized water, and used in control samples instead of toxic groundwater or south lake water. The samples were incubated at 37 °C for 30 min, and then centrifugated at 11 000 rpm for 10 min. 200 μL of the supernatants of samples were pipetted out and mixed with 200 μL of ferric solutions (pH = 0.8). The resultant 400 μL solutions were diluted to 1 mL with deionized water and the colors of which were recorded with a digital camera.

The groundwater was obtained from the well in our institute. South lake is a lake located at the city of Changchun.

All data given here are the mean of three replicates.

Instruments

The UV-vis absorption spectra were obtained with a Varian 50 Conc UV-visible Spectrophotometer.

Results

The feasibility of the proposed colorimetric microorganism biosensors

Scheme 1 shows the principle of toxicity detection based on the PB formation reaction and the ferricyanide-mediated bioreaction. In the presence of toxicants the respiratory activity of

cells is inhibited and therefore no ferrocyanide can be produced during incubation. The solution color cannot be changed from yellow (color of ferricyanide) to green (a mix of blue and yellow from PB and ferricyanide, respectively) by subsequently adding ferric solutions, because no PB is formed. In contrast, in the absence of toxicant, adequate ferrocyanide can be produced by cells during incubation, and the color change can thus occur by adding ferric ions due to the formation of PB. Therefore, toxicity can be detected by measuring the decrease in PB production with UV-vis absorption spectroscopy or directly observing the color changes by naked eye.

To demonstrate the feasibility of this proposed biosensor, the UV-vis absorption spectra and the photographs of ferric solutions with pH value of 0.80 before and after mixing with ferricyanide solutions upon different treatments are collected and shown in Fig. 1. The ferric solution is colorless and shows no absorption in the range from 500 to 800 nm (Fig. 1a). The addition of DCP to a mixture of ferric and ferricyanide does not result in the formation of PB,[22] as the color of the mixture is not changed and no absorption peak associated with PB can be observed (Fig. 1b). The mixture containing ferric ions and the ferricyanide upon 15 min incubation with *E. coli* in the presence of 50 ppm DCP, exhibited a yellow green color, indicating no or less PB is formed because of the DCP induced respiration inhibition (Fig. 1c). In contrast, the mixture containing ferric ions and the ferricyanide upon incubation without DCP, displays a green color with an obvious absorption peak at about 660 nm,[21] indicating more PB is formed (Fig. 1d). The results clearly demonstrate the toxicant-dependent production of PB and therefore the feasibility of the proposed biosensors for monitoring and detecting acute toxicity. It should be pointed that the concentration of resultant ferrocyanide produced by *E. coli* after 1 h incubation with 45 mM ferricyanide would be no more than 5 mM, and thus 5 mM ferric solution was chosen to completely convert the resultant ferrocyanide to PB. Moreover, we have tried to use a lower concentrated ferricyanide, but no obvious color change can be observed. Although it has been reported that ferricyanide could affect the viability of *E. coli*,[14] the influence of 45 mM ferricyanide on the viability of *E. coli* is not significant at 1 h incubation, which could be ignored due to the fact that, ferricyanide was added into both control and toxicity samples, respectively.[15,18]

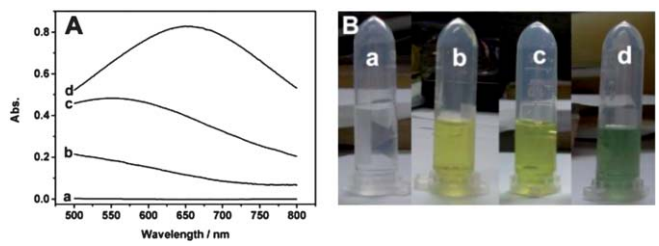

Fig. 1 UV-vis absorption (A) and color (B) changes of Fe^{3+} solutions. (a) Fe^{3+} solution; (b) Fe^{3+} solution after adding $[Fe(CN)_6]^{3-}$ and DCP; (c) and (d) Fe^{3+} solution after adding $[Fe(CN)_6]^{3-}$ upon incubation for 15 min with and without 50 ppm DCP, respectively. $OD_{600} = 12$, pH value of Fe^{3+} solution is 0.80.

The effects of *E. coli* concentration, pH value of ferric solution and incubation time on sensing toxicity

The production of ferrocyanide through the ferricyanide-mediated bioreaction and formation of PB *via* the chemical reaction between ferrocyanide and ferric ions, are related to the *E. coli* concentration and pH value of ferric solution, respectively. In order to investigate their effects on sensing toxicity, the assays were conducted with ferric solutions having different pH values at different *E. coli* levels. The incubation time is 30 min for all samples. Fig. 2A is the UV-vis absorption spectra of the resultant samples obtained with ferric solution of pH 2.20. No peaks assigned to PB can be observed for all samples, indicating no PB is formed, possibly because of the formation of ferric phosphate[27,28] (ESI†). The phosphate is from the PBS solution added during incubation. For ferric solution of pH 0.80 (Fig. 2B), the absorption intensity increases with the concentration of *E. coli* and an obvious absorption peak of PB at 660 nm is eventually observed at $OD_{600} = 12$ without DCP, indicating more ferrocyanide are actually needed for ferric solutions with pH values of 0.80 to produce PB. When decreasing the pH of the ferric solution to 0.08, the formation of ferric phosphate could be completely prevented. Therefore, PB can be formed in the whole *E. coli* concentration range, clearly demonstrated by the notable absorption peak of PB at about 700 nm for all samples in Fig. 2C. The different absorption peak positions of PB may be attributed to the different environments around them.[29,30] The absorption intensity changes (Abs_T/Abs_I) induced by 10 ppm DCP are plotted *versus* the concentration of *E. coli* (Fig. 2D), where Abs_T and Abs_I are the absorption intensity at 700 nm obtained with and without toxicants, respectively. The largest change, namely, the best sensitivity is obtained when the concentration of *E. coli* was $OD_{600} = 12$ and the pH value of ferric solution 0.80, which are thus used for the following experiments.

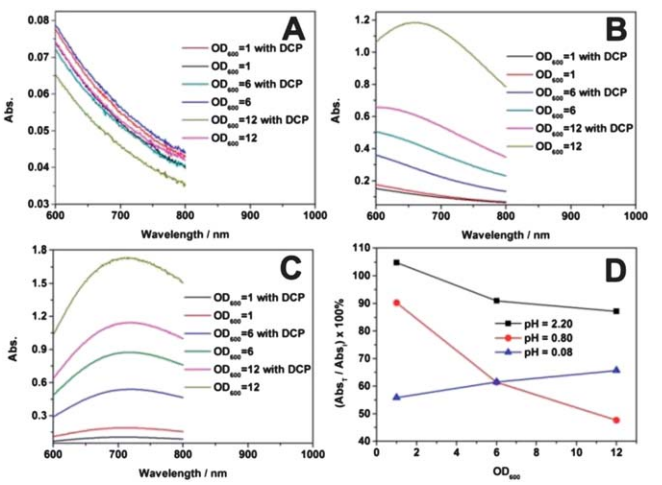

Fig. 2 (A–C) the effect of *E. coli* concentration on the UV-visible absorption changes of Fe^{3+} solutions of pH 2.20, 0.80 and 0.08, respectively. (D) The plots of (Abs_T/Abs_I) values *versus* the *E. coli* concentration obtained with Fe^{3+} solutions of different pHs. The concentrations of *E. coli* used are indicated in the figures, and the concentration DCP is 10 ppm. The incubation time is 30 min.

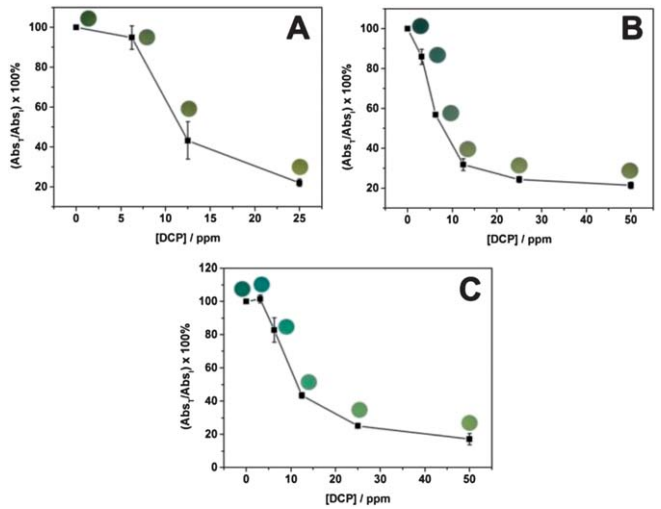

Fig. 3 The (Abs_T/Abs_I) values plotted against the DCP concentration at (A) 15, (B) 30 and (C) 60 min incubation. The corresponding sample colors are indicated in the figures. $OD_{600} = 12$, incubation time is 30 min, the pH value of Fe^{3+} solution is 0.80.

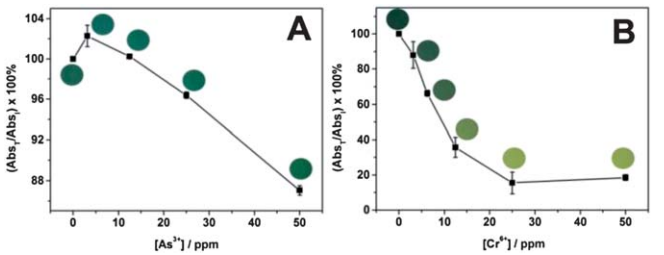

Fig. 4 The (Abs_T/Abs_I) values plotted against (A) As^{3+} and (B) Cr^{6+} concentrations. The corresponding sample colors are indicated in the figures. $OD_{600} = 12$, incubation time is 30 min, the pH value of Fe^{3+} solution is 0.80.

The effect of incubation time on sensing toxicity is further studied. Fig. 3 exhibits the (Abs_T/Abs_I) values plotted *versus* DCP concentration with different incubation times and the corresponding color of samples. The dose-dependent inhibition responses are obvious. The (Abs_T/Abs_I) value decreases with increase in DCP concentration and the lowest detectable concentrations for DCP are 12.5, 3.2 and 6.4 ppm at 15, 30 and 60 min incubation, respectively. The detection limit obtained at 30 min incubation is lower than that of Baroxymeter,[31] a portable toxicity detection systems also based on respiration inhibition. It should be pointed that the use of highly concentrated *E. coli* could cause the gradual degradation of DCP and consequently the decrease in DCP concentration during incubation.[32] Therefore, DCP could firstly serve as the toxicant to inhibit respiration and then nutrient to promote respiration during incubation because its concentration continuously decreases and its toxicity is dose-dependent. This may be why 3.2 ppm DCP inhibited respiration at 15 and 30 min incubation, but promoted respiration at 60 min incubation (Fig. 3). In fact, that the low concentrated DCP can promote respiration of microorganism has been observed before.[33] The color progression of samples from yellow green to dark green or blue, depending on the incubation time, could be observed with decreasing DCP concentration. This color change can be identified at DCP concentrations as low as 12.5, 12.5 and 25 ppm at 15, 30 and 60 min incubation, respectively. Obviously, the longer incubation time leads to the more ferrocyanide, and thus the higher DCP concentration at which the color change can occur. Based on these results, an incubation time of 30 min is adopted for sensing toxicity.

Sensing toxicity

To characterize the toxicity detection potential of the proposed biosensor, we examined the influence of two important toxicants, As^{3+} and Cr^{6+}, on PB formation. Fig. 4 presents the (Abs_T/Abs_I) values plotted against the concentration of As^{3+} and Cr^{6+}. The (Abs_T/Abs_I) value increases firstly and then decreases with increase in As^{3+} concentration from 3.2 to 50 ppm. The detection limit for As^{3+} is 25 ppm according to Fig. 4A. The color change from yellow green to dark green or blue does not occur, indicating the respiratory activity of *E. coli* is not seriously inhibited at As^{3+} concentration below 50 ppm. In contrast, the color change is obvious in the case of Cr^{6+}, and can be simply identified by naked eye at a concentration as low as 12.5 ppm. The (Abs_T/Abs_I) value decreases as the Cr^{6+} concentration increases and the lowest detectable concentration for Cr^{6+} is 3.2 ppm, as shown in Fig. 4B. Phenol is further tested, but there is no dose-dependent inhibition at the concentration range from 3.2 to 100 ppm, possibly because phenol can cause the cell membrane damage but not the respiration inhibition of *E. coli*.[3,34,35] This specificity of the response may be useful to detect the nature of the toxicant and to characterize its involvement in respiration inhibition.

To verify its practicality, the proposed biosensor has been applied to detect the acute toxicity of groundwater and south lake water. As shown in Fig. S4†, for the groundwater polluted by DCP, Cr^{6+}, Hg^{2+} and DCP + Cr^{6+} (polluted by DCP and Cr^{6+} simultaneously), the color changes occur at concentrations as low as 12.5, 12.5, 50 and 12.5 + 12.5 ppm, respectively. For south lake water polluted by DCP, Cr^{6+}, Hg^{2+} and DCP + Cr^{6+}, the color changes can be observed at concentrations as low as 12.5, 12.5, 25 and 6.25 + 6.25 ppm, respectively. The tested toxicants exhibit more toxicity in south lake water than in groundwater, which should be related to the different compositions of groundwater and south lake water. 4-Nitrophonel and 2-chlorophenol polluted groundwater and south lake water are further tested by this biosensor, and the color changes occur at 50 ppm 4-NP or 50 ppm 2-CP for south lake water and 100 ppm 4-NP for groundwater, as shown in Fig. S5.†

The proposed biosensor is compared with some toxicity detection sensors based on different comparison aspects such as EC_{50} value, requirement for special equipment, test time, and so on, which are listed in Table 1. This work is aimed at providing colorimetric toxicity sensors whose results can be obtained by direct observation. So the lowest concentrations (LC) of toxicants at which color changes occur are compared with EC_{50} (a generally used parameter for comparison) from other toxicity sensors. It can be seen that, the LC values of the

Table 1 Comparison of the LC values with the EC_{50} values of different toxicants

Test	Microorganism	EC_{50} (ppm) of the toxicants obtained by various toxicity biosensors					Matrix	Equipment	Test time (min)	Reference
		DCP	Cr^{6+}	Hg^{2+}	4-NP	4-CP				
Colorimetric biosensors	*E. coli*	12.5	12.5	50	100		Groundwater	No	30	This work
		12.5	12.5	25	50	50	South lake water			
Toxalert	*Vibrio fischeri*	4.42	87.0		11.6	1.45		Yes	5	38
Microtox	*Vibrio fischeri*	4.09	48.6		9.23	1.17		Yes	5	
Lumistox	*Vibrio fischeri*	4.80	216		3.17	1.14		Yes	5	
Microtox	*Photobacterium phosphoreum*		12.7	0.36			Sewage	Yes	15	39
Micredox	*Bacillus subtilis*	7.5					Distilled water	Yes	60	20
	Pseudomonas putida	8.5					Distilled water			
	Escherichia coli	7.0					Distilled water			
CellSense	Activated sludge	9.8					DI water	Yes	15–30	40
CellSense	*Vibrio fischeri*	37.5					DI water		15–30	
Baroxymeter		10		15				Yes		41
Respirometer	Activated sludge	12.97	19.36				Synthetic sewage	Yes	180	42
Respirometer	Activated sludge	5.19			149.9			Yes	60	43

test toxicants are comparable with the EC_{50} obtained by the biosensors based on respiration inhibition (Micredox, Cellsense, Baroxymeter and Respirometer), but are larger than the EC_{50} obtained with the biosensors based on fluorescent fading (Toxalert, Microtox and Lumistox). It is obvious that the sensitivity of sensors to toxicants is related to the toxicity nature of toxicants and the toxicity detection mechanism. The test time of this biosensor is 30 min, which is shorter than that of respirometer and Micredox. Importantly, this biosensor exhibits no requirement for special equipment, which can dramatically decrease the test cost. Given its sensitivity, test time, and test cost, this biosensor may have promising potential in environmental monitoring. Moreover, by employing the free-dried or sol–gel embedded *E. coli*,[36,37] this sensor can meet the requirement for on-demand use in environmental monitoring.

It is worth mentioning that colored samples cannot be tested using this biosensor by the naked eye, however, the formed PB can be spectroscopically measured as the difference in absorption at 700 nm of two tests, one of which incubates *E. coli* with ferricyanide and the other one incubates *E. coli* without ferricyanide. Such a differential measurement would practically eliminate the influence of interferences on sensing toxicity due to presence of colored species in the samples.

Conclusion

Here, we describe the design of a new colorimetric microorganism biosensor for monitoring and determining toxicity, by coupling the PB formation reaction and the ferricyanide-mediated bioreaction. The toxicity can be simply detected by measuring the decrease in PB production induced by toxicants. The detection limits for DCP, As^{3+}, and Cr^{6+} are 3.2, 25, and 3.2 ppm, respectively. Importantly, the toxicities of DCP and Cr^{6+} can be directly observed by naked eye at a concentration as low as 3.2 and 3.2 ppm, respectively. This biosensor has been successfully used to detect the acute toxicity of groundwater and south lake water by observing the color changes. This sensor is sensitive, cost-effective, and responds to low concentrations of toxicants within 30 min, and thus exhibits promising potential in monitoring and determining water toxicity.

Acknowledgements

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