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A signal-amplified whole-cell biosensor for sensitive detection of Hg^{2+} based on Hg^{2+}-enhanced reporter module

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ABSTRACT

A signal-amplified mercury sensing biosensor with desired sensitivity was developed through firstly using the GFP mutant with fluorescence increasing response towards Hg^{2+} as the reporter module. The developed biosensor showed response for Hg^{2+} in a relatively wide range of 1–10,000 nmol/L, and the detection limit was improved one or two orders of magnitude in comparison with most metal-sensing biosensors in similar constructs. In addition, the biosensor could distinguish Hg^{2+} easily from multiple metal ions and displayed strong adaptability to extensive pH conditions (pH 4.0–10.0). More importantly, the developed biosensor was able to provide an initial assessment of Hg^{2+} spiked in the environmental water with the recoveries between 85.70% and 112.50%. The signal-amplified strategy performed by the modified reporter module will be widely applicable to many other whole-cell biosensors, meeting the practical requirements with sufficient sensing performance.

Introduction

Due to the widely existing and significant toxicity of mercury pollution, it is a continuing goal to obtain novel detection techniques that are cost-effective, operational stable, and performance efficient for environmental mercury determination. The whole-cell biosensor based on micro-engineering strategies become more and more attractive due to their relatively simple and inexpensive sustainable production as well as operational stable (Belkin, 2003; Gui et al., 2017; Kim, 2018). However, the sensitivity of most biosensors is still needed improvement to meet the requirement of environmental samples monitoring.

In general, the whole-cell biosensor usually contains a signal sensing module for external signal input and a reporter module that convers the biological response into an output signal (Kim, 2018). Upon activated by the target molecules, the signal sensing modules will induce the expression of the downstream reporter genes that the measurable or visible signals will be output in a dose-dependent manner. This process plays a pivotal role in determining the sensitivity and selectivity of the biosensor. In previously reported studies, the artificial genetic circuits were introduced into the cellular biosensors that harbor sensing and reporter modules to improve both specificity and sensitivity (Shis and Bennett, 2013; Wang et al., 2013; Wu et al., 2009). However, the genetic circuits, including toggle switches, AND gates, and amplification modules via gene network cascades, inevitably increase the complexity of molecular building process of biosensor. Therefore, the simple methodologies are deserved to develop for effectively improving the sensitivity of the whole-cell biosensor.

Herein, the reported green fluorescent protein (GFP) mutant H148C (Jiang et al., 2015), which exhibited fluorescence enhancement in the presence of Hg^{2+}, was used as the reporter module in the developed whole-cell biosensor by fusing with the Hg^{2+}-dependent transcriptional regulator MerR through the bidirectional *mer* promoter (Ansari et al., 1992; Chang et al., 2015; Frantz and O'Halloran, 1990; Ralston and O'Halloran, 1990; Wang et al., 2016) (Fig. 1). Upon Hg^{2+} binding, the MerR dimer in the active state will facilitate a higher expression of GFP mutant. The expressed GFP mutant H148C showed fluorescence increasing response towards Hg^{2+}in a highly sensitive manner, which will further amplify the response signal of biosensor towards Hg^{2+}. Through the signal amplification directly by the reporter module, the sensitivity of biosensor towards Hg^{2+} was increased, which provides

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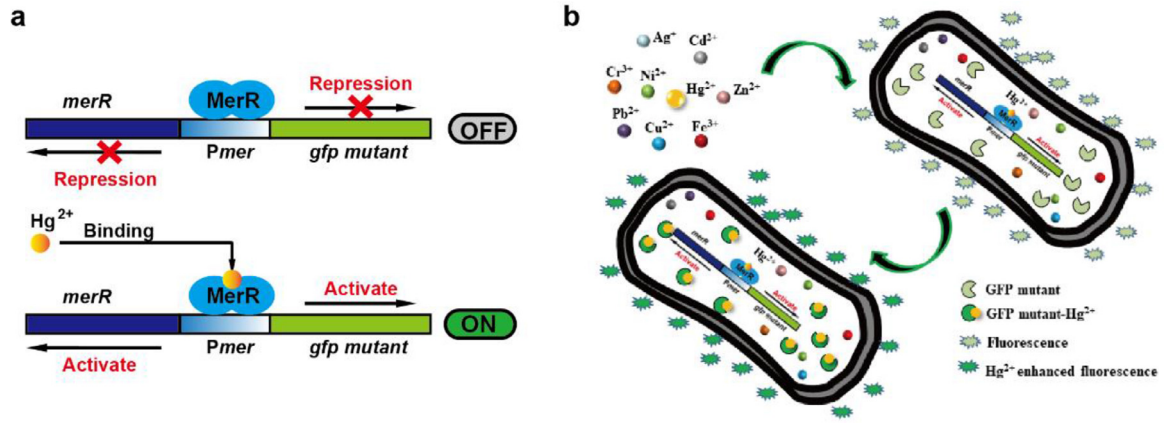

Fig. 1 – Design of the signal-amplified mercury sensing biosensor. (a) Genetic organization of the signal sensing and reporter modules of the biosensor. In the absence of Hg^{2+}, the expression was repressed for the reporter module of GFP mutant. Once Hg^{2+} recognized and bound by MerR dimer, the expression was activated for the reporter module of GFP mutant. (b) Biodetection of Hg^{2+} by the engineered *E. coli* biosensor cells with the transcription factor MerR regulated Hg^{2+}-lighted up GFP mutant as the reporter module.

an effective and convenient manner for improving sensor cell performance.

1. Materials and methods

1.1. Strains, plasmids, media, and reagents

The recombinant DNA manipulation and engineered biosensing system were performed in the *E. coli* DH5α strain [F^-, φ80d*lac*ZΔM15, Δ (*lac*ZYA-*arg*F) U169, *hsd*R17 ($r^-\kappa$, $m^+\kappa$), *rec*A1, *end*A1, *deo*R, *thi*-1, *sup*E44, *gyr*A96, *rel*A1, λ^-]. Plasmid pSB1A2, a pUC19-derived pMB1, is a high copy number plasmid carrying ampicillin resistance, which was used as the vector for biosensor construction. Cells of biosensor were grown in Luria-Bertani (LB) medium (1% (*w*/*V*) tryptone, 0.5% (*w*/*V*) yeast extract, and 1% (*w*/*V*) NaCl) containing 50 μg/mL of ampicillin. All the chemical reagents were purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. The analytical standard solutions of metal ions (Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, and Ag^+) are existed in the form of nitrates in 1 mol/L or 1% nitric acid, and the metal ion Cr^{3+} is existed in the form of chloride in 1 mol/L hydrochloric acid. The high purity deionized water (resistivity 18 MΩ•cm) for all experiments was produced by the Millipore water purification system.

1.2. Construction of the signal-amplified biosensor

The expression vector of biosensor was engineered following the steps shown in Supplementary Fig. S1. Firstly, the DNA fragment including *merR* gene and its bidirectional promoter *mer* (*merR*-P*mer*, GenBank: Z00027.1) was codon optimization and synthesized by Shanghai Sangon Biotechnology Co. Ltd., and it was amplified by PCR reaction with primer I and primer II. The DNA fragment encoding amino acids of GFP mutant H148C was amplified with primer III and primer IV. Then, the aforementioned two fragments were connected by overlap PCR using primer I and primer IV. The overlap PCR product was cloned into the XbaI and SpeI sites of pSB1A2 vector, which yielded the *merR*-P*mer*-*gfp mutant*-pSB1A2 construct. The final construct was confirmed by sequencing and transformed into *E. coli* DH5α cell by chemical method. The primers listed in Supplementary Table S1 were used for the construction of expression vector. The DNA and protein sequences used in the construction were attached in the Supporting Information. The control strain that harboring the plasmid *merR*-P*mer*-*gfp*-pSB1A2 was constructed in the same steps.

1.3. Cultivation of the signal-amplified biosensor

E. coli cells containing the final construct were grown in LB medium with 50 μg/mL of ampicillin. They were cultivated overnight at 37°C with constantly shaking at 250 r/min. The overnight bacteria cells were further cultivated in the LB medium after inoculation at a proportion of 1:100. The bacterial solution was continued to cultivate until an optical density of $OD_{600}\approx1.0$ reached. Then, the solutions of Hg^{2+} and other metal ions were added to the cultural medium for additional time before taking for analysis.

1.4. SDS-PAGE analysis of Hg^{2+}-induced expression of reporter module

The Hg^{2+}-induced expression of fluorescent protein, GFP mutant H148C, of the reporter module was identified by SDS-PAGE according to the following procedures. The cells of the biosensor were cultivated as descripted above. After induced by 1 μmol/L Hg^{2+} for additional time (0, 0.5, 1, 2, 3, and 4 hr), the cells with target protein expressed were harvested by centrifugation. Then, the harvested cells were resuspended in 10 mmol/L PBS buffer (pH 7.4) and mixed with 5 × SDS PAGE loading buffer. After heating at 95°C for 10 min, all the samples were loaded onto SDS-PAGE gel and electrophoresed for 60 min under constant current.

1.5. Fluorometric analysis of Hg^{2+}-induced expression of reporter module

To further identify Hg^{2+}-induced expression of GFP mutant H148C, the cell samples under the stimulation of 1 μmol/L $Hg^{2+}$for 0, 0.5, 1, 2, 3, 4, 5, and 6 hr were also applied for fluorometric analysis. They were harvested by centrifugation and resuspended in 10 mM PBS buffer (pH 7.4) with normalized OD_{600} = 1.0. The fluorescence of all the cell samples was measured by fluorescence spectrophotometer (Shimadzu RF-6000, Japan) with 488 nm for the excitation and 510 nm for the emission. As control, the cell samples in the absence of Hg^{2+}stimulation were cultivated and applied for fluorometric analysis in the same manner.

1.6. Mercury detection with the signal-amplified biosensor

For the evaluation of detection sensitivity towards Hg^{2+}, graded concentrations of Hg^{2+} (0–20,000 nmol/L) were added into the cultural medium. After induced by Hg^{2+} for 4 hr, the cell samples were taken for analysis. For the evaluation of detection selectivity towards Hg^{2+}, some other metal ions, such as Cr^{3+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and Ag^+were respectively added to replace Hg^{2+} into the cultural medium (100 and 1000 nmol/L). Further, the

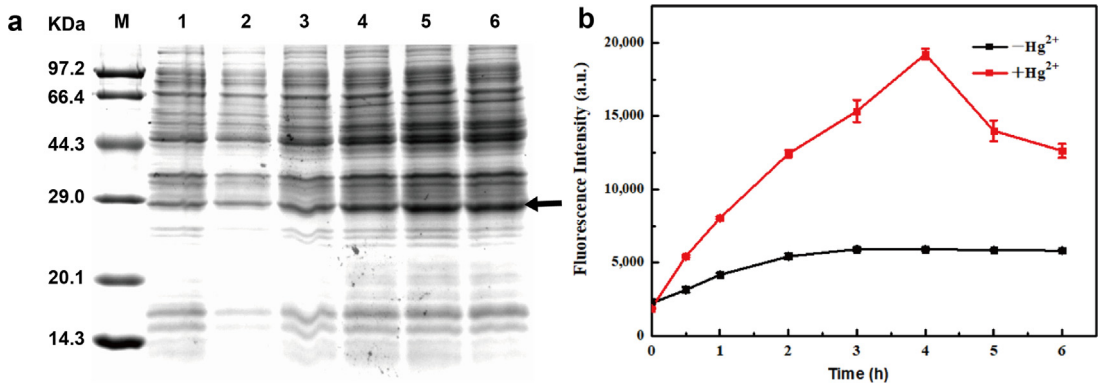

Fig. 2 – SDS-PAGE and fluorometric analyses of Hg^{2+}-induced expression of reporter gene in designed biosensor. (a) The expression identification of GFP mutant after the biosensor cell induced by 1 μmol/L Hg^{2+} on 14% SDS-PAGE (lane 1–6: 0, 0.5, 1, 2, 3, and 4 hr). (b) The fluorometric analysis of GFP mutant after the biosensor cell induced with/without Hg^{2+} for 0, 0.5, 1, 2, 3, 4, 5, and 6 hr.

mixture of Hg^{2+} and other metal ions were separately added to replace Hg^{2+} into the cultural medium, too. Both the concentrations of Hg^{2+} and other metal ions were 100 nmol/L. The cell samples were continued to cultivate for 4 hr before taking for analysis, too.

To evaluate the effect of pH values on the detection of Hg^{2+}, the overnight culture was continued to cultivate following a 1:100 inoculation in the cultural medium with different pH values (pH = 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0). These cultural mediums were prepared by buffers with different pH values. These buffers are 10 mmol/L citrate-sodium citrate (pH = 4.0), 10 mmol/L citrate-sodium citrate (pH = 5.0), 10 mmol/L citrate-sodium citrate (pH = 6.0), 10 mmol/L Tris–HCl (pH = 7.0), 10 mmol/L Tris–HCl (pH = 8.0), 10 mmol/L Na_2CO_3–$NaHCO_3$ (pH = 9.0), and 10 mmol/L Na_2CO_3–$NaHCO_3$ (pH = 10.0). The cells of biosensor were initiated by adding 1000 nmol/L Hg^{2+} into the cultural medium, and they were continued to cultivate for 4 hr before taking for analysis.

In the analysis of environmental water, the tap water and lake water samples were used to replace the high purity deionized water for the preparation of mercury solutions. Tap water was collected from municipal water supply system (Nanning, China). Lake water samples were collected from People's Park (Nanning, China) and Lion Mountain Park (Nanning, China). All the aforementioned cell samples were prepared by quintuplicate. They were harvested and applied for fluorometric analysis in the same procedures as descripted in the Section 1.5.

2. Results and discussion

2.1. Design of the signal-amplified biosensor

In most of the whole-cell biosensors, the signal sensing modules are developed using the metal-responsive transcriptional factors and their cognate regulatory regions (Kim, 2018). The transcription of reporter gene is under the control of transcriptional regulator through its cognate regulatory region. In this research, the Hg^{2+}-dependent transcriptional activator MerR, which exhibits highly sensitive and selective towards Hg^{2+}, was used as the signal sensing module (Ansari et al., 1992; Chang et al., 2015; Frantz and O'Halloran, 1990; Ivask et al., 2002; Ralston and O'Halloran, 1990). It could tightly control the downstream reporter gene through the bidirectional *mer* promoter (Fig. 1a). In the absence of Hg^{2+}, the expression of reporter gene will be repressed by MerR in a closed inactive state. Once Hg^{2+} was recognized and bound to MerR, higher expression of reporter gene will be induced in a dose-dependent manner.

Because the expression level of the reporter gene is designed to be proportional to the sensing signal, the reporter gene is the crucial factor for improving the sensitivity of signal sensing. Herein, the GFP mutant H148C with fluorescence increasing response towards Hg^{2+} was used as the reporter module. Once Hg^{2+} bounding, the fluorescence intensity of the GFP mutant will be further enhanced, which will amplify the response signal of biosensor towards Hg^{2+} (Fig. 1b). Compared to the amplifying genetic circuits used in previously studies, the signal amplification directly performed by the modified reporter module is more convenient in cell construction for improving the sensitivity of whole-cell biosensor (Shis and Bennett, 2013; Wan et al., 2019; Wang et al., 2013; Wu et al., 2009).

2.2. SDS-PAGE and fluorometric analyses of Hg^{2+}-induced expression of reporter module

The identification of Hg^{2+}-induced expression of GFP mutant was performed by SDS-PAGE as well as fluorometric analyses. As shown in Fig. 2a, after adding Hg^{2+}, the GFP mutant band of expression was observed and increased slightly with time near the theoretical value (calculated MW = 26.8 kDa) on the SDS-PAGE gel. It indicated that the reporter module of GFP mutant can be successfully induced by Hg^{2+} through the transcriptional factor MerR in the biosensor cell. With the expression of GFP mutant, the continuously increased fluorescence was observed for the cell samples induced by Hg^{2+} during the first 4 hr. After which, the fluorescence intensity of the cell samples began to reduce. By contrast, the cell samples without Hg^{2+} stimulation displayed weak fluorescence and the intensities of them barely had changes (Fig. 2b). In addition, the control strain (*E. coli* harboring the plasmid lacking the MerR sensor coding gene, P*mer*-GFP H148C) was successfully constructed and applied for fluorometric analysis. Compared with designed biosensor (MerR-P*mer*-GFP H148C), the cell samples of control strain displayed weak fluorescence in the presence of 1umol/L Hg^{2+}, and there did not exist obviously enhanced fluorescence with time went on (Supplementary Fig. S2). The results provided another evidence for the role of Hg^{2+}-binding MerR in inducing the expression of reporter module. With the measurable fluorescence of expressed GFP mutant, the content of Hg^{2+} can be determined accordingly, and the fluorescence intensity was determined for the cell samples induced by metal ions for 4 hr.

2.3. Mercury sensitivity detection with the designed biosensor

As shown in the biosensor response curve (Fig. 3a), a relatively wide sensing window for Hg^{2+} (1–10,000 nmol/L) was provided by

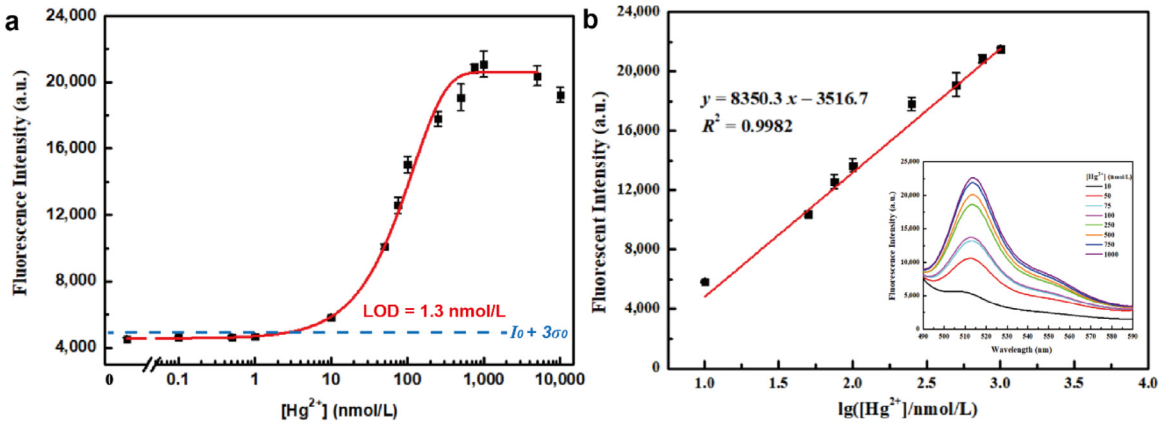

Fig. 3 – Response of the designed biosensor towards different concentrations of Hg^{2+}. (a) The response curve generated by the cells of biosensor induced with graded concentrations of Hg^{2+} in the range of 0.1–10,000 nmol/L. The curve was fitted by an exponential function with a coefficient of determination (R^2) of 0.996. An LOD of 1.3 nmol/L was obtained based on the fluorescence intensity ($I_0 + 3\sigma_0$) of a control sample ($[Hg^{2+}] = 0$) plus 3 times the standard deviation of the control (blue dashed line). (b) The relationship between the fluorescence signal and concentration of Hg^{2+} from 10 to 1000 nmol/L with the linear regression equation $y = 8350.3\ x – 3516.7$ ($R^2 = 0.9982$), where x and y represent the log concentration of Hg^{2+} and the fluorescence intensity at 510 nm. All the data are the means of five independent experiments. Error bars correspond to the standard deviations. Insert: Emission spectra produced by the biosensor cell induced with graded concentrations of Hg^{2+} in the range of 10–1000 nmol/L.

Table 1 – Comparisons of the whole-cell biosensors with GFP as reporter module.

Sensing module	Reporter module	Carrying cassette	Target	LOD (nmol/L)	Reference
MerR	GFP	Chromosome	Hg^{2+}	100	(Priyadarshi et al., 2012)
MerR	GFP	Plasmid	Hg^{2+}	25	(Huang et al., 2015)
MerR	GFP	Plasmid	Hg^{2+}	0.05	(Wan et al., 2019)
MerR	RFP	Plasmid	Hg^{2+}	1	(Cai et al., 2018)
MerR	PYO	Plasmid	Hg^{2+}	10	(Wang et al., 2020)
MerR	RFP	Plasmid	Hg^{2+}	50	(Guo et al., 2020)
MerR	GFP mutant	Plasmid	Hg^{2+}	1.3	This work
ArsR	GFP	Plasmid	As^{3+}	67	(Siddiki et al., 2011)
CadC	GFP	Plasmid	Cd^{2+}	10	(Siddiki et al., 2011)
GolS	GFP	Plasmid	Au^{3+}	33	(Cerminati et al., 2011)
CadR	GFP	Plasmid	Cd^{2+}	10	(Wu et al., 2009)

*LOD: Limit of detection.

the designed biosensor. In the range of 1–1000 nmol/L, the biosensor showed progressively increased response signal towards Hg^{2+}, and the limit of detection was estimated to be as low as 1.3 nmol/L (Fig. 3a). It still displayed strong response to Hg^{2+} with the concentration was in the range of 1000–10,000 nmol/L. However, with the concentration of Hg^{2+} continued increasing, the fluorescence signal of the biosensor was decreased to an extremely low level (data not shown). In the range of 10–1000 nmol/L, a good linear relationship between the fluorescence intensity and the log concentration of Hg^{2+} was also demonstrated after regression analysis with the correlation coefficient of $R^2 = 0.9982$ (Fig. 3b).

Bu using signal amplified function through the reporter module, the detection limit of the engineered biosensor was improved up to ~5-fold in comparison with control biosensor (Supplementary Fig. S3). It was also much lower than most previously reported mercury-sensing biosensors in similar constructs, which also used the regulatory protein gene *merR* as sensing module and the fluorescent protein gene *gfp* as reporter module (Table 1). The limit of the detection was 100 nmol/L with the aforementioned sensing cassette integrated into the chromosome of *E. coli* cell in a single copy number (Priyadarshi et al., 2012). When the sensing cassette was carried by plasmid, the limit of the detection was improved to 25 nmol/L (Huang et al., 2015). The detection limit was greatly improved in the research of Wan et al. through employing the cascaded signal amplifying methodology. However, the multilayered transcriptional amplifiers inevitably increased the complexity of plasmid circuit construction. The designed biosensor also had higher sensitivity than the majority of metal-sensing biosensors employing the fluorescent protein GFP as well as other reporters as the reporter module (Table 1). The safety level for Hg^{2+}in drinking water was 5–30 nmol/L recommended by WHO and other governmental protection agencies worldwide (Cerminati et al., 2015). With the sufficient sensitivity, the present biosensor has great potential in the field application of mercury pollution monitoring.

2.4. Mercury selectivity detection with the designed biosensor

Unlike in response to Hg^{2+}, the developed biosensor displayed weak response signals for other metal ions such as Cr^{3+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and Ag^{+} (Fig. 4a). In addition, the response signal of biosensor towards Hg^{2+} was not greatly interfered by the aforementioned metal ions (Fig. 4b). The good performances in selectivity of biosensor was attributed to the high selectivity of transcriptional factor MerR and reporter module GFP mutant H148C towards Hg^{2+}. Traditionally, when discharged into the environment, the mercury pollution is always companied by other heavy metal ions. The good selectivity made the engineered biosensor be practically feasible for on-site assessment of Hg^{2+} contamination in the real water samples.

2.5. Effect of pH values on mercury sensing of the designed biosensor

As shown in Fig. 4c, the response signals of biosensor towards Hg^{2+} were also not greatly influenced by the varying pH values

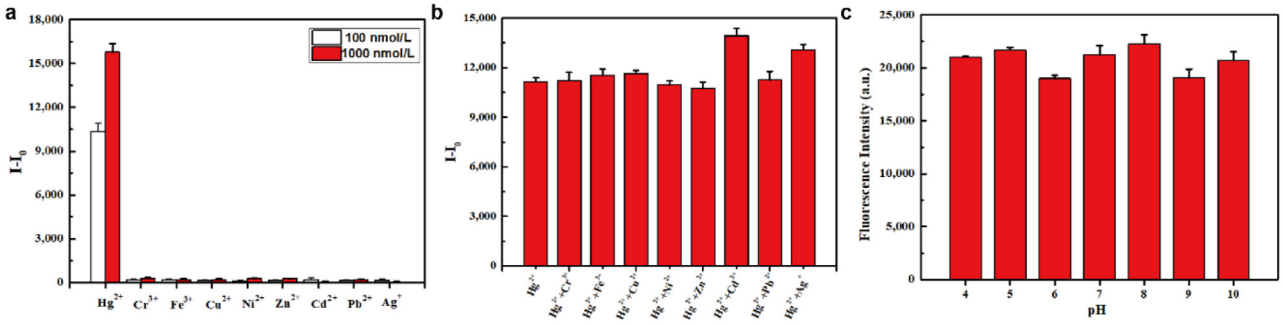

Fig. 4 – Response of the designed biosensor towards different metal ions and pH values. (a) Signals of biosensor towards different metal ions at the concentration of 100 and 1000 nmol/L. (b) Signals of biosensor towards 100 nmol/L Hg^{2+} in the presence of 100 nmol/L Cr^{3+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and Ag^{+}. In (a) and (b), the I-I_0 means the net fluorescence intensity generated by different metal ions. I_0 and I were the fluorescence intensity of the biosensor in the absence and in the presence of different metal ions. (c) Response of biosensor towards 1000 nmol/L Hg^{2+} under different pH values. All the data are the means of five independent experiments. Error bars correspond to the standard deviations.

Table 2 – Evaluation of Hg^{2+} pollution in environmental water by developed biosensor.

Sample	Added (nmol/L)	Found (nmol/L)	Recovery (%)	R.S.D (%)
Tap water	50	52.00±5.78	104.00	11.12
	100	103.94±2.52	103.94	2.43
	500	556.25±15.17	111.25	2.73
	750	732.08±56.86	97.61	7.76
Lake water 1	50	42.85±2.22	85.70	5.19
	100	99.81±7.72	99.81	7.73
	500	491.62±83.22	98.32	16.93
	750	763.92±76.11	101.86	9.96
Lake water 2	50	56.25±4.54	112.50	8.07
	100	110.61±8.84	110.61	7.99
	500	490.24±40.32	98.05	8.22
	750	754.80±38.61	100.64	5.11

*R.S.D: Relative standard deviation.

(pH 4.0–10.0). It still showed obvious and intense detection signals towards Hg^{2+} whether in acidic or alkaline pH environment, which meant the whole-cell based biosensor will be qualified for a wide range of practical application. In this respect, the present whole-cell biosensor had significant advantage over the most detection methods that only work in the specific pH environment (Gu et al., 2011; Syshchyk et al., 2015; Wang et al., 2018). For the most cell-free systems made from biological molecules, such as nucleic acids, peptides, enzymes, antibodies, and proteins, the detection of mercury ion were often compromised with the pH environment in the filed application. For this cellular biosensor, the engineered biosensing module performed its function by employing *E. coli* cells as the platform. Due to internal periplasmic proteins and amino acid catabolism, the *E.coli* cells can grow over a wide range of pHs (Stancik et al., 2002). As a result, the engineered biosensing system in *E. coli* cells could perform detectability well in a relatively wide pH range.

2.6. Application of the designed biosensor in environmental water detection

As is well-known, the water systems in the natural environment include abundant microorganisms and potentially complicated chemicals. The developed biosensor could preliminarily evaluate the concentration of Hg^{2+} spiked in the environmental water (including tap water and lake water) as shown in Table 2. It indicated that the biosensor could resist the complex environment and apply as a powerful tool for on-site mercury detecting in real-world water samples. Although the testing speed of an individual biosensor was not particularly fast, the whole-cell biosensor could be prepared by simple, fast, and inexpensive cultivation processes, and multiple sample assays could be performed in series. On the other hand, the tested samples didn't need complicated and tedious pre-processing. These greatly facilitated the biosensor for high throughput determination and revealed great economic and time advantages over the traditional measurements such as inductively coupled plasma-mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), and atomic fluorescence spectrometry (AFS). However, the accuracy of the developed biosensor needs further improvement to compare favorably with the traditional measurements.

3. Conclusions

In this research, a signal-amplified biosensor for the detection of Hg^{2+} was successfully developed by replacing the conventional reporter gene *gfp* in most whole-cell biosensors by its mutant with fluorescence increasing response towards Hg^{2+}. The signal-amplified biosensor had lower detection limit than the most mercury sensing biosensor as well as other metal ions' biosensors in similar constructs. This is a simple and effective strategy for improving the biosensor's sensitivity, and more biochemical systems can be engineered to meet the environmental sample detection requirements based on above design philosophy.

Declaration of competing interest

All of the authors have read and approved the paper, and they declare no competing financial interests. The work has not been published previously nor is it being considered by any other peer-reviewed journal.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.03.020.

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