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Improvement of a highly sensitive and specific whole-cell biosensor by adding a positive feedback amplifier

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ABSTRACT

In this study, we designed a Cd^{2+} whole-cell biosensor with both positive and negative feedback cascade amplifiers in *Pseudomonas putida* KT2440 (LTCM) based on our previous design with only a negative feedback amplifier (TCM). The results showed that the newly developed biosensor LTCM was greatly improved compared to TCM. Firstly, the linear response range of LTCM was expanded while the maximum linear response range was raised from 0.05 to 0.1 μM. Meanwhile, adding a positive feedback amplifier further increased the fluorescence output signal of LTCM 1.11–2.64 times under the same culture conditions. Moreover, the response time of LTCM for detection of practical samples was reduced from 6 to 4 h. At the same time, LTCM still retained very high sensitivity and specificity, while its lowest detection limit was 0.1 nM Cd^{2+} and the specificity was 23.29 (compared to 0.1 nM and 17.55 in TCM, respectively). In summary, the positive and negative feedback cascade amplifiers effectively improved the performance of the biosensor LTCM, resulting in a greater linear response range, higher output signal intensity, and shorter response time than TCM while retaining comparable sensitivity and specificity, indicating better potential for practical applications.

1. Introduction

Cadmium (Cd) is a toxic transition metal that endangers the growth of animals, plants, and human health [1]. Long-term cadmium exposure can cause various types of cancer, osteoporosis, as well as liver and kidney diseases [2]. The epidemic of Itai - Itai disease in Japan is the most severe public health hazard caused by cadmium pollution [3]. Normally, Cd is present at low levels in the environment, but continuous human activities have significantly increased cadmium accumulation in the environment [4]. Especially the rapid industrialization in China during the past few decades has resulted in the widespread release and accumulation of cadmium, and effective interventions are urgently needed to control the damage caused by cadmium pollution [5]. The World Health Organization (WHO) has set a strict standard for the permissible Cd^{2+} concentration in drinking water of only 0.027 μM, and it is necessary to develop efficient, low-cost, and sensitive methods for real-time Cd^{2+} detection to ensure that these limits are not breached.

Currently, atomic absorption spectrophotometry, atomic fluorescence spectrometry, and inductively coupled plasma mass spectrometry are commonly used to measure the Cd^{2+} concentration [6–8]. These methods have excellent sensitivity and selectivity, but they require complex and time-consuming procedures, which makes them difficult to satisfy the requirements of simple, fast, and real-time monitoring [8,9]. As a simple and rapid detection method, biosensing has attracted increasing attention. Different kinds of biosensors were designed based on enzymes, antibodies, or living cells [10–12]. With the development of synthetic biology, naturally occurring regulatory elements are increasingly being utilized to construct modular gene circuits. The gene circuits of whole-cell biosensors (WCBs) can be divided into recognition elements, amplification elements, and output elements. Many kinds of WCBs were designed de novo using these modular elements [13].

CadR is a well-known transcriptional regulator that can specifically identify Cd^{2+} as part of the cadmium-resistance operon of *Pseudomonas putida* 06909 [14]. In the absence of Cd^{2+}, CadR binds at its specific site

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of the P_{cad} promoter and prevents the transcription of downstream genes. Conversely, CadR binds to Cd^{2+} and alters the promoter so that the transcription of downstream genes can be activated [15]. Many recent studies on cadmium WCBs utilized the P_{cad} promoter and CadR. However, there are still some unsolved disadvantages that limit their practical application to complex samples because they do not meet the high sensitivity and specificity requirements of environmental monitoring [16,17].

In previous studies, our group has accumulated some experience in construction and optimization of WCBs, some of which exhibited the ability to detect other metal ions, such as Pb^{2+} or As^{3+} [18–20]. Based on these studies, we developed multiple possible combinations of these modular elements that provide inspiration for the design of WCB gene circuits. Different combinations of designed circuits and host cells resulted in a variety of basic biosensors. These biosensors were screened, and then the best among them was further optimized [19]. Bereza-Malcolm et al. found that the native transcriptional machinery is closely related to biosensor's performance: a biosensor is more effective when its sensing module is more homologous to the host cell [21–23]. And the same phenomenon existed in our experiments. In preliminary experiments, we used *E. coli* DH5α or *P. putida* KT2440 as host cells carrying the sensing module based on the P_{cad} promoter and *cadR* gene combined with the *mcherry* (red-fluorescent protein gene) or *gfpuv* (green-fluorescent protein gene), which was used as the detection circuit to construct Cd^{2+} biosensors. Among them, the biosensor CM with *P. putida* KT2440 as the host cell was selected for further optimization due to its better performance. Since the specificity of CadR protein was not high enough, the negative feedback amplifier was introduced into the circuit to improve the biosensor's performance by controlling the intracellular CadR protein concentration. At the same time, changes in the relative concentrations of intracellular CadR protein and Cd^{2+} could also affect the sensitivity of the biosensor [24]. Therefore, we obtained the WCB TCM with higher sensitivity and specificity, as well as a lower detection limit than CM. Moreover, its tolerance to Cd^{2+} was also increased, providing a detectable signal in a shorter time [25].

Although the TCM has made great progress, there are still some problems. For example, its response time is long, and its linear response range is narrow, which may affect the accuracy of actual detection. Furthermore, a stronger fluorescence signal is also conducive to the detection of Cd^{2+}. In view of this, different schemes were considered to improve the performance of TCM and make it suitable for practical environmental samples. In our previous study on As^{3+} detection, we found that adding a positive feedback amplifier could enhance the fluorescence intensity and increase the detection range [18]. Therefore, this work introduced a positive feedback amplifier based on LuxR autoregulatory elements to the TCM, so that positive and negative feedback cascade amplifiers were constructed to further optimize the TCM [26]. The comparison of the biosensor with only negative feedback and the one with both positive and negative feedback in this work provides valuable insights for the development of WCBs in the future.

2. Materials and methods

2.1. Bacterial strains, reagents, and growth conditions

The designed WCBs were constructed and characterized in *P. putida* KT2440. The cells were cultured in LB broth (5 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone), and kanamycin (Kan, 50 mg/L) was added to the medium as needed. Solid plates were made using the same medium with the addition of 15 g/L agar. Unless otherwise indicated, all experiments were performed at 30 °C.

PCR reagents, restriction endonucleases, and the Basic Seamless Cloning and Assembly Kit were purchased from TransGen Biotech (China). $CdCl_2$, $Pb(NO_3)_2$, $ZnCl_2$, $CuCl_2$, and $NaAsO_2$ were purchased from Shandong Western Chemical Industry Co. Ltd, China. Anhydrotetracycline hydrochloride (aTc ≥98% purity) was purchased from Aladdin (China). PCR primer synthesis and sequencing were performed by Genewiz (China).

2.2. Construction of the sensor plasmids

The *cadR* coding sequence and the P_{cad} promoter that controls its expression were amplified from the genome of *P. putida* KT2440. The *tetR* coding sequence and the P_{lteto1} promoter were amplified from the genome of *E. coli* DH5α. The plasmid pGN68 served as a template for the amplification of the P_{luxI} promoter and the *luxR* coding sequence. The *mcherry* gene was amplified from the pAM1 plasmid. The terminator (BBa_B0015) sequence was obtained from the iGEM Registry, and was used to terminate the transcription of each separate part of the designed sequence. The detailed sequences of the mentioned genes were listed in the supplementary material. All genes were synthesized and optimized by Genewiz (China). The broad-host-range shuttle plasmid pBBR1MCS-2 was used as a vector to construct the cadmium WCB gene circuits, which contained the positive feedback gene circuit (Fig. S1).

2.3. Growth curves of the WCB strains

In order to compare the WCB containing the positive feedback with the WCB with only the negative feedback, growth curves were recorded at different Cd^{2+} concentrations. The WCB stains were grown overnight in LB medium containing kanamycin at 30 °C and 220 rpm. The OD_{600} of the overnight cultures was adjusted to 1.0 with fresh LB medium, after which 1 mL of the diluted culture was used to inoculate 100 mL of fresh LB medium. Samples were taken every hour, and when the OD_{600} value reached 0.6–0.8, $CdCl_2$ solution was added to the shake flasks at different final concentrations (0, 0.1, 1, 10, 100, 200, 300, 400, 500, 600 μM). Furthermore, the aTc solution was added at the final concentration of 0.4 mg/L. After the addition of Cd^{2+}, samples were taken every 2 h. The OD_{600} was measured by UV spectrophotometry.

2.4. Time-dependent response of the WCBs

The seed culture was prepared as the same as described in 2.3, inoculated into 10 mL of fresh LB medium at a volume ratio of 1%, and then incubated at 30 °C and 220 rpm. When the OD_{600} value reached 0.6–0.8, $CdCl_2$ solution at different final concentrations (0, 0.01, 0.02, 0.04, 0.05, 0.1, 1, 10 μM) was added to the test tubes with the 0.4 mg/L aTc. Every 2 h, 200 μL samples were transferred from test tubes to a 96-well plate to measure the OD_{600} and relative fluorescence value (RFU) using the microplate reader (infinite 200Pro; TECAN, Switzerland), and the experiment was performed for 10 h.

2.5. Dose-dependent response to cadmium

In order to investigate the effect of Cd^{2+} concentration on the fluorescence of the WCBs, different final concentrations of $CdCl_2$ (0, 0.00001, 0.0001, 0.001, 0.01, 0.02, 0.04, 0.05, 0.1, 0.5, 1, 10 μM) and 0.4 mg/L aTc were added to the test tubes when the OD_{600} of the bacterial culture reached 0.6–0.8. The growth conditions and sampling operation were the same as before, OD_{600} and RFU were measured after 8 h of incubation as described above.

2.6. Specificity of the WCBs

Other common heavy metal ions in the environment were used to test the specificity of the WCBs. When the bacteria were at the exponential phase, different kinds of heavy metal solution (Cd^{2+}, Pd^{2+}, Zn^{2+}, Cu^{2+}, As^{3+}) were added to the culture at different final concentrations (0.01, 0.1, 1, 10 μM), along with 0.4 mg/L aTc as above. The culture was incubated under the same conditions for 8 h, after which the OD_{600} and RFU were determined.

2.7. Data analysis

The fluorescence intensity was measured using excitation/emission wavelengths of 580/610 nm, and the optical density was measured at 600 nm. All experiments were also conducted with the unmodified *P. putida* KT2440 as a control strain.

The fluorescence induction rates (FIRs) were calculated using the formula FIR = AFU/BFU. The fluorescence value (AFU) was defined as the RFU divided by the sample absorbance, and the fluorescence value of the control (BFU) was defined as the RFU of the negative control (unmodified *P. putida* KT2440 strain) divided by its absorbance. The BFU was defined as 1.0, and the other data were normalized to the BFU.

The calculations of important indicators of WCBs (including the response time, detection limit, sensitivity, and specificity) were the same as in our previous study [25].

2.8. Analysis of spiked samples

River water samples (collected from Longfeng River, Wuqing District, Tianjin) were filtered and used to prepare the LB medium with 50 mg/L kanamycin. Different final concentrations of cadmium ions (0.01, 0.027, 0.045, 0.050 μM) were then added to bacterial culture in the test tubes and incubated until the OD_{600} of the bacterial culture reached 0.6–0.8. Medium and high concentrations of cadmium (5, 10, 50, 100, and 200 μM) were also added to the bacterial cultures to test the performance of the LTCM. At the same time, a standard curve was generated by culturing biosensors in normal LB medium with different cadmium concentrations (0.01, 0.02, 0.04, and 0.05 μM). These experiments were carried out at room temperature and the samples were cultured for 4 h.

3. Results

3.1. Design and construction of the biosensor

The Cd^{2+} WCB with negative feedback (TCM) was constructed in our previous study, representing a significant improvement over the basic sensor CM [25]. In order to further improve the performance of TCM, the P_{luxI} promoter and the *luxR* coding sequence were integrated into the gene circuit of TCM as a positive feedback amplifier (Fig. 1). The *luxR* sequence was added to the downstream of the P_{cad} promoter. The negative feedback consisting of the P_{lteto1} promoter and *tetR* gene keeps the concentration of CadR at a low level, and in the presence of Cd^{2+}, it binds to the P_{cad} promoter to activate the expression the downstream gene *luxR*. The resulting LuxR can further upregulate P_{luxI}, increasing the expression of *mcherry* and *luxR*. The product can continue to upregulate P_{luxI} to realize the purpose of improving the output intensity of the red fluorescent protein mCherry. The WCB with this positive feedback amplifier was called LTCM, and compared to TCM from our previous study in the following analysis.

3.2. Growth curves of the WCB strains

To study whether adding the positive feedback amplifier could improve the tolerance of the LTCM to cadmium ions, the growth curves of TCM and LTCM were evaluated. The WCBs were cultured as described previously. As shown in Fig. 2, the TCM and LTCM both entered the exponential phase after approximately 2 h. The OD_{600} value of TCM reached 0.6–0.8 after 3.5 h, while LTCM took longer to reach the same stage. These results indicated that the complex gene circuit may have an impact on cell growth, which was proved by the overall growth trend of TCM and LTCM. Then the tolerance of the TCM and LTCM to Cd^{2+} were compared by recording growth curves in the presence of different concentrations of Cd^{2+}. At Cd^{2+} concentrations of 300–600 μM, the growth of both TCM and LTCM was significantly inhibited. At Cd^{2+} concentrations below 200 μM, the impact on the growth of TCM and LTCM was

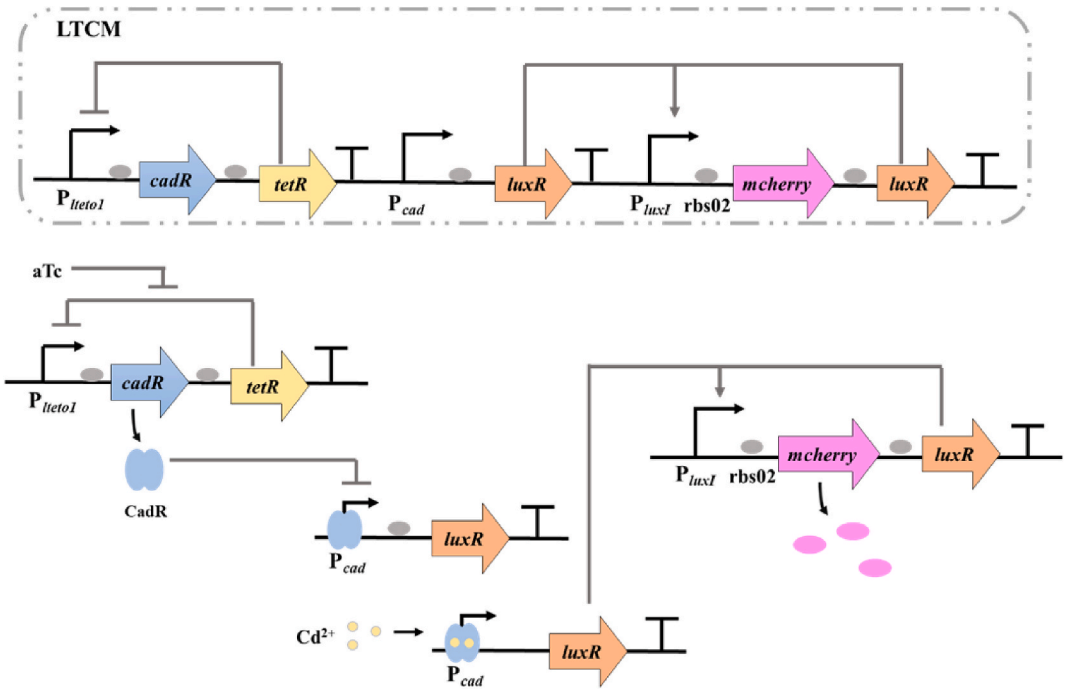

Fig. 1. Schematic diagram of a Cd^{2+} WCB with positive and negative feedback cascade amplifiers. The recognition element of the Cd^{2+} WCB LTCM consists of the promoter P_{lteto1}, the regulator *cadR*, and *cadR*-regulated promoter P_{cad}. The negative feedback loop uses the tetracycline repressor protein TetR to regulate the specific promoter P_{lteto1}. The positive feedback loop consists of the regulator *luxR* and *luxR*-regulated promoter P_{luxI}. The positive feedback loop enhances the transcription of the reporter gene *mcherry*.

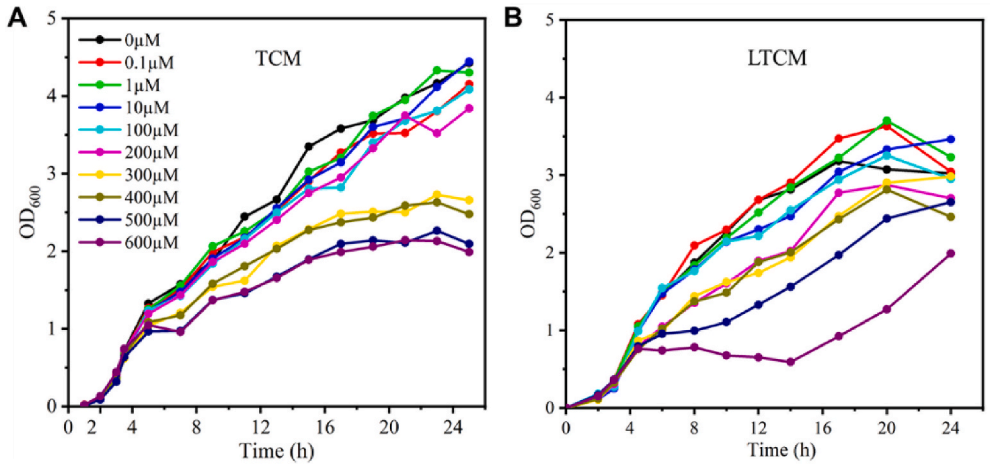

Fig. 2. Growth curves of the two WCB strains at various Cd^{2+} concentrations. (A) With negative feedback, WCB TCM; (B) With positive and negative feedback, WCB LTCM.

not significant. Overall, the time it took for LTCM to enter the stable phase was delayed, which proved that the positive feedback amplifier could improve the strain's tolerance to Cd^{2+}. To allow a direct comparison with the TCM and reduce the effect of Cd^{2+} on the growth of the WCBs, 0–10 μM Cd^{2+} was selected for subsequent experiments.

3.3. Time-dependent response

For practical applications, the response time of WCBs should be kept as short as possible. At the same time, the addition of a positive feedback amplifier may result in the enhancement of background signals, which is also affected by the response time of the WCBs. As can be seen in Fig. 3,

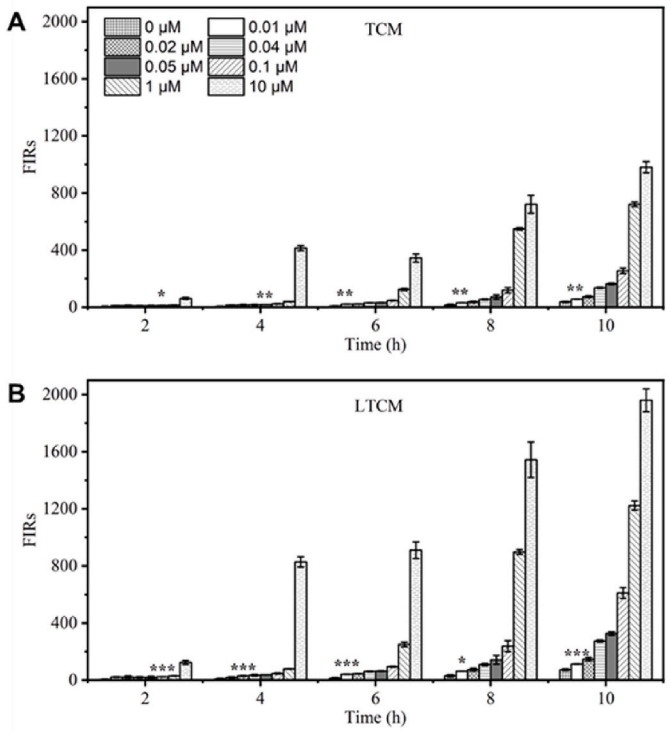

Fig. 3. Time-dependent response of Cd^{2+} biosensors (A) without and (B) with a positive feedback amplifier. The TCM and LTCM biosensor strains were grown in medium containing 0, 0.00001, 0.0001, 0.001, 0.01, 0.02, 0.04, 0.05, 0.1, 1, or 10 μM Cd^{2+}. The error bars indicate the standard deviations of triplicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the fluorescence response of LTCM was much higher than that of TCM at the corresponding time points, while the OD_{600} of LTCM was lower than that of TCM after the same culture time. Although the background signal was enhanced, it did not influence the detection. According to our previous study, the defined response time of TCM was 4 h, but its response time for the detection of practical samples was 6 h [25]. After adding the positive feedback amplifier, the LTCM produced a significant fluorescence response to 0.1 μM Cd^{2+} at 2 h, and it met the WHO requirement after being cultured for 4 h, producing a significant response to 0.02 μM Cd^{2+}, when the LOD of TCM was merely 0.05 μM at 4 h. Its LOD increased 2.5-fold compared to TCM, and there was a 33% reduction in the response time of LTCM, which proved that its Cd^{2+}detection ability was improved. In addition, the fluorescence intensity of the LTCM increased by 1.11 – 2.64-fold during detection. A clear red fluorescence could be observed in the experiment, and the fluorescence intensity was positively correlated with the Cd^{2+} concentration. (Fig. S2). This showed that the aim of adding the positive feedback amplifier was partly achieved.

3.4. Dose-dependent fluorescence response

To study the quantitative relationship between the fluorescence intensity and the Cd^{2+} concentration, the biosensors were incubated in the presence of 0–10 μM Cd^{2+} for 8 h. According to Fig. 4the dose-dependent relationship of the TCM and the LTCM showed a positive correlation. According to the significance analysis, their lowest limit of detection (LOD) at 8 h were both 0.0001 μM Cd^{2+}, which was well below the WHO limit of Cd^{2+} in drinking water. When cultured for 8 h with 10 μM Cd^{2+}, the fluorescence produced by LTCM was 2.14 times higher than that of TCM under the same conditions, and the output signal intensity was greatly enhanced.

Previous analysis showed that the linear response range of TCM was 0.0001–0.05 μM and its sensitivity was 678.07. Similarly, the linear fitting analysis was performed on the data of LTCM. The linear response range of the LTCM was 0.0001–0.1 μM and its sensitivity was 1397.1. The sensitivity of LTCM was approximately 2.06 times higher than that of TCM, and the linear response range was effectively expanded. These data further showed that the performance of LTCM was further improved compared to TCM.

3.5. Specificity of the WCBs

Since environmental samples usually contain many different metal ions in addition to the targeted cadmium ions, the TCM and LTCM were cultured for 8 h with different concentrations of other ions, including

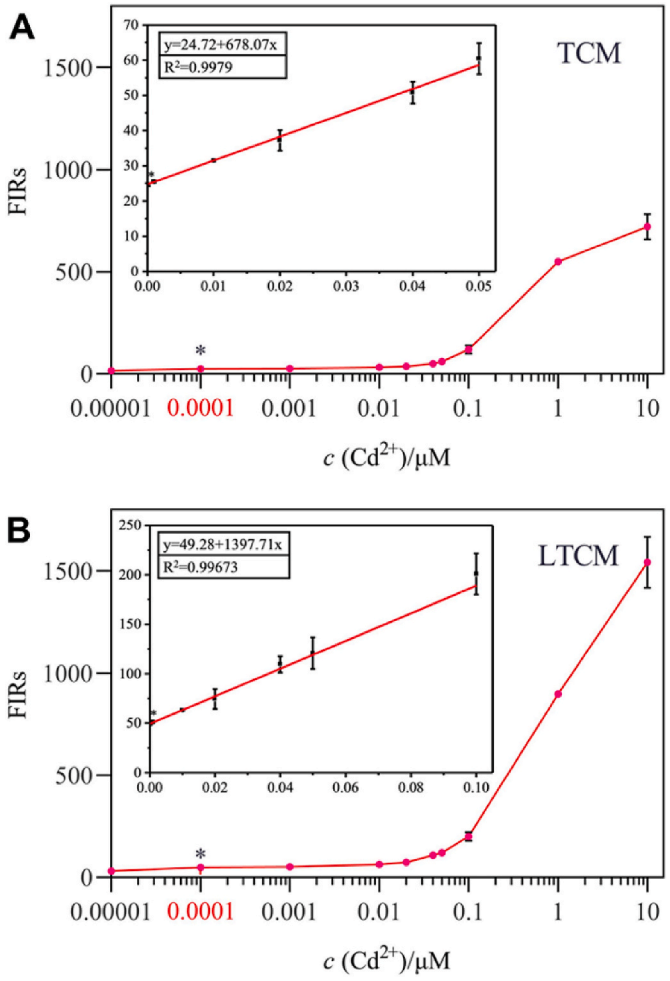

Fig. 4. Dose-dependent fluorescence response and linear fitting curves of the biosensor TCM (A) and biosensor LTCM (B). The error bars represent the standard deviations from triplicate experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

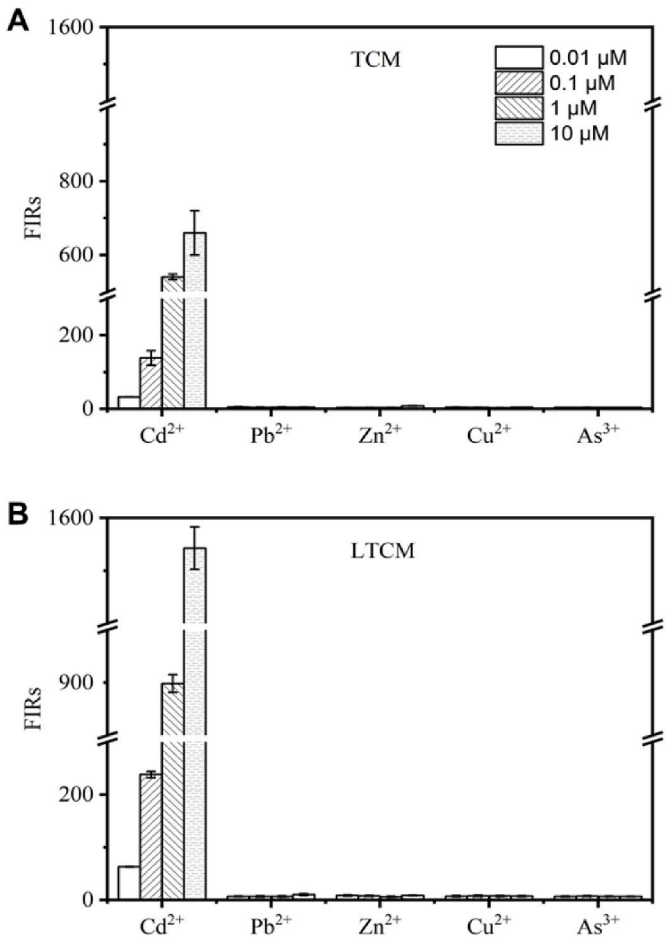

Fig. 5. The Cd^{2+} specificity of the biosensor TCM (A) and the biosensor LTCM with the positive and negative feedback cascade amplifiers (B). The fluorescence intensity of the biosensor was measured by incubating the cells for 8 h in the presence of 0.01, 0.1, 1, or 10 μM Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, or As^{3+}. The error bars represent standard deviations from triplicate experiments.

Pb^{2+}, Zn^{2+}, Cu^{2+}, and As^{3+} to test their specificity to Cd^{2+}. Compared with the TCM, the fluorescence response of the LTCM to Cd^{2+} was significantly enhanced. The fluorescence intensity of LTCM in the presence of 0.1 μM Cd^{2+} was 23.29, 28.14, 33.23, and 35.69 times higher than in the presence of 10 μM Pd^{2+}, Zn^{2+}, Cu^{2+}, and As^{3+}, respectively (Fig. 5). According to the definition, the specificity of LTCM was 23.29, which was a 33% improvement compared to TCM (17.55). Compared to other indicators, the addition of the positive feedback amplifier had little effect on the specificity of the LTCM.

3.6. Analysis of spiked samples

In previous experiments, the prepared Cd^{2+} solution was used as the pollutant to verify the performance of the designed biosensor. To determine whether other substances of the actual samples would affect the performance of LTCM, natural river water was collected for detection. Firstly, the river water sample was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS), which revealed that the Cd^{2+} concentration of the original samples was 0.00054 μM. Then, the original sample was processed. The TCM was cultured for 6 h and the LTCM was cultured for 4 h to determine the Cd^{2+} concentration. The linear equations Y = 18.10713 + 294.92341X (TCM) and Y = 12.6362 + 512.16476X (LTCM, Fig. S3) were used to evaluate the Cd^{2+} concentration of the spiked river water samples. As can be seen in Table 1, the Cd^{2+} recovery of LTCM was between 99.92% and 101.53%, which was consistent with the actual concentration of the sample. Similarly, the Cd^{2+} recovery of TCM was between 98.08% and 101.11%, indicating that it was comparable to LTCM, albeit with lower precision. Because the linear response range of LTCM was 0.0001–0.1 μM, it may not be suitable for samples with high Cd^{2+} concentration. Therefore, the performance of LTCM at higher Cd^{2+} concentrations (5, 10, 50, 100 and 200 μM) was tested, and its recovery rates were 96.37%, 98.53%, 99.37%, 102.13% and 105.23%, respectively (Table S1). This proved that the LTCM could not only quantitively detect Cd^{2+} with high sensitivity and stability at low Cd^{2+} concentrations, but maintain appropriate precision at medium and high concentrations.

4. Discussion

In recent years, extensive studies have been done on using WCBs to detect the toxic heavy metal ions, including Cd^{2+}, Pb^{2+}, Hg^{2+}, and As^{3+} [27–29]. Part of earlier studies mainly focused on whether biosensors could assess the toxicity of heavy metal ions. At the same time, many groups noticed the need for assessment in real environmental samples instead of well-defined laboratory mixtures [30]. However, environmental samples often contain more than one heavy metal ion, making it crucial to design WCBs with high sensitivity and specificity. Different strategies have been used to improve the performance of WCBs, including enhancing the reporter gene, truncating the CadR protein, and adding regulatory circuits [16,17,31].

With the development of synthetic biology, many studies have been

Table 1
Accuracy and reliability of the constructed biosensor TCM and LTCM in the measurement of cadmium in real river water samples.

Samples	WCBs	ICP-MS (μM)	Cd^{2+}added (μM)	FIRs[a]	Estimated (μM)	Recovery (%)
River water	TCM	0.00054	0.01	21.21 ± 0.52	0.01066	101.11%
		0.00054	0.027[b]	26.01 ± 0.55	0.02727	99.02%
		0.00054	0.045[c]	31.24 ± 0.66	0.04467	98.08%
		0.00054	0.05	33.28 ± 0.95	0.05101	100.92%
	LTCM	0.00054	0.027	26.78 ± 2.21	0.02761	100.24%
		0.00054	0.045	36.32 ± 3.13	0.04624	101.53%
		0.00054	0.05	38.49 ± 4.16	0.05048	99.89%
		0.00054	0.1	64.09 ± 3.59	0.10046	99.92%

[a] Mean FIR, value ± standard deviation (n = 3).
[b] Mean 0.027 μM is the legal limit for cadmium ions in drinking water set by the WHO.
[c] Mean 0.045 μM is the legal limit for cadmium ions specified in the national sanitary standard of China for domestic drinking water (GB 5749–2006).

conducted on the components of biosensors, including host cells, plasmids, sensing modules, and reporter modules [32]. Many factors need to be considered in the design of biosensors to minimize mutual effects between the various components. Kim et al. suggested that bioparts or genes with the potential for cross-reactivity should be removed from the genome of host cells in the design step [32]. And Wu et al. used the mutant strain of *P. putida* 06909 with *cadR* gene knocked out as the host cell to design a Cd^{2+} biosensor. The strain without the *cadR* gene lost its cadmium resistance: cell growth was totally inhibited with the addition of 0.1 μM Cd^{2+}. At the meantime, no obvious improvement in the biosensor's performance was observed. The authors also pointed out that the use of high-copy plasmids could largely avoid the genome effect on the detection [33]. In line with these, *P. putida* KT2440 and a high-copy plasmid pBBR1MCS-2 were used in our experiments. Not only were no significant effects observed in the experiments, but the *P. putida* KT2440 played a positive role due to its cadmium resistance.

In the initial optimization of the biosensor CM, we did not consider the use of the positive feedback amplifier, because our preliminary experiments showed that the improvement of the biosensors' specificity was not significant by only adding the positive feedback amplifier [18–20]. Therefore, we introduced the negative feedback amplifier into the circuit and obtained the biosensor TCM [25]. Although the detection limit, sensitivity, and specificity of TCM could meet the requirements, its response time and linear response range could be further improved. Based on this analysis, we considered further optimization of the TCM. Since Ferrell et al. demonstrated that the positive and negative feedback cascades could achieve a wide tunable frequency by calculation and the LuxR positive feedback amplifier played a important role in our previous studies, we introduced the LuxR positive feedback amplifier into the TCM to further enhance its fluorescence response intensity, reduce its response time, and broaden its linear response range [18,19,34]. However, the positive feedback amplifier promotes the overexpression of mCherry, and the complex regulatory circuits may affect the growth of LTCM. Fortunately, it maintained a satisfactory performance in terms of output signal intensity, response time, and response range.

The results showed that the performance of the positive and negative feedback cascade whole-cell biosensor LTCM was further improved. As described in the results section, the tolerance of LTCM to Cd^{2+} was improved. Because of the enhancement of the fluorescence output signal, the detection limit of the LTCM increased 2.5 times at 4 h, reaching a minimum of 0.02 μM. At the same time, the response time of the LTCM was reduced by 2 h, and the fluorescence at high Cd^{2+} concentrations can be clearly distinguished. Moreover, the specificity of LTCM was also improved. The fluorescence intensity of LTCM at 0.1 μM Cd^{2+} was 23.29–35.69 times higher than that of LTCM at 10 μM Pd^{2+}, Zn^{2+}, Cu^{2+}, and As^{3+}. Its specificity is 23.29, compared to only 17.55 in TCM. Finally, LTCM showed good potential for practical applications by reproducibly and precisely determining the Cd^{2+} concentrations of spiked river water samples.

A comparison between our study and other biosensors (Table 2) clearly shows that the lowest detection limit of our design is lower than previous reports, and the specificity of our design is also outstanding. After modification, the linear response range of LTCM was doubled from 0.05 to 0.1 μM. Based on experience, a sensor with a wide linear range is not necessarily with high sensitivity generally. Although the linear response range of LTCM is inferior to some other studies, our WCB exhibited the best performance at low Cd^{2+} concentrations because of its high sensitivity and selectivity, while also ensuring accurate detection

Table 2
Performance of the WCB developed in this study and other microbial heavy metal biosensors.

WCBs	LOD (μM)	Specificity	Detection range (μM)	Reference
P. putida 06909 (P_{tac}-*cadR-ter*-P_{cadR}-*lacI*^{q}-*gfp*)	0.01	–	0.01–0.04[b]	[33]
E. coli TOP10 (P_{cadR}-*cadR*TC10, P_{cadR}-*gfp*)	1.3	–	1.3–72[b]	[17]
E. coli TOP10 (pCadC-G-CadR-R)	eGFP: 0.05 mCherry: 0.1	–	0.05–400[b]	[38]
E. coli TOP10 (pP_{cad}-RFP or pP_{cad}-RFP-GFP or pP_{cad}-RFP-GFP-*lacZα*)	0.1	–	0.1–1.56[b]	[39]
E. coli DH5α (*bla*-OP-*cadC-gfpmut3a-rep-cat)*	0.0445	–	0.089–0.445[c]	[40]
P. aeruginosa PAO1 (pBB*cadRgfp-rfp*)	0.8897	–	26.7–355.9[a]	[21]
E. coli Judel (pSB1k3-CadR-Kana-GFP)	0.0040	1.97[a]	0–0.1335[c]	[41]
E. coli TOP10 (pPcad-vio)	0.049	–	0.024–0.78[d]	[42]
P. putida KT2440 (P_{lteto1}-rbs-*tetR*-rbs-*cadR*-T-P_{cad}-rbs02-*mcherry*-T)	0.00001	17.55[a]	0.00001–0.05[c]	[25]
P. putida KT2440 (P_{lteto1}-rbs-*cadR*-rbs-*tetR*-T-P_{cad}-rbs-*luxR*-T-P_{luxI}-rbs02-*mcherry*-rbs-*luxR*-T)	0.00001	23.29[a]	0.00001–0.1[c]	This study

- Indicates that the specificity of these WCBs was preliminarily assessed, but was not quantitatively analyzed.
[a] Indicates that the the specificity of the biosensor was determined based on the lowest ratio of the its fluorescence response to Cd^{2+}at 0.1 μM to the fluorescence response to other heavy metal ions at 10 μM.
[b] Indicates that the detection range was not fitted, and was calculated by different methods.
[c] Indicates that the linear fitting analysis was performed on the data and the detection range is the linear response range.
[d] Indicates that the data were subjected to regression analysis and the detection range is the range of its quadratic regression equation.

within its linear response range. In addition, the response data of some sensors in Table 2 were not subjected to regression fitting, making it difficult to clearly compare their performance. For example, the detection limit of the indigoidine-based whole-cell biosensor designed by Hui et al. was 0.024 μM, and the quantifiable concentration range was 6.25–200 μM. Its LOD is lower than the WHO standard, but the WHO standard is not within its quantifiable concentration range [35]. By contrast, the LTCM can satisfy both these requirements at the same time. Moreover, its performance in the determination of medium or high Cd^{2+} concentrations has also been confirmed.

In addition, both TCM and LTCM showed promising performance in the analysis of actual environmental samples, but only LTCM was able to reach the standard set by WHO within the defined response time. Nevertheless, because the response time of TCM for the detection of practical samples (6 h) was longer than that of LTCM (4 h), TCM showed a lower detection limit than LTCM, and this phenomenon was in agreement with the time-dependent response. It can be seen from Fig. 3 that the detection limit of LTCM can also reach 0.01 μM if the incubation period is prolonged to 6 h. Therefore, the length of detection time can be selected according to different practical requirements. If the aim is to estimate whether the samples conform to WHO standards, the measurement can be completed within the response time, and if the requirement is to accurately detect Cd^{2+} at low concentrations, we can culture the biosensor for a longer time to reach the lowest detection limit.

In spite of these encouraging results, there is still much room for improvement in our research. Although their sensitivity is affected, immobilization of WCBs can improve the survival of the cells in practical environments and promote long-term cell viability. Previously, we immobilized WCBs with sodium alginate, but this method greatly influenced their performance. Rathnayake et al. designed a biosensor immobilized in silica matrix, which enabled it to maintain a stable fluorescence signal (90%) up to 4 weeks of storage. This sensor's lowest detection limit of Cd^{2+} was 1.42×10^{-4} mg/L (0.001 μM), indicating that various materials have different degrees of impact on biosensor performance [36]. In future studies, further experiments need to be carried out to identify immobilization methods with minimal impact on the performance of biosensors and find suitable materials for long-term preservation in actual environments. Microfluidic technology can also be used to design portable biosensor arrays for the in situ detection of Cd^{2+}. He et al. selected 16 cadmium-sensing transcription factors from the GenBank database and attempted to reduce interfering ions in the cytoplasm of WCBs by overexpressing corresponding exporters. At the same time, they developed a simple quantitative cadmium detection method using their microbial sensor, a smartphone, and low-tech equipment for practical on-site applications [37]. This inspires us that our research can be combined with common devices such as smartphones to achieve low-cost and real-time detection. However, portable devices are affected by different factors in the field, including light intensity, mode of recording, and the quality of the equipment. To be able to use a smartphone instead of a microplate reader to quantify Cd^{2+} in samples, the WCB needs further optimization to increase its visual signal output, which should be a focus of future studies. In addition, we would like to design a biosensor to detect mixed heavy metal ions in future work, which may include more than one gene circuit. In such case, each gene circuit should be as simple as possible to reduce the pressure on cell. We will continue to explore methods of optimization to achieve our goals using as simple circuits as possible.

5. Conclusions

Overall, our findings prove that the positive and negative feedback cascade biosensor LTCM performs better than the negative feedback biosensor TCM in various aspects, including detection limit, sensitivity, and specificity. Although the response times of the two biosensors are the same, LTCM produces a stronger fluorescence signal, demonstrating the ability of positive feedback to improve the output signal and enhance the response range. And our study demonstrates the significant effect of positive and negative feedback cascade amplifiers in biosensor gene circuits. The LTCM may serve as an inspiration for the design of other WCBs and is expected to be applied for practical detection in the future.

CRediT authorship contribution statement

Shuting Hu: Formal analysis, Writing – original draft, Writing – review & editing. **Guangbao Zhang:** Conceptualization, Methodology, Investigation. **Xiaoqiang Jia:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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