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Construction of cadmium whole-cell biosensors and circuit amplification

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Abstract

Owing to the prevalence of cadmium contamination and its serious hazards, it is important to establish an efficient and low-cost monitoring technique for the detection of the heavy metal cadmium. In this study, we first designed 30 cadmium whole-cell biosensors (WCBs) using different combinations of detection elements, reporting elements, and the host. The best performing WCB KT-5-R with *Pseudomonas putida* KT2440 as the host and composed of CadR and mCherry was selected for further analysis and engineering. In order to enhance its sensitivity, a positive feedback amplifier was added or the gene dosage of the reporter gene was increased. The WCB with the T7RNAP amplification module, p2T7RNAPmut-68, had the best performance and improved tolerance to cadmium with a detection limit of 0.01 μM, which is the WHO standard. It also showed excellent specificity toward cadmium when assayed with mixed metal ions. This study demonstrated the power of circuit engineering in WCB design and provided valuable insights for the development of other WCBs.

Key points

- *KT-5-R was selected after prescreening and engineered for better performance.*
- *Using multi-copy reporters and the T7RNAP amplifier greatly improved the performance.*
- *p2T7RNAPmut-68 had a detection limit of 0.01 μM and improved tolerance to cadmium.*

Keywords Cadmium · Circuit amplification · Sensitivity · Specificity · Whole-cell biosensor (WCB)

Introduction

Cadmium is a non-essential element of the human body, generally with a very low content, and under normal environmental conditions will not affect human health. When the environment is contaminated by cadmium, cadmium levels can be increased in organisms and brought into the human body through the food chain, causing chronic poisoning. Cadmium ions are highly toxic and difficult to degrade, making people susceptible to a range of health problems (Hu et al.

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2017). Cadmium ingestion can cause bone softening and osteoporosis; the most famous case is the Japanese "itai-itai disease" (Bhattacharyya 2009). In addition, as an endocrine disruptor, cadmium ions have an impact on the human reproductive system (de Angelis et al. 2017). Cadmium as a mutagen, despite its weak mutagenicity, is still teratogenic, carcinogenic, and genotoxic, and also interferes with major DNA repair pathways (Martelli et al. 2006). The target organs for cadmium carcinogenesis include the pancreas, pituitary gland, liver, adrenal gland, and prostate, and the hematopoietic system (Abbas et al. 2018; Akerstrom et al. 2013).

The World Health Organization (WHO) has set a limit value of 0.027 μM for cadmium in drinking water, but industrial activities release cadmium into water at much higher concentrations than the specified limits, which leads to environmental pollution problems and increased risks to human health (WHO 2011). At present, the internationally recognized methods to determine the cadmium concentration include atomic absorption spectrophotometry, dithizone spectrophotometry, atomic fluorescence spectroscopy, and anodic dissolution voltammetry (Feng and Liu 2010; Hou et al. 2001; Iu et al. 1979; Kumar et al. 2017; Rosolina et al. 2015; Santos et al. 2015; Ure et al. 1978; WHO 1992; Zhou et al. 2019). These methods are complicated and require complex pretreatment and handling of the samples by well-trained professionals and the use of costly equipment and reagents. To overcome these limitations, enzymes, antibodies, and microbial cell biosensors are developed as low-cost alternative methods to detect cadmium in drinking water (Bereza-Malcolm et al. 2015; van der Meer and Belkin 2010).

Whole-cell biosensors (WCBs) typically use microbial cells as the biometric element, which generate measurable outputs, such as fluorescence and luminescence, in response to environmental input signals like the concentration of the analyte to be detected. The response time, detection limit, sensitivity, and specificity are important indicators of sensor performance. In case of cadmium WCBs, the time to produce a significant change in the fluorescence response ($p < 0.05$) at low Cd^{2+} concentrations (≤ 0.05 μM) was defined as the response time of the sensor. The Cd^{2+} concentration that causes a significant change in fluorescence response ($p < 0.05$) was defined as the detection limit of the sensor (Bereza-Malcolm et al. 2017; Hou et al. 2015; Liao et al. 2006). The slope of the linear detection curve of the fluorescence response to the cadmium ion concentration was defined as the sensitivity of the sensor (Guo et al. 2019; Pola-Lopez et al. 2018). The minimum ratio of the fluorescence response of the sensor to Cd^{2+} at a concentration of 0.1 μM to its fluorescence response to other heavy metal ions at a concentration of 10 μM was defined as the specificity of the sensor. WCBs have been extensively studied in order to detect toxic heavy metals in the environment, including cadmium, lead, mercury, and arsenic (Jain and Ali 2000; Jia et al. 2020; Nachman et al. 2005; Nistala et al. 2010; Wongsasuluk et al. 2014). Although many WCBs have been constructed for cadmium detection, most suffer from poor sensitivity or specificity (Bereza-Malcolm et al. 2017; Hou et al. 2015; Hynninen et al. 2010; Liao et al. 2006; Tao et al. 2013; Wu et al. 2009).

Currently, the detection limits, sensitivity, and specificity are the major limiting factors hindering the application of WCBs for measuring cadmium concentration and their performance needs to be improved. Regulating the concentration of intracellular metal-responsive transcription factors has been used to improve the sensitivity of biosensors (Guo et al. 2019; Wang et al. 2015). Hou et al. constructed a series of cadmium WCBs using CadR and promoters of different strengths (Hou et al. 2015). The expression level of CadR was found to be critical and deviation from the optimal level resulted in WCBs with reduced sensitivity. Removing the metal-extruding proteins is another strategy often used to improve the detection sensitivity of heavy metal WCBs but failed to work in the case of Cd/Zn/Pb WCBs (Hynninen and Virta 2010). Hynninen et al. deleted the Cd/Zn/Pb exclusion proteins CadA1, CadA2, CzcA1, and CzcA2 from the *Pseudomonas putida* KT2440 genome and used them as chassis cells to construct the biosensor KT2440.2431 (pDNPczc1lux). Compared to the wild-type KT2440 (pDNPczc1lux), the Pb^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+} detection limits are 45 times, 12 times, 10 times, and 3 times lower, respectively (Hynninen et al. 2010). In 2017, Bereza-Malcolm et al. assembled multiple cadmium biosensors by cloning a single-output cadmium biosensor element cadRgfp and constitutively expressing mrfp1 using a broad-host vector. They were tested in different gram-negative bacteria, including *Pseudomonas aeruginosa* PAO1, *Shewanella* MR-1, NCR3, LCR17, and *Escherichia coli* DH5α. The detection limits of Cd^{2+} ranged from 0.1 to 10 μg mL^{-1}, and did not meet the WHO standard for detecting cadmium ions in drinking water (Bereza-Malcolm et al. 2017). He et al. selected 16 cadmium-sensing transcription factors from the GenBank database and used the expression of green fluorescent protein as the readout. The resulting cadmium biosensor was highly specific and showed a detection limit of 3 nM (He et al. 2021).

Signal amplification has been proved as an effective way to improve the sensitivity and specificity of WCBs. Increasing the copy number of the reporting element or adding the T7RNAP amplification module decreased the detection limit of the sensor (Kim et al. 2016; Tani et al. 2009). Kim et al. increased the sensitivity of the sensor using the CadC-T7 gene circuit (Kim et al. 2016). In this gene circuit, in the presence of Cd^{2+}, CadC is released from cadO upstream of CadC-T7RNAP and *egfp*, and T7RNAP is produced before EGFP is expressed from the T7 promoter. Owing to strict transcriptional control, the sensitivity of the CadC-T7 microbial sensor to Cd^{2+} and Pb^{2+} increased by 1.9-fold and 2.6-fold, respectively, when incubated in test tubes. The sensor pVLCD1 constructed by Liao et al. has GFP under the control of the

promoter P_{cad} of the *Staphylococcus aureus* plasmid pI258 which is regulated by CadC. The sensor has a detection limit of 0.1 nM and a detection limit of 0.1 nM–1 mM for Cd^{2+} (Liao et al. 2006). Hou et al. constructed similar WCBs pcadCluc and pzntRluc with detection limits less than 0.1 and 0.05 μM for Cd^{2+} and Pb^{2+}, respectively. Another WCB they developed parsRluc has a detection limit of 5.0 μM for Cd^{2+} and less than 0.1 μM for As^{3+}, which cannot achieve specific detection of Cd^{2+} (Hou et al. 2015).

In this study, a series of genetic circuits were assembled using detection elements and reporting elements from different sources and introduced into different chassis cells. The four metrics of response time, detection limit, sensitivity, and specificity were used to analyze and determine the optimal combination of the designed gene circuit and host. The performance of the WCBs was further improved using the following strategies: adding a positive feedback amplification module, increasing the copy number of reporting elements, and adding a T7RNAP amplification module, to construct cadmium WCBs with high sensitivity and specificity and provide design ideas for constructing and optimizing other heavy metal WCBs.

Materials and methods

Bacterial strains, reagents, and growth conditions

As shown in Table S1, the construction and characterization of the designed WCBs were performed in *E. coli* DH5α (F^- *φ*80d*lacZ*Δ*M15* Δ[*lacZYA-argF*]*U169 deoR recA1 endA1 hsdR17*[r_k^-, m_k^+] *supE44* λ^- *thi*$^-$*1 gyrA96 relA1*), *E. coli* MG1655 (ATCC 10798), and *P. putida* KT2440 (ATCC 47054). Cells were grown in LB broth (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract). Solid plates were made using the same medium with 1.5% (w/v) agar. *E. coli* strains were grown at 37°C, whereas *P. putida* strains were grown at 30°C. The antibiotics kanamycin (Kan), gentamicin (Gen), and chloramphenicol (CmR) were used at a concentration of 50 μg/mL. PCR reagents, restriction endonucleases, and T4 DNA ligase were purchased from TransGen Biotech. $NaAsO_2$, $Pb(NO_3)_2$, $ZnCl_2$, $CuCl_2$, and $CdCl_2$ were purchased from Shandong Western Chemical Industry Co. Ltd. China. Oligo primer synthesis and sequencing were performed by Genewiz (China).

Fluorescence measurement

Fluorescence was characterized using a fluorescence microplate reader (M2; SpectraMax, USA). In total, 200 μL of each subculture was transferred to a 96-well microplate. The optical density at 600 nm and the fluorescence intensity with an excitation/emission of 580/610 nm were measured. All experiments were performed in triplicates, and the *E. coli* DH5α (DSM6897), *E. coli* MG1655 (ATCC 10798), and *P. putida* KT2440 (ATCC 47054) strains were used as the blank control (Jia et al. 2019).

The fluorescence induction ratios (FIRs) are defined as FIR = AFU/BFU, where the AFU (arbitrary units of fluorescence) is the relative fluorescence unit (RFU) divided by the optical density at 600 nm of the same sample. The background fluorescence unit (BFU) is defined as the RFU of the culture containing no metal divided by its optical density at 600 nm. The FIRs of all other samples were normalized to BFU.

Design and construction of cadmium WCBs

The templates for amplifying the terminator ter and T7RNAP (GenBank: CP053602.1) were the plasmid pGN68 and the *E. coli* BL21 genome. The template for the gene *luxR* (Gene ID: 58212468) was the plasmid pGN68, which was then optimized by the *P. putida* codon. The template for the gene mCherry (GenBank: MN781140.1) was the plasmid pAM1, which was used for the expression in *E. coli*. It was optimized by the *P. putida codon* for the expression in *P. putida* KT2440 (Table S2).

The gene sources of cadmium-specific proteins and their corresponding promoters are shown in Table S1. The design of amplification circuits for cadmium WCBs is shown in Fig. 1. All constructs were confirmed by PCR/gel electrophoresis and Sanger sequencing.

Pre-screening of cadmium WCBs

Fluorescence response measurements of cadmium WCBs at different Cd^{2+} concentrations were performed for WCBs prescreening. Individual colonies of *P. putida* KT2440 containing WCB plasmids were incubated in LB medium containing antibiotics overnight at 30°C and 220 rpm. The cultures were then diluted with LB medium to an OD_{600} of 1 and inoculated at 1% into fresh LB liquid medium without antibiotics. When OD_{600} reached 0.6–0.8, Cd^{2+} solutions at concentrations of 0, 0.01 μM, 0.1 μM, 1 μM, 10 μM, and 100 μM were added to tubes and the bacteria were incubated in a shaker for 12 h. The RFU and absorbance OD_{600} of the samples were determined by a fluorescence microplate reader using 200 μL of bacterial solution in a 96-well plate.

Growth of cadmium WCBs

To understand the toxic effects of cadmium on the engineered strains, the growth curves of the constructed WCBs at different cadmium concentrations were measured. Cd^{2+} solutions with final concentrations of 0, 0.1 μM, 1 μM, 10 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, and 600 μM were added to the subcultures with OD_{600} of 0.6–0.8 and then they

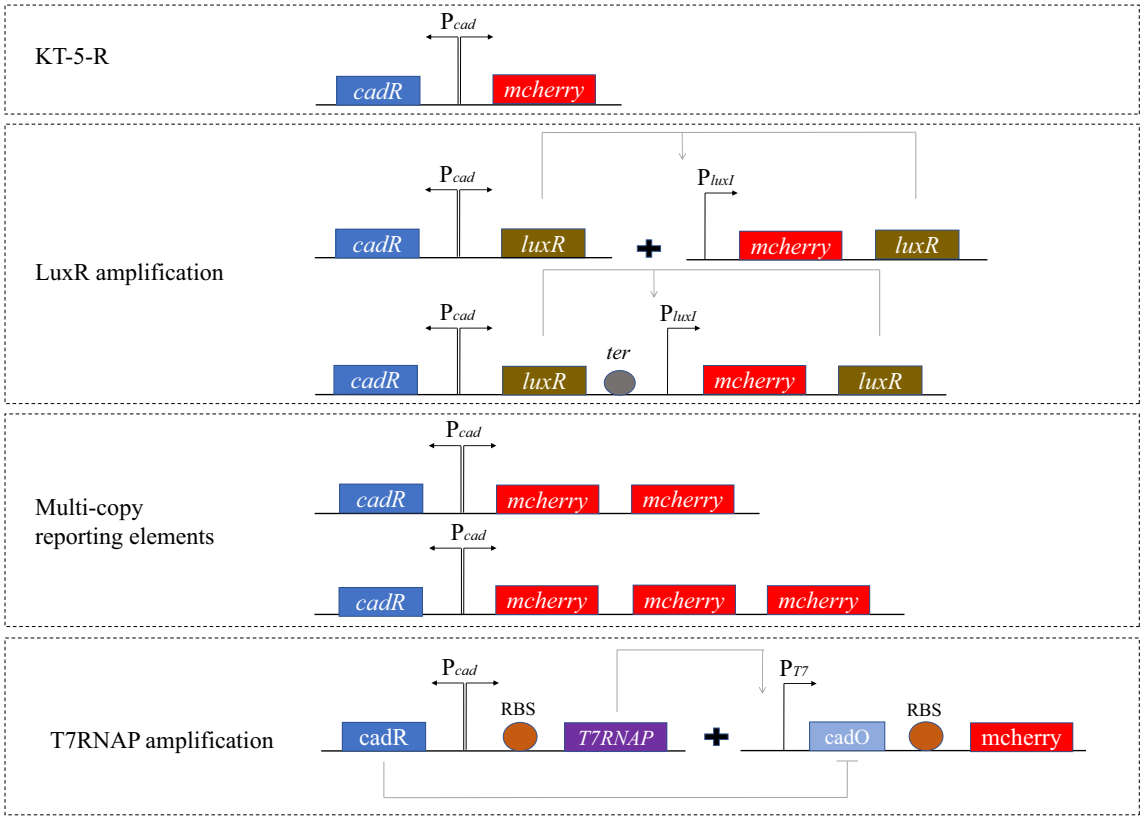

Fig. 1 Design of amplification circuits for cadmium WCBs

were incubated at 30°C and 220 rpm. Samples were taken every 1 h before the addition of Cd^{2+} and every 2 h after the addition of Cd^{2+} to measure the OD_{600}.

Dose-dependent response to cadmium

Analysis of the fluorescence responses of gene circuits at different Cd^{2+} concentrations was carried out. Cd^{2+} solutions at final concentrations of 0, 0.01 μM, 0.02 μM, 0.04 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM, and 100 μM were added to the subcultures with OD_{600} of 0.6–0.8 and then they were incubated at 30°C and 220 rpm. After 8 h, 200 μL of the bacterial liquid was taken into a 96-well plate, and the RFU and OD_{600} of the sample were measured with a fluorescence microplate reader.

Time-dependent response to cadmium

The response time of a WCB is an essential factor for its practical application. In addition, a potential issue using a genetic amplifier in WCBs is that the basal-level expression, either from the sensing module or the amplifying module, may be self-reinforcing and cause a high level of false-positive signals over time. Cd^{2+} at final concentrations of 0, 0.01 μM, 0.02 μM, 0.04 μM, 0.05 μM, 0.1 μM, 1 μM, and 10 μM was added to the subcultures with OD_{600} of 0.6–0.8 and then they were incubated at 30°C and 220 rpm. The RFU and OD_{600} of the samples were determined by a fluorescence microplate reader by taking 200 μL of the bacterial solution in 96-well plates every 2 h for 12 h.

Specificity of the WCBs

In addition to the sensitivity and strength of the output signal, specificity toward cadmium is an important factor to evaluate the WCB. Cd^{2+}, Pd^{2+}, Zn^{2+}, Cu^{2+}, and As^{3+} at final concentrations of 0.01 μM, 0.1 μM, 1 μM, and 10 μM were added to the subcultures with OD_{600} of 0.6–0.8 and then they were incubated at 30°C and 220 rpm with shaking. After 8 h, 200 μL of the bacterial liquid was placed in a 96-well plate, and the RFU and OD_{600} of the sample were measured with a fluorescence microplate reader.

Results

Selection of the cadmium WCB

We initially constructed a series of cadmium WCBs using combinations of the host, the cadmium-detecting transcription factors CadC and CadR, and the reporting gene *mCherry*—due to the excellent photostability and non-toxicity (Shaner

et al. 2004; Shaner et al. 2005). The genetic circuits were further engineered using different configurations among the transcription factor, the promoter, and the reporting gene (Table S1). The constructed WCBs were pre-screened, and the detection limits are shown in Table S3. Only three WCBs expressed in *P. putida* KT2440, that is, P-5-R, KT-5-R, and KT-8-A, had a detection limit of 0.1 μM. Although KT-5-R and KT-8-A had the same gene source and similar gene circuit design, KT-5-R had a better fluorescence response gradient. Therefore, P-5-R and KT-5-R were selected for further performance analysis.

The growth curves of all WCBs in presence of Cd^{2+} at different concentrations were shown in Fig. S1. Three replicated samples were assayed for each data point. Compared with the control without Cd^{2+}, P-5-R and KT-5-R showed a similar growth pattern until the mid-stationary phase with Cd^{2+} lower than 10 μM. However, their growth was inhibited when the concentration of Cd^{2+} was above 100 μM. Therefore, 0–100 μM of Cd^{2+} was used when tested with the fluorescence signal.

P-5-R and KT-5-R had the best fluorescence responses in the presence of 50 μM Cd^{2+} (Fig. 2a, b). Linear fitting was performed on the cadmium concentration–fluorescence response curve, and the linear response ranges of P-5-R and KT-5-R were 0.5–5 μM and 0.04–0.5 μM, with sensitivities of 5.3 μM^{-1} and 309.7 μM^{-1}, respectively. The fluorescence responses of P-5-R and KT-5-R gradually increased with time (Fig. 2c). The detection limit of P-5-R decreased to 1 μM after 8 h of incubation and 0.04 μM after 12 h, so its response time was 12 h. The detection limit of KT-5-R decreased to 1 μM after 4 h of incubation, 0.1 μM after 6 h, and 0.04 μM after 8 h, so its response time was 8 h. When Cd^{2+} was added at concentrations of 0.1 μM, 1 μM, and 10 μM, the fluorescence response of KT-5-R was significantly higher than the response to the other heavy metal ions (Fig. 2d). The specificities of P-5-R and KT-5-R were 0.7 and 5.9, respectively.

Overall, KT-5-R performed better than P-5-R (Fig. 2) and was thus selected for subsequent circuit amplification.

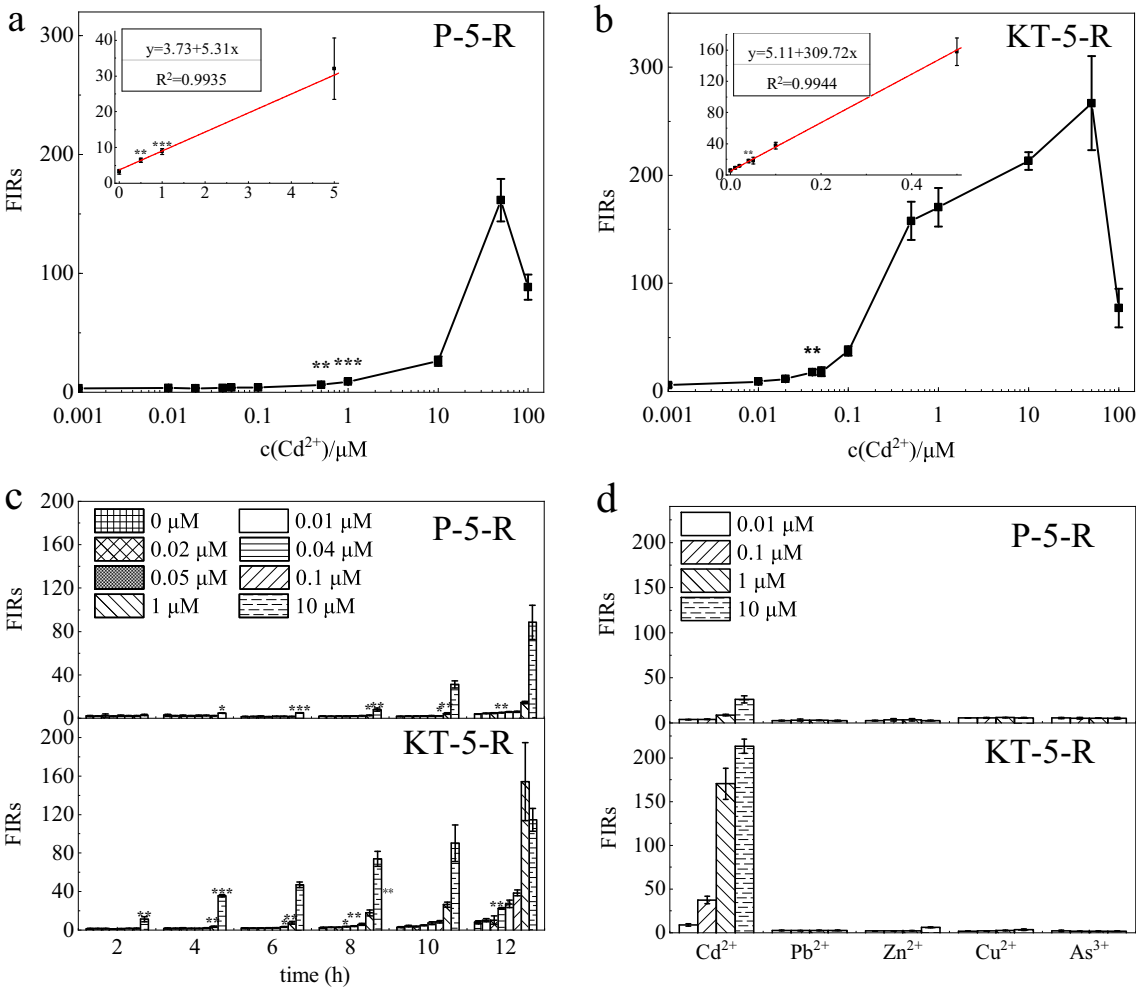

Fig. 2 **a** Time-dependent fluorescence responses of sensor P-5-R, **b** dose-dependent fluorescence responses of sensor KT-5-R, **c** time-dependent fluorescence responses of sensors P-5-R and KT-5-R, and **d** specificities of sensors P-5-R and KT-5-R (statistical significance was expressed as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Each experiment was repeated three times and standard deviations were shown as error bars)

Pre-screening of cadmium WCBs with signal amplification

It is widely known that a positive feedback loop or increasing gene dosage of the reporter could amplify the signal of a WCB. We used two different feedback circuits in this work. One was based on the transcription activator LuxR and the other was based on the T7RNAP amplification module. In addition, we also doubled or tripled the gene copy number of the reporter mCherry to enhance the output signal. As shown in Fig. 1, in the second design, the ligand-independent mutant of the positive regulator LuxR and its cognate promoter were introduced between the input Cd^{2+} and the output *mCherry* to add a positive feedback loop for the amplification of the output signal. Another strategy we used was to increase the copy number of the reporter gene *mCherry*, resulting in KT-5-R/Double and KT-5-R/Triple. Lastly, T7RNAP was introduced and put under the control of Cd^{2+} responsive promoter, while the reporter gene *mCherry* was placed after a T7 promoter with the cadO operator, making it under the control of both T7RNAP and CadR.

The detection limit, sensitivity, and specificity of these WCBs were assessed using the same methods mentioned above. As shown in Table S4, the detection limit of the WCB with the positive feedback module was 1 μM, a 25-fold increase over KT-5-R. The detection limits of the WCBs with the LuxR positive feedback module, multi-copy reporting elements, and T7RNAP amplification module were 1 μM, 0.02 μM, and 0.01 μM, respectively.

The circuits with increased copy number of the reporting component and containing the T7RNAP amplifier module demonstrated noticeable improvement and were further evaluated.

Growth of cadmium WCBs with signal amplification

To understand the toxic effects of cadmium on the engineered strains, the growth curves of the three selected WCBs constructed at different cadmium concentrations were measured. The growth trend of KT-5-R/Double was similar at different Cd^{2+} concentration gradients (Fig. S2). KT-5-R/Triple had little effect on cell growth in the presence of 0–200 μM Cd^{2+}. When the Cd^{2+} concentration was less than 400 μM, the growth status of p2T7RNAPmut-68 was almost identical to that without the addition of Cd^{2+}, indicating that the addition of 0–400 μM of Cd^{2+} had little effect on the cell growth. Compared with KT-5-R, p2T7RNAPmut-68 showed an increased tolerance to Cd^{2+} and a prolonged time to steady state. It is possible that the double plasmid system increased the metabolic load of the cells, resulting in the slow growth of the WCB.

When the Cd^{2+} concentration was more significant than 200 μM, the inhibition in growth for each WCB was more pronounced. The higher the Cd^{2+} concentration, the more toxic the effect on the cells. KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 all took longer to enter the stationary phase than KT-5-R under the same condition. It is possible that the increase in the metabolic load of the host cell results in an increase in the time for the cell to reach steady state.

Dose-dependent response to cadmium

We compared the sensitivity of the three selected WCBs using dose-dependent response experiments: KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 (Fig. 3). Their sensitivities were 328.1 μM^{-1}, 85.86 μM^{-1}, and 388.7 μM^{-1}, respectively. KT-5-R was 3.6 times more sensitive than KT-5-R/Triple, whereas KT-5-R/Double and p2T7RNAPmut were 1.1 times and 1.3 times more sensitive than KT-5-R. We found that including a double copy of the reporting element did not change the WCB sensitivity significantly, including a triple copy of the reporting element reduced the WCB sensitivity, and adding a T7RNAP amplification module increased the sensitivity of the WCB.

Time-dependent response to cadmium

The detection limits of the three selected WCBs were evaluated using time-dependent response experiments. The fluorescence responses of KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 gradually increased with time (Fig. 4). After 2 h of incubation with Cd^{2+}, the detection limit of p2T7RNAPmut-68 decreased from 1 to 0.05 μM; after 4 h of incubation with Cd^{2+}, the detection limit of KT-5-R/Double decreased from 10 to 0.02 μM; and after 6 h of incubation with Cd^{2+}, the detection limit of the KT-5-R/Triple reached 0.04 μM and the detection limit of p2T7RNAPmut-68 decreased to 0.01 μM (lower than the WHO detection limit of 0.027 μM for cadmium in drinking water). After 8 h of incubation with Cd^{2+}, the detection limit of KT-5-R/Triple reached 0.02 μM. In the presence of low concentrations of Cd^{2+} (≤ 0.05 μM), the time to produce a significant difference in the fluorescence responses of KT-5-R/Double and p2T7RNAPmut-68 was shortened by 4 h compared to KT-5-R, and that of KT-5-R/Triple was shortened by 2 h.

Specificity of cadmium WCBs

Although most WCBs require a high degree of specificity to ensure reliable and accurate results, WCBs using promiscuous transcription factors allow the detection of a greater number of heavy metal ions. The responses of these WCBs constructed in this study to different ions were compared to evaluate their specificity toward Cd^{2+}. The fluorescence responses of KT-5-R, KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 to different heavy metal are shown in Table S5. The

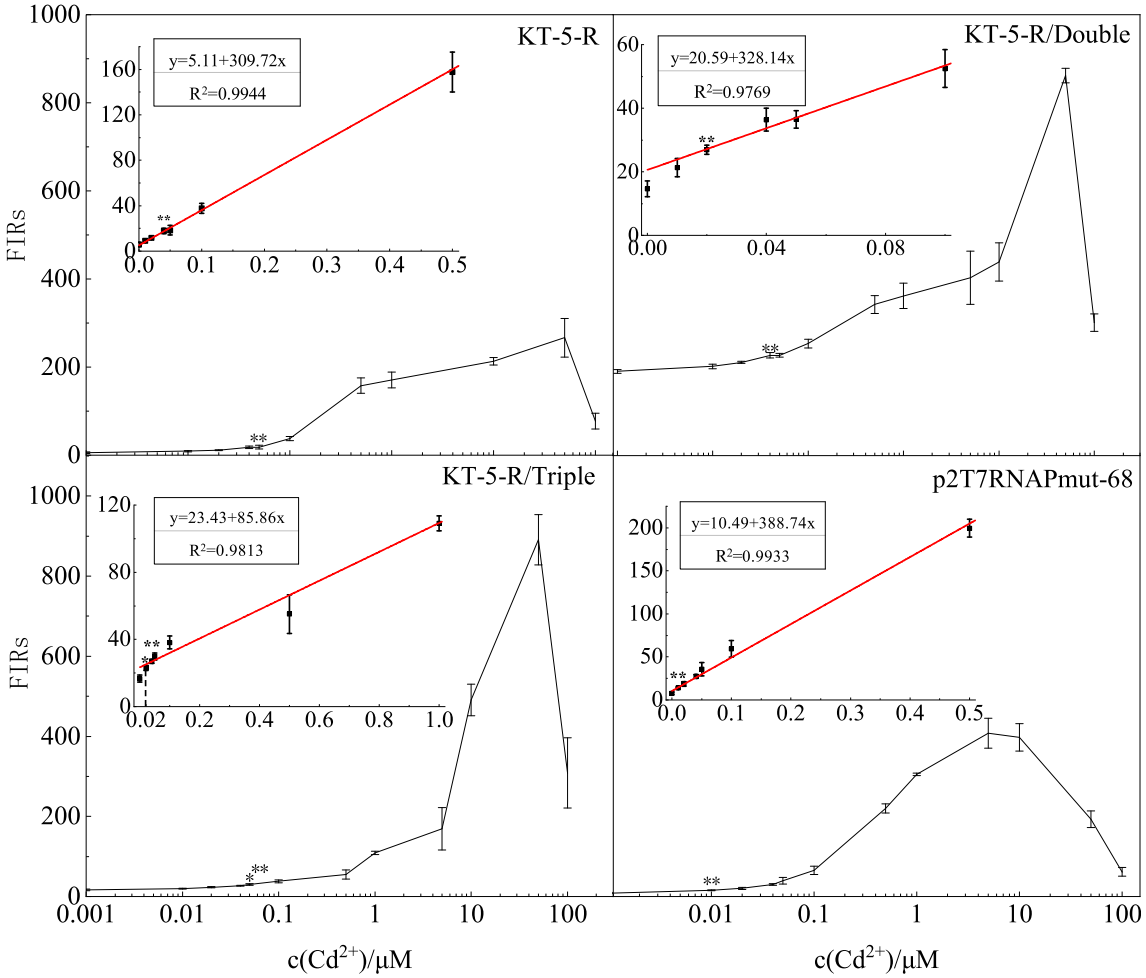

Fig. 3 Dose-dependent fluorescence response of KT-5-R, KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 (statistical significance was expressed as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Each experiment was repeated three times and standard deviations were shown as error bars)

specificities of the KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 were 2.8, 6.1, and 7.6, respectively (Fig. 5). KT-5-R was 2.1 times more sensitive than KT-5-R/Double, the specificity of KT-5-R/Triple did not increase significantly over KT-5-R, and p2T7RNAPmut was 1.3 times more sensitive than KT-5-R.

Discussion

WCBs have been extensively studied in order to detect toxic heavy metals in the environment, including cadmium, lead, mercury, and arsenic (Jain and Ali 2000; Jia et al. 2020; Nachman et al. 2005; Wongsasuluk et al. 2014). Although many WCBs have been constructed for cadmium detection, most suffer from poor sensitivity or specificity (Bereza-Malcolm et al. 2017; Hou et al. 2015; Hynninen et al. 2010; Liao et al. 2006; Tao et al. 2013; Wu et al. 2009). In this study, we developed cadmium WCBs with superior performance in detection limit, sensitivity, and specificity by systematical design/screening of WCBs and circuit engineering.

Initially, 30 cadmium WCBs were constructed using different combinations of the host, the cadmium-detecting transcription factors CadC and CadR, and the reporting gene *mCherry* (Table S1). Two WCBs, P-5-R and KT-5-R, were selected for further characterization and engineering after initial screening of detection limits (Table S3). The detection element CadR in P-5-R and KT-5-R was derived from the genome of *P. putida* 06909 and *P. putida* KT2440, respectively. The amino acid sequences of the CadR proteins from *P. putida* KT2440 and *P. putida* 06909 were 97% identical with only five mismatches: N24Q, E33D, A76S, S117A, and D135E, none of which was at the functional sites (Liu et al. 2019). Since *P. putida* KT2440 was used as the host, it is expected that KT-5-R may perform better in its native host compared with P-5-R heterologously expressed in *P. putida* KT2440. Our results are consistent with this speculation.

Circuit engineering was utilized to further improve the performance of KT-5-R by introducing a positive feedback module, increasing the copy number of the reporter, or introducing the T7RNAP amplification module (Fig. 1). The detection limits of the WCBs with the positive feedback module,

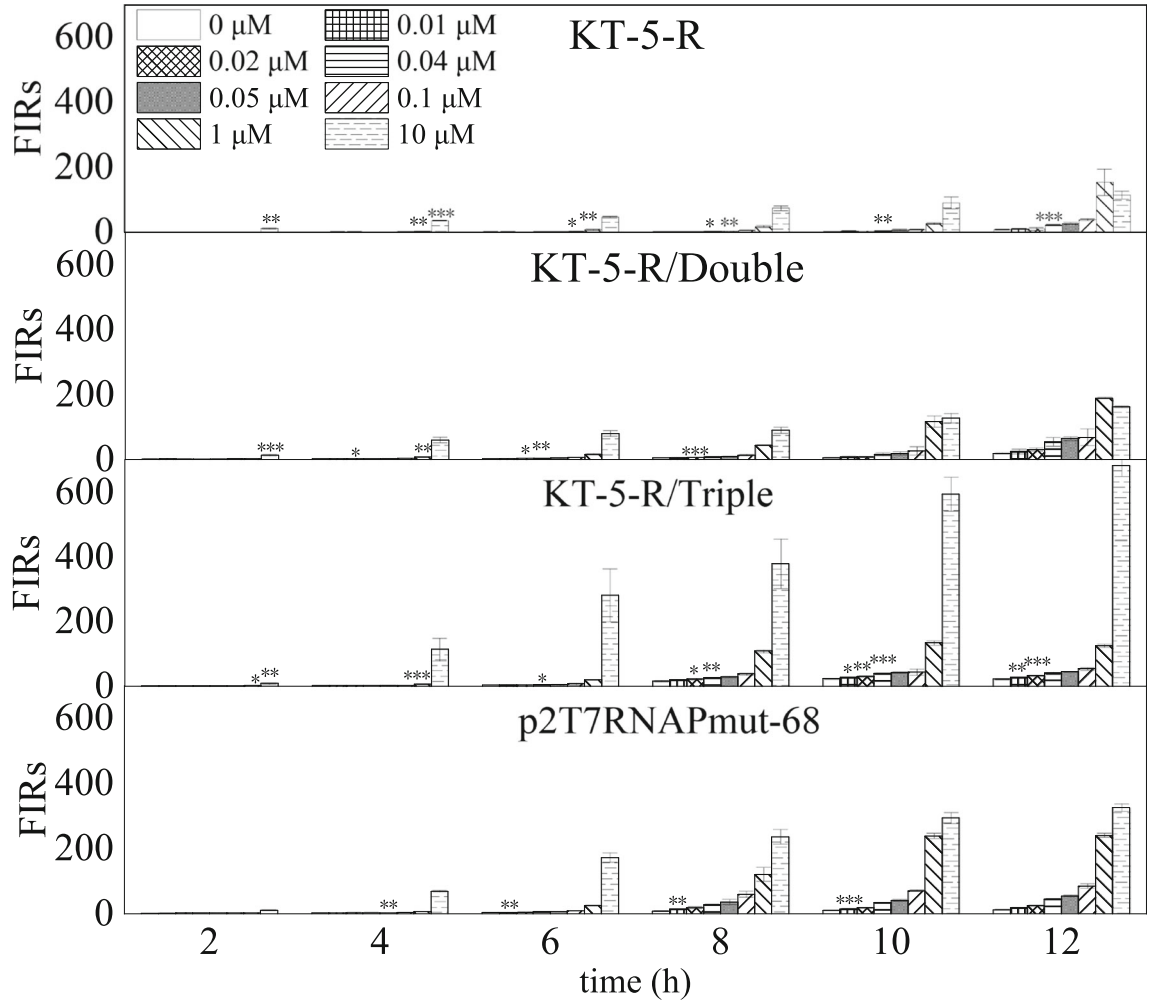

Fig. 4 Time-dependent fluorescence response of KT-5-R, KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 (statistical significance was expressed as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Each experiment was repeated three times and standard deviations were shown as error bars)

multi-copy reporting elements, and T7RNAP amplification module were 1 μM, 0.02 μM, and 0.01 μM, respectively (Fig. 3). Nistala et al. constructed a positive feedback-based amplifier to demonstrate the modular functionality of signal amplifiers (Nistala et al. 2010). Jia et al. coupled an arsenic WCB to a positive feedback circuit modulation module to construct a WCB that reduced the detection limit of As^{3+} and increased the sensitivity by a factor of 20 (Jia et al. 2019). However, the LuxR positive feedback amplification system did not show an enhanced signal in the KT-5-R-based sensor amplification circuit. It is possible that when the Cd^{2+} concentration is lower, the detection circuit expresses a lower concentration of the LuxR variant protein, which is not sufficient to activate the *mCherry* gene downstream of the P_{luxI} promoter transcription in the reporter circuit, and the red fluorescent protein mCherry is not expressed, so adding a positive feedback module instead raises the detection limit for Cd^{2+}. Similar to previous studies, increasing the copy number of the reporting element or adding the T7RNAP amplification module decreased the detection limit of the sensor (Kim et al. 2016; Tani et al. 2009).

Three engineered WCBs were selected for further comparison, KT-5-R/Double, KT-5-R/Trible, and p2T7RNAPmut-68. The sensitivities of KT-5-R/Double, KT-5-R/Trible, and p2T7RNAPmut-68 were 328.1 μM^{-1}, 85.86 μM^{-1}, and 388.7 μM^{-1}, respectively (Fig. 3). KT-5-R was 3.6 times more sensitive than KT-5-R/Triple, whereas KT-5-R/Double and p2T7RNAPmut were 1.1 times and 1.3 times more sensitive than KT-5-R. Including a double copy of the reporting element did not change the WCB sensitivity, including a triple copy of the reporting element reduced the WCB sensitivity, and adding a T7RNAP amplification module increased the sensitivity of the WCB. The response times of the KT-5-R/Double, KT-5-R/Trible, and p2T7RNAPmut-68 were 4 h, 6 h, and 4 h, respectively (Fig. 4). Compared to KT-5-R, all three WCBs significantly reduced the detection time for cadmium.

Although most WCBs require a high degree of target specificity to ensure reliable and accurate results, the core

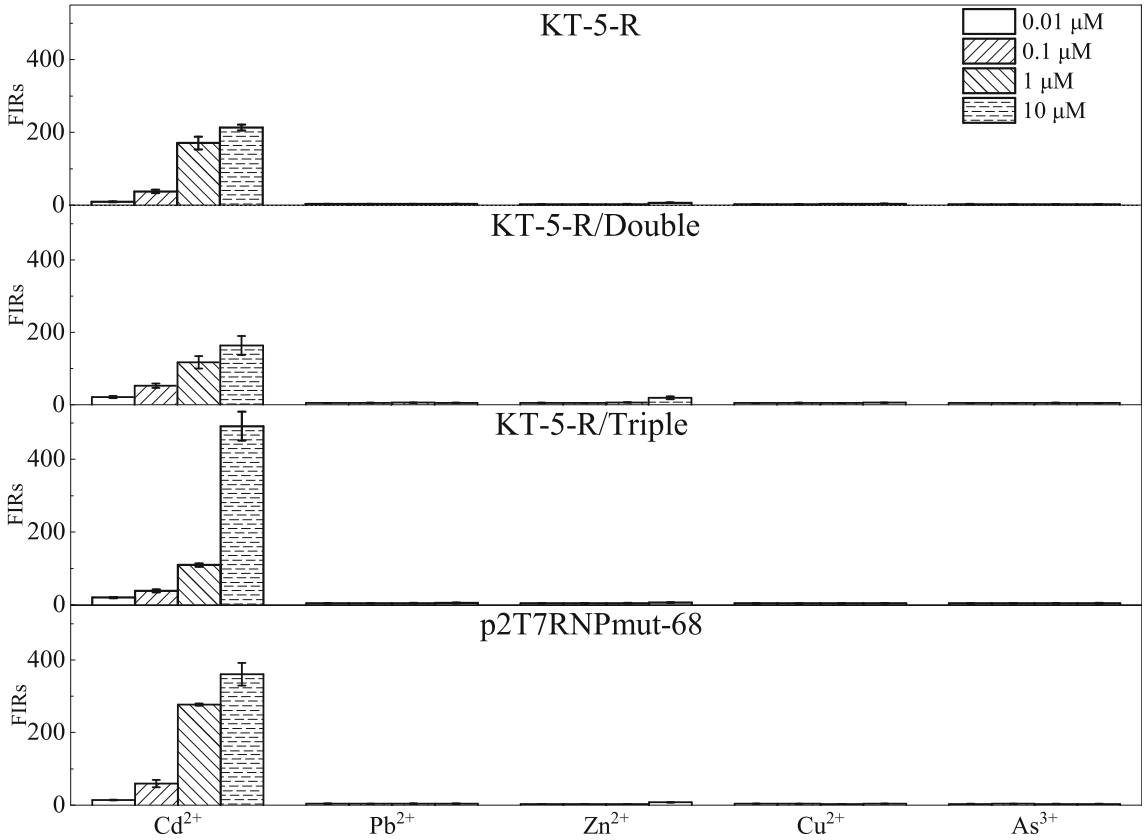

Fig. 5 Specificity of KT-5-R, KT-5-R/Double, KT-5-R/Triple, and p2T7RNAPmut-68 (each experiment was repeated three times and standard deviations were shown as error bars)

component of the heavy metal WCBs, the natural metal-responsive transcription factor, is promiscuous, thus allowing the cell to respond to a greater number of heavy metal ions. The specificities of the KT-5-R/Double, KT-5-R/Trible, and p2T7RNAPmut-68 were 2.8, 6.1, and 7.6, respectively (Fig. 5). KT-5-R was 2.1 times more sensitive than KT-5-R/Double, the specificity of KT-5-R/Triple did not increase significantly over KT-5-R, and p2T7RNAPmut was 1.3 times more sensitive than KT-5-R. The presence of 10 μM Zn^{2+} produced a certain fluorescence response because the CadR protein has two different types of functional sites: metal-binding site I (S1 and S1′) and metal-binding site II (S2 and S2′) (Liu et al. 2019). Metal-binding site II is located in the middle of the molecule and is formed by two histidine residues (His87 and His90 in the α5 helix), one glutamate residue (Glu62 in the α4 helix), variable ligands from the His tail region, three histidine residues, and one glutamate residue arranged in a tetrahedral structure that binds to Cd^{2+} and Zn^{2+} but not to Pb^{2+}.

We found that the WCB with the T7RNAP showed no fluorescence during initial characterization. This is likely due to disrupted gene expression from the hybrid promoter composed of P_{T7} promoter and the cadO operator which is adjacent to the RNAP binding site. In the presence of Cd^{2+}, the cadO operator downstream of the P_{T7} promoter forms a complex structure with the CadR protein and Cd^{2+}, allowing the initiation of transcription from the P_{T7} promoter. The complex cadO/CadR/Cd^{2+} may form a spatial barrier which prevents the binding of T7RNAP to the P_{T7} promoter. T7RNAP has 883 amino acids and consists of an N-terminal domain (residues 1–325) and a polymerase domain (residues 326–883), and the two subdomains form a gap for DNA template binding (Fig S3, Fig S4). In particular, residues 93–101 in the N-terminal domain are active sites (green) that interact with the DNA template. A series of truncated T7RNAP were made to replace the wild-type T7PNAP. One of them, with the first 39 amino acids deleted, functioned together with cadO in the circuit we designed. It is speculated that this T7RNAP has a smaller size which allows it to access the P_{T7} promoter in the presence of the complex cadO/CadR/Cd^{2+}. Therefore, it was used in this work instead of the wild-type T7RNAP.

In conclusion, we have successfully developed WCBs for detecting Cd^{2+} with improved sensitivity and specificity compared with previous WCBs. The best performing WCB is the p2T7RNAPmut-68 in the host *P. putida* KT2440, which is built on the transcription factor CadR and its cognate promoter, a mutant of the T7RNAP, the hybrid T7-cadO promoter, and the fluorescent reporter mCherry. Compared with previous studies, it showed lower detection limits (Bereza-Malcolm et al. 2017; Hou et al. 2015; Hynninen et al. 2010) and higher

sensitivity and specificity without a significant increase in background noise (Hou et al. 2015; Liao et al. 2006; Tao et al. 2013). Although the field application of the designed WCBs to detect cadmium in real water samples is limited by the culture of WCBs in the samples, particularly wastewater with other toxic compounds which may inhibit bacterial growth, the strategy we developed to systematically design, screen, and engineer WCBs to improve the performance provides valuable insights to the development of in situ detection of cadmium and other heavy metal ions as some of our strategy can be adapted to the optimization of cell free systems as sensors which are independent of cell culture and allow the assay of real water samples.

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Code availability Not applicable

Author contribution J XQ and W K conceived and designed study. M YB collected data. J XQ, L T, M YB, and W K analyzed data. L T wrote the manuscript and created the figures.

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Data availability Supplementary Information is available.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate The authors agree to participate.

Consent for publication The authors agree to submit the article.

Conflict of interest The authors declare no competing interests.

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