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ENVIRONMENTAL BIOTECHNOLOGY

Indigoidine biosynthesis triggered by the heavy metal-responsive transcription regulator: a visual whole-cell biosensor

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Abstract

During the last few decades, whole-cell biosensors have attracted increasing attention for their enormous potential in monitoring bioavailable heavy metal contaminations in the ecosystem. Visual and measurable output signals by employing natural pigments have been demonstrated to offer another potential choice to indicate the existence of bioavailable heavy metals in recent years. The biosynthesis of the blue pigment indigoidine has been achieved in *E. coli* following heterologous expression of both BpsA (a single-module non-ribosomal peptide synthetase) and PcpS (a PPTase to activate *apo*-BpsA). Moreover, we demonstrated herein the development of the indigoidine-based whole-cell biosensors to detect bioavailable Hg(II) and Pb(II) in water samples by employing metal-responsive transcriptional regulator MerR and PbrR as the sensory elements, and the indigoidine biosynthesis gene cluster as a reporter element. The resulting indigoidine-based biosensors presented a good selectivity and high sensitivity to target metal ions. High concentration of target metal exposure could be clearly recognized by the naked eye due to the color change by the secretion of indigoidine, and quantified by measuring the absorbance of the culture supernatants at 600 nm. Dose–response relationships existed between the exposure concentrations of target heavy metals and the production of indigoidine. Although fairly good linear relationships were obtained in a relatively limited concentration range of the concentrations of heavy metal ions, these findings suggest that genetically controlled indigoidine biosynthesis triggered by the MerR family transcriptional regulator can enable a sensitive, visual, and qualitative whole-cell biosensor for bioindicating the presence of bioaccessible heavy metal in environmental water samples.

Key points

- *Biosynthesis pathway of indigoidine reconstructed in a high copy number plasmid in E. coli.*
- *Visual and colorimetric detection of Hg(II) and Pb(II) by manipulation of indigoidine biosynthesis through MerR family metalloregulator.*
- *Enhanced detection sensitivity toward Hg(II) and Pb(II) achieved using novel pigment-based whole-cell biosensors.*

Keywords Whole-cell biosensor · Pigment biosynthesis · Mercury · Lead · Visual detection

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Introduction

Most heavy metals are usually naturally occurring elements which are spread into the environment by both natural processes and anthropogenic activities. Bioavailable and bioaccessible forms of heavy metals pose serious ecological and health risks to organisms worldwide (Vareda et al. 2019). However, instrumental analysis provides the total amount of heavy metals that exist in the ecosystem, rather than the bioaccessible amount of heavy metal ions (Magrisso et al. 2008).

To overcome the disadvantages of traditional instrumental methods in failing to provide information about the biological effects of heavy metal pollutants, a substantial number of

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artificial biological devices have been designed and developed. Although a few molecule-based biosensors have been successfully developed with rapid, sensitive, and selective determination of heavy metal ions, these noncellular systems do not distinguish the bioavailable portion of analytes (Lu et al. 2003; Qian et al. 2009; Zhu and Zhang 2014). Environmental microorganisms have evolved numerous detoxification molecular mechanisms to counteract the toxic effects of heavy metals of both natural and anthropogenic origins. The wide adaptation to different environments makes microorganisms very powerful tools when designing and developing novel whole-cell biosensors to assess the impact of heavy metal on the organisms (Jung and Lee 2019).

MerR family metalloregulators have been demonstrated to play important roles in heavy metal resistance via regulating the transcription and translation of a series of detoxification-related genes. The MerR family of transcriptional activators are found in different bacteria, and mainly includes MerR, CadR, PbrR, CueR, and ZntR which sense Hg(II), Cd(II), Pb(II), Cu(I), and Zn(II), respectively (Brocklehurst et al. 2003; Brown et al. 2003; Hui et al. 2018a; Hui et al. 2018b; Lee et al. 2001). The extraordinary fidelity, sensory, and regulatory capacities of MerR family regulators have made them as a source of powerful biological parts for the assembly of whole-cell biosensors (Jung and Lee 2019). Among MerR family proteins, well-characterized MerR, PbrR, and CadR have been used as sensing modules to develop many classical heavy metal biosensors, and the output modules containing luciferase, fluorescent protein, and β-galactosidase were usually inserted downstream of the promoter/operator region to replace the detoxification genes (Bereza-Malcolm et al. 2016; Cai et al. 2018; Guo et al. 2019a; Guo et al. 2021; Hansen and Sorensen 2000; Kumar et al. 2017; Zhang et al. 2021). The resultant output signals including luminescence, fluorescence, and enzymatic activity can be detected quantitatively by various types of spectrophotometers.

With the rapid development of synthetic biology, microorganisms can be programmed to produce visible signals such as pigments which are recognized by the naked eye (Hicks et al. 2020). It would minimize the need for analytical equipment, and enable a low cost, portable measurement for heavy metal ions. The whole-cell biosensors sense bioavailable heavy metal ions and respond by producing pigmented metabolites, such as the purple violacein for Pb(II) (Hui et al. 2020a), red deinoxanthin for Cd(II) (Joe et al. 2012), orange β-carotene for Zn(II) (McNerney et al. 2019), and yellow betaxanthin for Cu(II) (Chen et al. 2017), allowing for mini-equipment determination of bioavailable fractions of heavy metals.

Indigoidine belongs to a family of non-ribosomal peptide natural products. Unlike mRNA-encoded peptides that are assembled on the ribosomes using proteinogenic amino acids as substrates, each non-ribosomal peptide is biosynthesized by a specific non-ribosomal peptide synthetase (NPRS) (Takahashi et al. 2007). Indigoidine is a powerful radical scavenger for the producing bacteria. As a potential therapeutic candidate, indigoidine has been heterologously biosynthesized in several bacteria successfully (Brachmann et al. 2012; Xu et al. 2015; Yu et al. 2013). The key enzymes involved in the biosynthesis of indigoidine have been well characterized previously (Takahashi et al. 2007). Indigoidine is synthesized through the condensation of two molecules of L-glutamine catalyzed by the indigoidine synthetase, a single module NPRS. The 4′-phosphopantetheinyl transferase (PPTase) is necessary for the activation of indigoidine synthetase by mediating post-translational modification. Co-expression of indigoidine synthetase and PPTase is usually necessary for the biosynthesis of indigoidine. Besides industrial fermentation for the production of pigment (Xu et al. 2015), indigoidine has been employed as a reporter for precise quantitative analysis of gene expression in both bacterial and mammalian cells (Muller et al. 2012), and for measurement of L-glutamine in biological samples (Brown et al. 2017). A modified substrate-free blue/white screening system, as an alternative to the classic lacZ-based blue/white system, has also been developed by using indigoidine which colors bacterial colonies in blue (Xie et al. 2017).

This work presented here focuses on the development of novel indigoidine-based whole-cell biosensors employing MerR family regulators as sensing modules and a dicistronic NRPS-PPTase unit as a reporter module, with bright blue color for bioindicating bioavailable Hg(II) and Pb(II). The reporter molecule indigoidine is a pigment with charming blue color which is recognized by the naked eye at a high concentration, and quantified by a colorimetric method at a low concentration after a simple centrifugal process.

Material and methods

Bacterial strains, plasmids, and agents

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* BL21(DE3)plysS was used as the host for the biosynthesis of indigoidine under the control of IPTG-inducible T7 lac promoter. *E. coli* TOP10 was used as the host for gene cloning and for harboring artificial heavy metal-responsive genetic systems. Bacterial strains were routinely cultured aerobically in standard Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), supplemented with 50 μg/mL ampicillin when necessary. NaCl-free LB broth (SLB) was also used to avoid AgCl precipitation in metal selectivity assay as described elsewhere (Stoyanov and Brown 2003). If not mentioned otherwise, the growth condition is at 37 °C with rotation at 250 rpm in a shaker. Stock solutions of $CuSO_4$, $CrCl_3$, $NiSO_4$, $MnSO_4$, $Pb(NO_3)_2$, $ZnSO_4$, $CdCl_2$, $AgNO_3$, and $HgCl_2$ were freshly

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Genotypes or description	Origin
Bacterial strains		
BL21(DE3)pLysS	F$^-$ *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
TOP10	F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1	Invitrogen
Plasmids		
pET-21a	Amp^R, T7 promoter, lac operator	Novagen
pT7lac-ind	pET-21a derivative containing the indigoidine biosynthetic module (a *bspA* and *pcpS* dicistronic cassette) inserted as a *Nde*I/*Sac*I fragment	This study
pPmer-ind	pT7lac-ind derivative containing *merR* and Pmer divergent promoter region cloned into *Bgl*II and *Xba*I sites	This study
pPpbr-ind	pT7lac-ind derivative containing *pbrR* and Ppbr divergent promoter region cloned into *Bgl*II and *Xba*I sites	This study
pT-Pmer	T vector carrying *merR* and Pmer divergent promoter region	(Zhang et al. 2021)
pT-Ppbr	T vector carrying *pbrR* and Ppbr divergent promoter region	(Guo et al. 2019b)
Primers		
F-mer	GAAGATCTCTAAGGCATAGCTGACC	This study
R-mer	GCTCTAGAACGTTGGCCCTTTTG	This study
F-pbr	GAAGATCTTTACCCAGATGTTTGA	This study
R-pbr	GCTCTAGAGAGTAACTCCTGAAAATC	This study

prepared with the analytical grade chemicals. All oligonucleotide primers and synthetic DNA fragments were synthesized by Sangon Biotech (Shanghai, China).

Genetic assembly of artificial heavy metal-responsive operons

The strategy used for assembling artificial heavy metal-responsive operons and the molecular mechanism of biosensing with indigoidine as a visible signal is summarized in Fig. 1. To reconstruct the indigoidine biosynthetic pathway, a dicistronic genetic fragment of 4631 bp encoding two open reading frames designated as *bpsA* derived from *Streptomyces lavendulae* (Genbank: AB240063.1) and *pcpS* derived from *Pseudomonas aeruginosa* PAO1 (Genbank: NP_249856.1) was synthesized after being modified for the *E. coli* codon preference (Fig. S1). The synthetic indigoidine biosynthesis module (Genbank: MW629112) was first inserted into the *Nde*I and *Sac*I sites of pET-21a to generate pT7lac-ind. The genetic element including the *merR* gene and its divergent *mer* promoter was amplified from pT-Pmer by PCR using primer pairs F-mer and R-mer. Then, the amplified DNA fragment was cloned into the *Bgl*II-*Xba*I site of the pT7lac-ind to yield pPmer-ind, which is designed as a Hg(II) biosensing construct with indigoidine as the output signal. Similarly, the PCR-amplified *pbrR*-divergent *pbr* promoter fragment from pT-Ppbr using primer pairs F-pbr and R-pbr, was cloned into the *Bgl*II-*Xba*I site of the pT7lac-ind, in replacement of the T7 lac promoter, to generate pPpbr-ind, a Pb(II) biosensing construct with indigoidine as the output signal. The recombinant plasmids used in this study were all sequenced for verification by Sangon Biotech (Shanghai, China). Sequence and annotation of the inserted fragments are shown in Fig. S2.

Visible light absorption spectrum of heterologous biosynthetic indigoidine

Freshly transformed *E. coli* BL21(DE3)plysS/pT7lac-ind colony was inoculated into 3 mL LB medium in a 15-mL bioreaction tube with a vent cap (Jet Bio-Filtration, Guangzhou, China), and then cultured at 37 °C for 12 h. Thirty microliters of overnight culture was inoculated into 3 mL fresh LB medium. The culture was grown at 37 °C for 3 h with rotation at 250 rpm, and then induced with 0.1 mM IPTG at 30 °C for 5 h. The culture supernatant was obtained by centrifuged at 5000 *g* for 5 min. The visible light absorption spectrum of the resultant supernatant was finally obtained by utilizing a microplate reader (BioTek Epoch, USA). A 400-700 nm scanning wavelength range with an interval of 5 nm was set.

Analysis of response characteristic of heavy metal whole-cell biosensors

The plasmids pPmer-ind and pPpbr-ind were transformed into *E. coli* TOP10 competent cells. A single transformed colony was inoculated in 3 mL of LB broth and grown as a preculture at 37 °C for 12 h. Overnight culture was subcultured using 1% v/v inoculum to 3 mL of fresh LB broth and grown

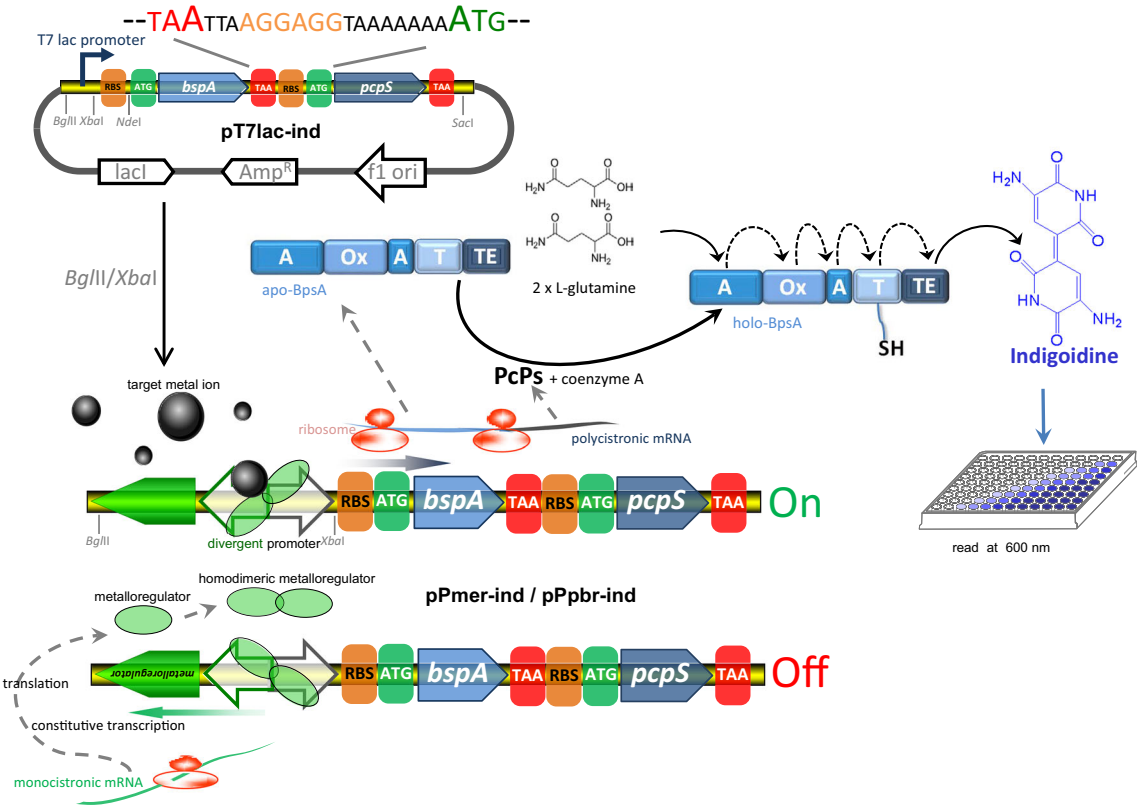


Fig. 1 Metabolically engineered biosensors with the indigoidine output. The indigoidine biosynthesis module was artificially synthesized, and placed under the control of the T7 lac promoter (pT7lac-ind), the *mer* promoter (pPmer-ind), and the *pbr* promoter (pPpbr-ind), respectively. After *Streptomyces lavendulae* non-ribosomal peptidesynthase (NRPS) BpsA and *Pseudomonas aeruginosa* PAO1 4′-phosphopantetheinyl transferase (PPTase) PcpS were transcribed as a dicistronic unit and translated into two enzymes with target heavy metals exposure, an inactive apo-BpsA was converted into an active holo-BpsA by PcpS-mediated transfer of a moiety derived from coenzyme A to the thiolation domain of BpsA. Then, one molecule of indigoidine was synthesized with two endogenous L-glutamines as substrates by a sequential catalytic reaction mediated by an adenylation (A) domain interrupted by an oxidation (Ox) domain, a thiolation (T) domain, and a thioesterase (TE) domain. The resultant indigoidine was conveniently recognized by the naked eye at a high concentration, and quantitatively measured at 600 nm over a wide concentration range

at 37 °C until the OD_{600} value reached about 0.4. Then, a final concentration of 1 μM Hg(II) and 30 μM Pb(II) was supplemented into TOP10/pPmer-ind culture and TOP10/pPpbr-ind culture, respectively. The culture supernatant was prepared and analyzed by taking a sample at regular time intervals from the induced culture.

Detection specificity test

Individual transformed colonies were grown overnight in LB or SLB broth at 37 °C. The overnight culture was then inoculated into fresh LB or SLB broth using 1% v/v inoculum, and grown to exponential growth phase (OD_{600} is about 0.4). To evaluate the detection selectivity of heavy metal indigoidine-based biosensor, a final concentration of 1 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), or Hg(II) was added to TOP10/pPmer-ind in LB medium, and a final concentration of 1 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), Hg(II), and Ag(I), a mixture of 1 μM of various metal ions without Hg(II), or a mixture of 1 μM of various metal ions including Hg(II) was added to TOP10/pPmer-ind in SLB medium, followed by incubation at 37 °C for 3 h before indigoidine content and cell density were determined. A final concentration of 30 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), or Hg(II) was supplemented into TOP10/pPpbr-ind in LB medium, and a final concentration of 30 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), Hg(II), and Ag(I), a mixture of 30 μM of various metal ions without Pb(II), or a mixture of 30 μM of various metal ions including Pb(II) was added to TOP10/pPpbr-ind in SLB medium. After incubation at 37 °C for 3 h, the indigoidine content and cell density were determined.

To evaluate the influence of various non-target metal ions on the response of indigoidine-based biosensor toward target heavy metal ion, the mixture of 1 μM Hg(II) with other different metal ions at the concentration of 1 μM was added to TOP10/pPmer-ind in LB medium at the exponential growth phase, and the mixture of 30 μM Pb(II) with other different metal ions at the same concentration was supplemented into TOP10/pPpbr-ind in LB medium at the exponential growth

phase. After incubation at 37 °C for 3 h, the indigoidine content and cell density were determined.

Detection sensitivity assay

Individually transformed TOP10/pPmer-ind and TOP10/pPpbr-ind colonies were inoculated and grown overnight in LB broth at 37 °C. To evaluate the detection sensitivity of logarithmic phase bacterial cultures toward target heavy metal, the overnight culture was then inoculated into fresh LB broth using 1% v/v inoculum, and grown at 37 °C for 3 h. A final concentration of 0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, or 33.3 μM Hg(II) was supplemented into the early-log phase culture of TOP10/pPmer-ind, followed by a 3-h culture. A final concentration of 0, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, 33.3, 66.7, 133.4, or 266.8 μM Pb(II) was supplemented into the early-log phase culture of TOP10/pPpbr-ind, followed by a 3-h culture before the indigoidine content and cell density were determined.

To evaluate the detection sensitivity of lag-phase culture of TOP10/pPpbr-ind toward Pb(II), 30 μL of the overnight culture was directly used to inoculate 3 mL of fresh LB medium supplemented with 0, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, 33.3, or 66.7 μM Pb(II). After incubation at 37 °C for 7 h, the indigoidine content and cell density were determined.

Detection of environmental water samples

To examine the effectiveness of the indigoidine-based biosensors to detect bioavailable target metal ions in environmental water samples, recombinant TOP10/pPmer-ind and TOP10/pPpbr-ind were grown in LB medium prepared with purified water, tap water, and lake water collected from a local lake as described previously (Hui et al. 2020a). Exponential-phase LB-cultures of TOP10/pPmer-ind were spiked with 0, 0.016, 0.033, 0.065, 0.13, or 0.26 μM Hg(II). Exponential-phase LB-cultures of TOP10/pPpbr-ind were spiked with 0, 1.04, 2.08, 4.17, 8.3, or 16.7 μM Pb(II). After an extra incubation at 37 °C for 3 h, the indigoidine content and cell density were determined.

Water-soluble indigoidine analysis

One milliliter of induced culture was centrifuged at 5000 *g* for 5 min. Aliquots of 200 μL supernatant were transferred into a 96-well microplate, and measured in a BioTek Epoch microplate reader for absorbance at 600 nm. The cell pellets were washed three times with ddH_2O to remove the residual pigment, and resuspended in 1 mL ddH_2O. Then, the OD_{600} value of cell suspension was read at 600 nm. The absorbance of the culture supernatant containing indigoidine was finally normalized by the OD_{600} value of the cell suspension.

Results

Validation for signal output of the indigoidine biosynthesis module

The indigoidine biosynthetic gene cluster located on the plasmid pT7lac-ind was regulated by T7 lac promoter. After a 5-h induction with 0.1 mM IPTG at 30 °C, recombinant BL21(DE3)plysS/pT7lac-ind culture developed a deep blue color (Fig. 2). Blue culture supernatant was easily obtained by centrifugation, and the color of resultant cell pellets was still gray white (data not shown). The results demonstrated that indigoidine was heterogeneously biosynthesized and mostly secreted into the culture. Visible light absorption spectroscopy can usually be applied to determine the maximum absorption wavelength of pigment (Wang et al. 2020; Zhang et al. 2014). Compared with the uninduced culture supernatant, an absorption peak located at near 600 nm was found in the induced culture supernatant. The detection wavelength of indigoidine in culture supernatant was then set at 600 nm in the following studies.

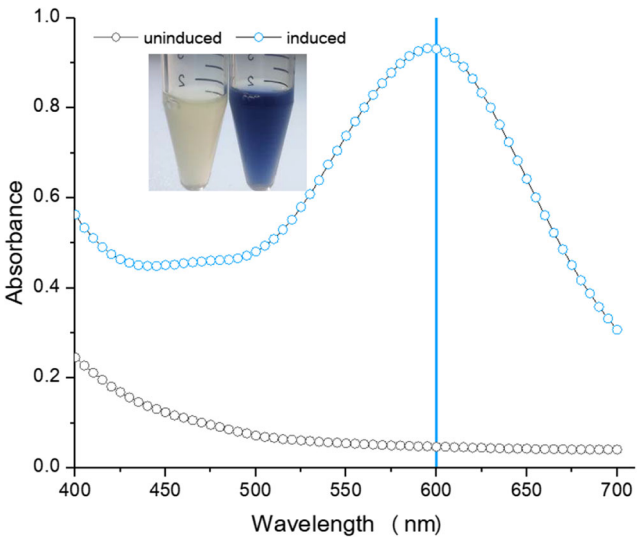


Fig. 2 The visible absorption spectrum of water-soluble blue pigment indigoidine secreted by IPTG-induced BL21(DE3)plysS/pT7lac-ind. Exponential phase LB-culture of BL21(DE3)plysS/pT7lac-ind was induced with 0.1 mM IPTG at 30 °C for 5 h. The indigoidine secreted into the culture shows a deep blue color, and the maximum absorption wavelength is located around 600 nm. The assay was done at least three independent times with similar results. The representative result from one assay is shown here

Response characteristics of the indigoidine-based biosensors toward target heavy metal ions

The response strength of whole-cell biosensor was determined by both the response rate of the sensing element and the maturation time of the reporter product (Bereza-Malcolm et al. 2015; Hansen and Sorensen 2000; Zhang et al. 2021). To find the suitable induction time for the two kinds of indigoidine-based biosensors, exponential-phase TOP10/pPmer-ind and TOP10/pPpbr-ind were exposed to 1 μM Hg(II) and 30 μM Pb(II), respectively. Time–response curves of the indigoidine-based biosensors toward their cognate metal ions are shown in Fig. 3a. The color of blue pigment in both of the culture supernatants deepened with the extension of induction time. After a 4-h induction, not only was the blue color not deepened any more, but also the blue color even tended to lighten (Fig. 3b). Considering the situation, a 3-h induction was finally chosen for color indicating heavy metal exposure.

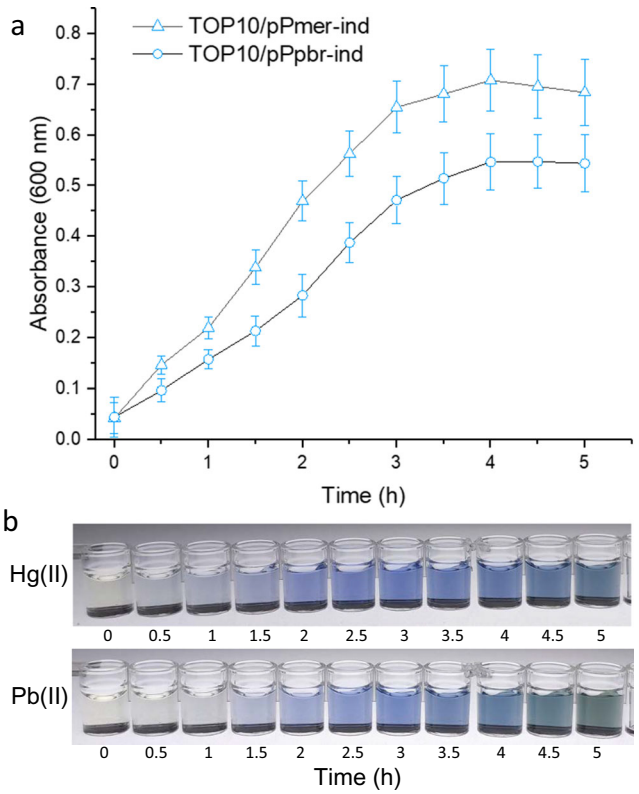


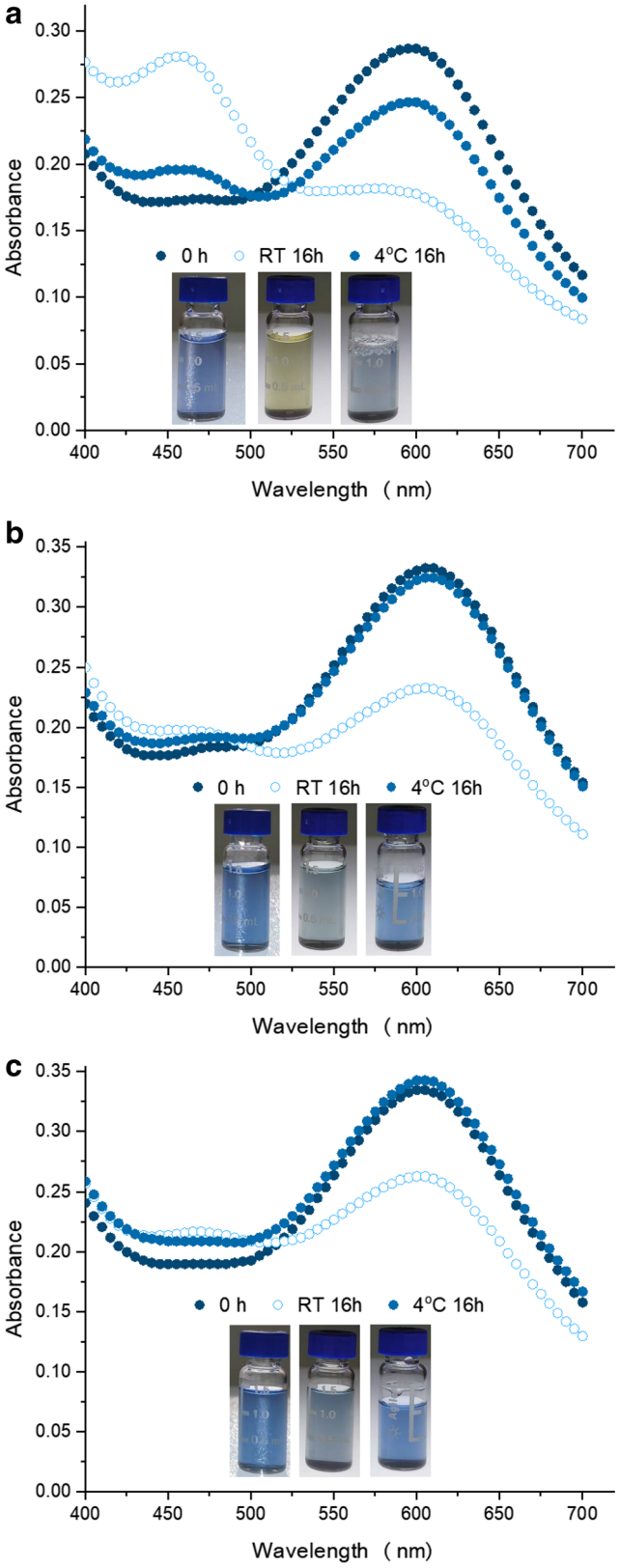
Fig. 3 Time–response curves of two kinds of biosensors toward target heavy metal ions. Exponential phase LB-cultures of recombinant TOP10/pPmer-ind and TOP10/pPpbr-ind were induced with 1 μM Hg(II) and 30 μM Pb(II) at 37 °C for 5 h. The culture supernatants containing indigoidine were prepared at regular time intervals, and read at 600 nm in a microplate reader (**a**). The results are shown as the mean of three independent assays ± the standard deviation. (**b**) The culture supernatants were obtained at regular intervals throughout the whole target heavy metal induction process. Shown is one representative of three independent experiments with similar results

To further investigate the effect of different solvents and temperature on the stability of indigoidine, the indigoidine was dissolved in the aqueous phase, 50% dimethyl sulfoxide (DMSO), and 50% ethanol, respectively. The visible absorption spectrums of three samples were compared during preservation. The results are shown in Fig. 4. Preservation temperature was demonstrated to be a key factor affecting the stability of indigoidine. The blue color in the aqueous phase completely faded after a 16-h preservation at room temperature, and the corresponding absorption peak at 600 nm almost disappeared (Fig. 4). Both the blue color degree and the absorption peak at 600 nm in 50% DMSO (Fig. 4b) and 50% ethanol (Fig. 4c) significantly decreased after a 16-h preservation at room temperature. However, the blue color degree and the absorption peak at 600 nm were almost unchanged after a 16-h preservation at 4 °C.

Responses of the indigoidine-based biosensors incubated in LB culture medium toward different heavy metal ions

To verify the selectivity of the indigoidine-based biosensors, recombinant TOP10/pPmer-ind and TOP10/pPpbr-ind were exposed to eight metal ions in LB culture medium (Fig. 5). The accumulation of indigoidine was determined by spectrophotometry from the culture supernatants of TOP10/pPmer-ind at exponential growth phase exposed to 1 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), or Hg(II) for 3 h. TOP10/pPmer-ind produced visible blue pigment in response to Hg(II) (Fig. 5a). We further investigated the response of the indigoidine-based biosensor toward higher concentrations of metal ions using TOP10/pPpbr-ind. After TOP10/pPpbr-ind at exponential growth phase was exposed to 30 μM Cu(II), Cr(III), Ni(II), Mn(II), Pb(II), Zn(II), Cd(II), or Hg(II) for 3 h, the amount of indigoidine in culture supernatants was determined. As expected, visible blue pigment was only found to be accumulated in the Pb(II) exposure group (Fig. 5c). Here, it was demonstrated that the metal selectivity of the indigoidine-based biosensor corresponded to the sensing element employed in the genetic construct.

To investigate the performance of the indigoidine-based biosensors toward target heavy metals in the presence of various interference metal ions in low and high concentration, LB-incubated exponential-phase TOP10/pPmer-ind was exposed to the mixture of 1 μM Hg(II) with other metal ions (Fig. 5b), and LB-incubated exponential-phase TOP10/pPpbr-ind was exposed to the mixture of 30 μM Pb(II) with other metal ions (Fig. 5d). The performance of the indigoidine-based biosensors was demonstrated to be not significantly influenced by other interference metal ions. A similar accumulation of



◀ **Fig. 4** The stability of indigoidine in three different solutions. The freshly prepared culture supernatants containing indigoidine were mixed with an equal volume of purified water (**a**), DMSO (**b**), or ethanol (**c**). The visible-light absorption properties of three different samples were compared before and after, stored at 4 °C or at room temperature (RT) for 16 h. A representative result and image of three independent experiments with similar results are shown

indigoidine was observed in all groups with target metal ion exposure combined with interference metal ions.

Specificity of the indigoidine-based biosensors incubated in SLB culture medium toward target heavy metal ions

It is well known that silver chloride is insoluble in water, and chlorizated salts formed by other metal ions except for mercury is completely ionized and dissolved in the aqueous solution. Although mercury chloride has a tendency to exist in its molecular form in aqueous solution, bioavailable Hg(II) can still be well sensed by whole-cell biosensors (Du et al. 2019; Zhang et al. 2021). To avoid the possible effect of sodium chloride involved in LB culture medium on the performance of two developed biosensors, the specificity of indigoidine-based biosensors was further investigated in SLB culture medium. As shown in Fig. 6, both whole-cell biosensors responded silently to nontarget metal ions separately or in combination. Interestingly, the response of TOP10/pPmer-ind toward Hg(II) was not significantly decreased when low concentration of various metal ions coexisted in the culture medium (Fig. 6a), and the response of TOP10/pPpbr-ind toward Pb(II) was still relatively strong when high concentration of various metal ions coexisted in the culture medium (Fig. 6b). Compared with the responses in LB culture medium, the responses of both whole-cell biosensors in SLB culture medium toward target metal ions slightly decreased possibly due to the absence of medium components. Thus, the LB culture medium was chosen in the following studies.

Sensitivity of the indigoidine-based biosensors toward target heavy metal ions

A great advantage of using a pigment as a reporter is that even a low-level expression of the pigment biosynthetic enzymes can, over time, catalyze the synthesis of enough pigment to produce a detectable signal (Lim et al. 2015). The limit of detection was determined by exposing exponential-phase TOP10/pPmer-ind to 0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, and 33.3 μM Hg(II) for 3 h at 37 °C. To investigate the adverse effects of Hg(II) and indigoidine on the growth of biosensor cell, the bacterial cell density was first determined. As shown in Fig. S3a, no significant growth inhibition was observed with less than 4.17 μM Hg(II) exposure. Exposures of 8.3 μM Hg(II) and above exerted significant adverse influences on the growth of the biosensor cell. Engineered

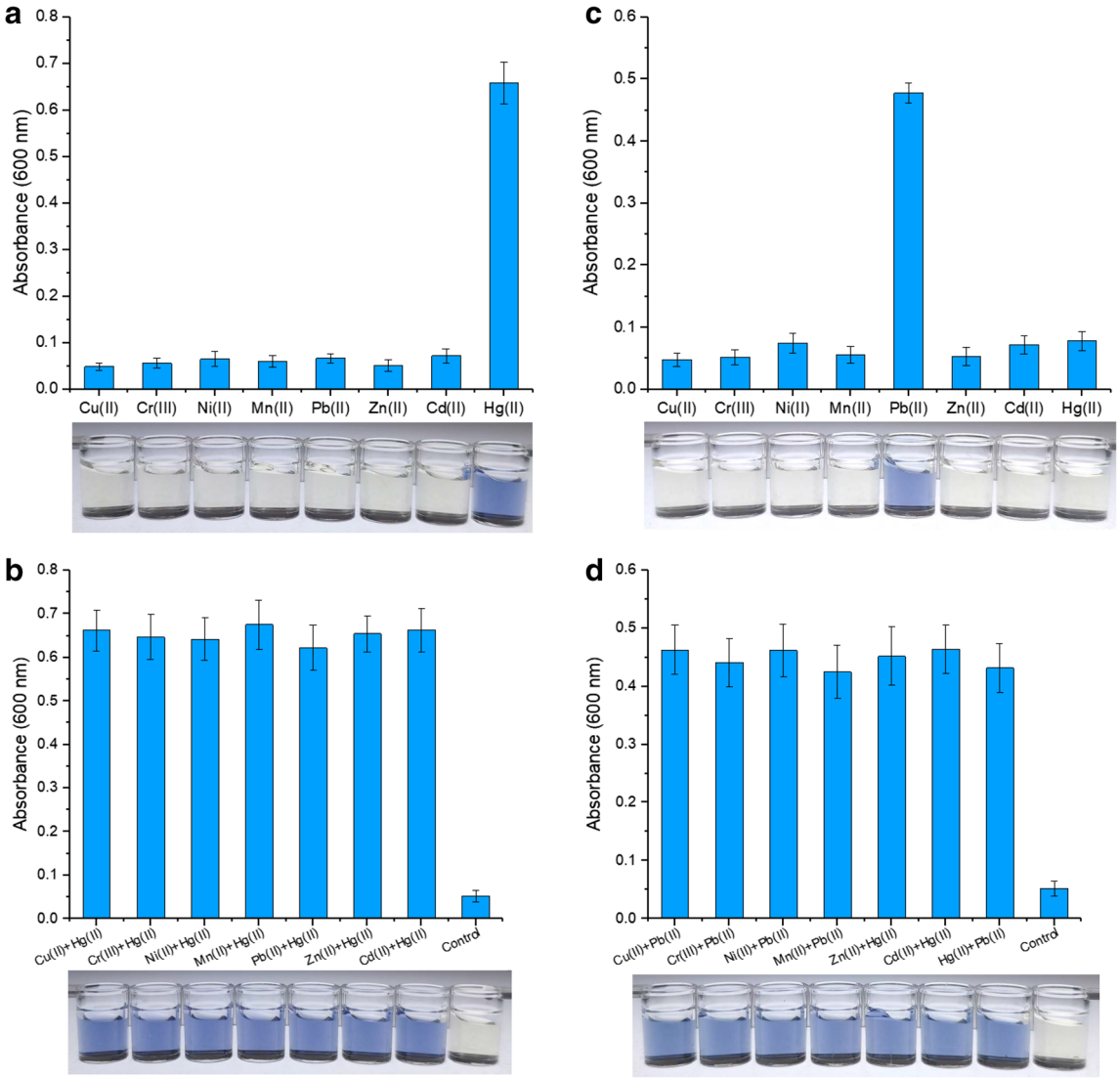


Fig. 5 The responses of engineered biosensors toward different metal ions in LB culture. LB-incubated exponential-phase cultures of TOP10/pPmer-ind were exposed to 1 μM various metal ions alone (**a**) or in combination with Hg(II) (**b**) at 37 °C for 3 h. LB-incubated exponential-phase cultures of TOP10/pPpbr-ind were exposed to 30 μM various metal ions alone (**c**) or in combination with Pb(II) (**d**) at 37 °C for 3 h. The control groups were supplemented with no metal ion. The culture supernatants containing indigoidine were collected and detected by visible light absorbance at 600 nm, and normalized to bacterial cell density at 600 nm. The results represent the average of three independent assays ± the standard deviation. A representative image (bottom) of three independent experiments with similar results is shown

whole-cell biosensor TOP10/pPmer-ind was found to respond to the lowest concentration of Hg(II) at 0.008 μM in a colorimetric method (Fig. 7d), and at 0.033 μM viewed by the naked eye (Fig. 7e). The output signal of indigoidine was significantly increased between 0.008 and 0.13 μM (Fig. 7b). The output signal of indigoidine showed a downward trend with exposures greater than 4.17 μM Hg(II), probably owing to the cytotoxicity of bioavailable Hg(II) (Fig. 7a). The limit of detection was adopted as the lowest Hg(II) concentration that induced a significant increase of indigoidine-derived signal (background + 3 × SD) as described previously (Kumar et al. 2017). It can then be concluded that the limit of detection of exponential-phase TOP10/pPmer-ind is 0.008 μM Hg(II). Furthermore, a good linear relation between output signal of indigoidine and concentration of Hg(II) was observed within 0.004 and 0.065 μM Hg(II) exposure (Fig. 7c).

Exponential-phase TOP10/pPpbr-ind was exposed to 0, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, 33.3, 66.7, 133.4, and 266.8 μM Pb(II). After a 3-h induction at 37 °C, bacterial cell density was first determined. Different from the Hg(II) induction group, up to 266.8 μM Pb(II) induction exerted no significant adverse effects on the growth of TOP10/pPpbr-ind (Fig. S3b). The culture supernatants containing indigoidine were prepared and quantified in a colorimetric method. As shown in Fig. 8d, recombinant TOP10/pPpbr-ind could respond to

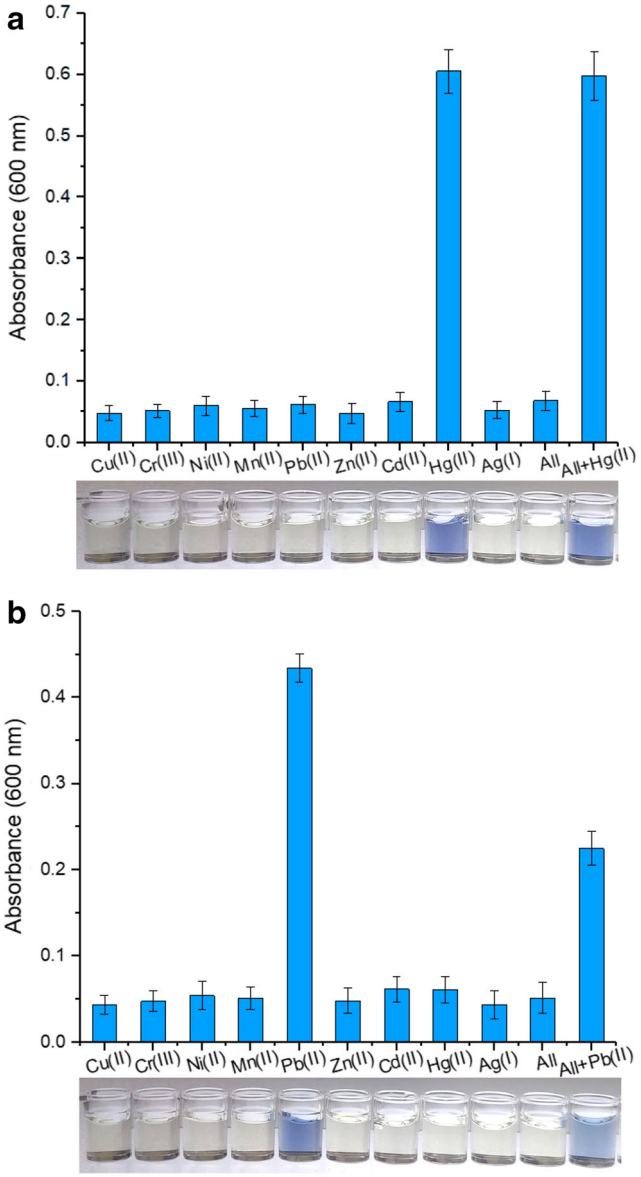


Fig. 6 The responses of engineered biosensors toward different metal ions in SLB culture. (**a**) SLB-incubated exponential-phase cultures of TOP10/pPmer-ind were exposed to 1 μM of nine different metal ions alone, in combination without Hg(II), or in combination with Hg(II) at 37 °C for 3 h. (**b**) SLB-incubated exponential-phase cultures of TOP10/pPpbr-ind were exposed to 30 μM of nine different metal ions alone, in combination without Pb(II), or in combination with Pb(II) at 37 °C for 3 h. The culture supernatants containing indigoidine were collected and detected by visible light absorbance at 600 nm, and normalized to bacterial cell density at 600 nm. The results represent the average of three independent assays ± the standard deviation. A representative image (bottom) of three independent experiments with similar results is shown

concentrations as low as 0.033 μM Pb(II) in a colorimetric method. However, a significant visible color change was found in the culture supernatants with exposures greater than 4.17 μM Pb(II) (Fig. 8e). The output signal of indigoidine was significantly increased in the culture supernatants of TOP10/pPpbr-ind exposed to 0.033–33.3 μM Pb(II) (Fig. 8b). The output signal of indigoidine remained stable at exposures greater than 33.3 μM Pb(II) (Fig. 8a). The limit of detection of exponential-phase TOP10/pPpbr-ind was 0.065 μM Pb(II), and a good linear relation between output signal of indigoidine and Pb(II) exposure concentration was found to range between 0.26 and 8.3 μM of Pb(II) (Fig. 8c).

The detection sensitivity of TOP10/pPpbr-ind at lag phase toward Pb(II)

The production amount of reporter is also determined by biosensor cell cycle upon heavy metal exposure, and it is attributed to the differences in metabolic capacity, bioavailable heavy metal concentration per cell, residual nutrients in medium, and so on (Hui et al. 2020a; Kim et al. 2018; Lim et al. 2015). The influence of growth phase of indigoidine-based biosensor on the limit of detection was then studied. Non-growing TOP10/pPpbr-ind was directly exposed to 0, 0.008, 0.016, 0.033, 0.065, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.3, 16.7, 33.3, and 66.7 μM Pb(II) for 7 h at 37 °C. The culture supernatants in each group were separated and quantified in a colorimetric method. TOP10/pPpbr-ind at lag phase was found to respond to the lowest concentration of Pb(II) at 0.008 μM in a colorimetric method (Fig. 9d), and at 2.08 μM viewed by the naked eye (Fig. 9e). The output signal of indigoidine was significantly increased between 0.008 and 16.7 μM (Fig. 9b), and remained stable with higher than 16.7 μM Pb(II) exposure (Fig. 9a). The limit of detection of lag-phase TOP10/pPpbr-ind was 0.008 μM Pb(II), and a good linear relation between the output signal of indigoidine and Pb(II) exposure concentration was found between 0.13 and 4.17 μM (Fig. 9c).

Performance of the indigoidine-based biosensors in environmental water samples

A substantial number of microorganisms, organic and inorganic pollutants coexist in aquatic ecosystems. To validate the value of the developed biosensors to sense bioavailable fractions of heavy metal in environmental water samples, exponential-phase TOP10/pPmer-ind was exposed to 0, 0.016, 0.033, 0.065, 0.13, and 0.26 μM Hg(II) dispersed in LB medium prepared using purified water, tap water, and lake water. Exponential-phase TOP10/pPpbr-ind was exposed to 0, 1.04, 2.08, 4.17, 8.3, and 16.7 μM Pb(II) dispersed in LB medium prepared using three kinds of water samples as mentioned above. As shown in Fig. 10, a nearly identical increase in pigment signals correlated with an increase in spiked heavy metal concentration was observed in three water samples. Furthermore, higher RSD values were observed in the environmental water-treated group. It suggests that bioavailable toxic heavy metal spiked in the environmental water samples can be preliminarily evaluated by monitoring the blue color

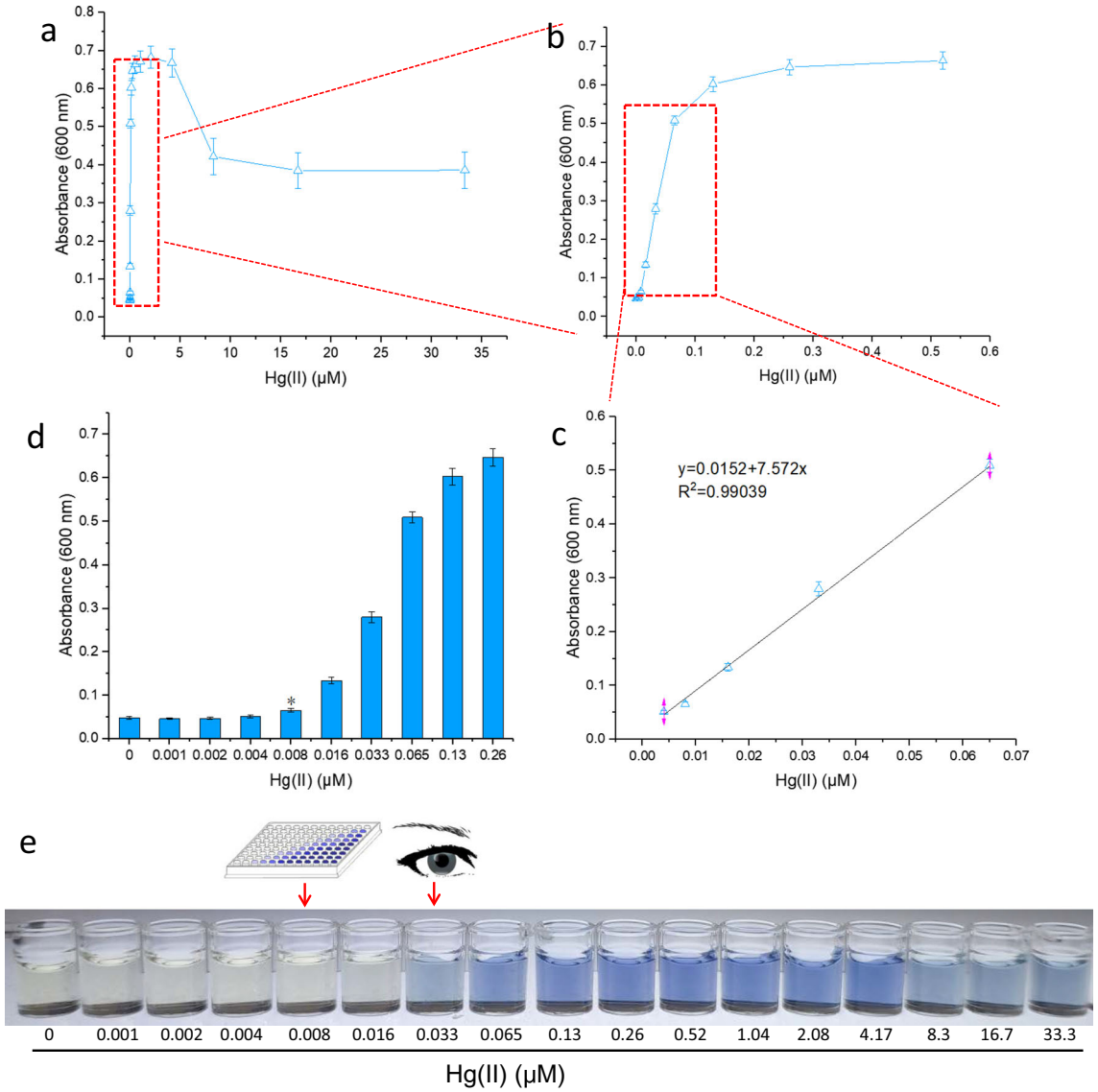


Fig. 7 The performance of exponential-phase culture of TOP10/pPmer-ind induced with increased concentrations of Hg(II). Exponential-phase cultures of TOP10/pPmer-ind were induced with increased concentrations of Hg(II) at 37 °C for 3 h. The concentrations of dose–response curve with Hg(II) range from 0 to 33.3 μM (**a**), and the concentrations of the dose–response curve with Hg(II) range from 0 to 0.52 μM (**b**). (**c**) A linear response to Hg(II) was in the concentration range of 0.004 to 0.065 μM (R^2=0.99039). (**d**) The detection sensitivity of TOP10/pPmer-ind. The asterisk represents a statistically significant difference (two-tailed *t* test, $P<0.05$) compared with the control group with no Hg(II) exposure. Error bars represent the standard deviation from at least triplicate measurements. (**e**) The representative picture of the culture supernatants is shown, and the visible detection limit and the instrumental detection limit are all shown

change, and different water matrixes exert a certain effects on the stability of the indigoidine-based biosensor.

Discussion

The two main reporter systems differ in their detection properties, which range from the substrate-free fluorescent signals, to enzymatic signals scoring the conversion of chromogenic substrates, chemiluminescent substrates, and bioluminescent substrates (Magrisso et al. 2008). A substantial number of whole-cell biosensors using fluorescent proteins, β-galactosidase, and luciferase as reporters have been largely documented in heavy metal biosensing (Gupta et al. 2019). Recently, some natural pigments with bright color as the novel reporters have been demonstrated to have an overwhelming advantage over traditional reporters in heavy metal biosensing. Genetic control of pigment production has been demonstrated to enable a fast-responding and minimal-equipment biosensor in many studies (McNerney et al. 2019; Wang et al. 2020; Watstein and Styczynski 2018). In the present work, we present the design and construct of metabolically engineered

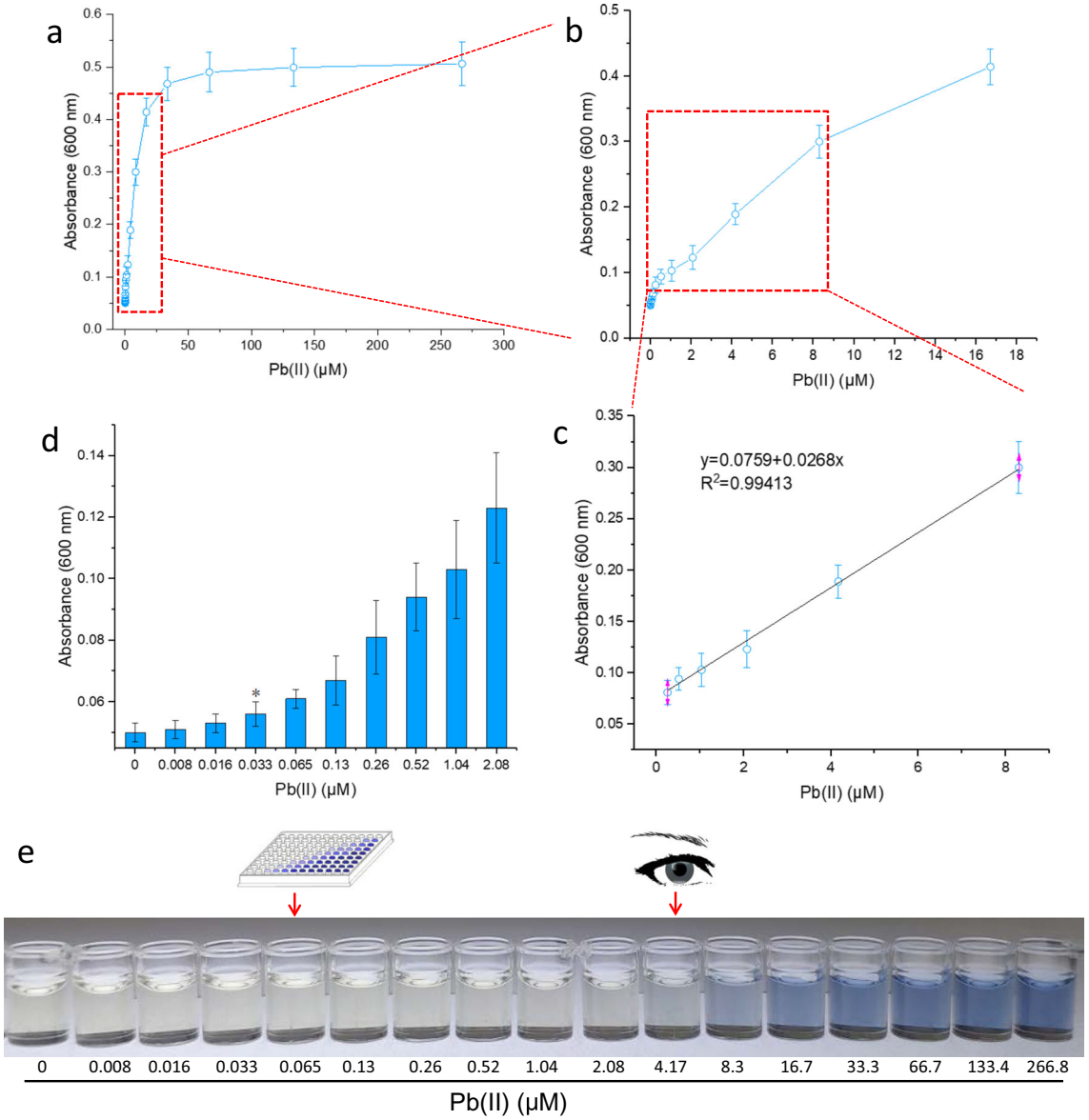


Fig. 8 The performance of exponential-phase culture of TOP10/pPpbr-ind induced with increased concentrations of Pb(II). Exponential-phase cultures of TOP10/pPpbr-ind were induced with increased concentrations of Pb(II) at 37 °C for 3 h. Whole-cell biosensor dose–response curve with Pb(II) concentrations ranging from 0 to 266.8 μM (**a**), and dose–response curve with Pb(II) concentrations ranging from 0 to 16.7 μM (**b**). (**c**) A linear response to Pb(II) was in the concentration range of 0.26 to 8.3 μM (R^2=0.99413). (**d**) The detection sensitivity of TOP10/pPpbr-ind. The asterisk represents a statistically significant difference (two-tailed *t* test, $P<0.05$) compared with the control group with no Pb(II) exposure. Error bars represent the standard deviation from at least triplicate measurements. (**e**) The representative picture of the culture supernatants is shown, and the visible detection limit and the instrumental detection limit are all shown

indigoidine-based biosensors, which are capable of responding to bioavailable Hg(II) and Pb(II), with potential application to detect heavy metal in environmental water.

Indigoidine is usually biosynthesized by condensation of two units of L-glutamine by a NRPS which is always activated by a PPTase (Takahashi et al. 2007). Compared to other NRPSs, BpsA is a small and simple enzyme, and has been demonstrated to be well suited for use in quantitative in vitro and in vivo assays (Brown et al. 2017). *Pseudomonas aeruginosa* carrier protein synthase (PcpS) is a well-characterized PPTase (Finking et al. 2002), which was chosen in this study. Coding sequences of BpsA and PcpS were fused to generate a dicistronic expression module. The decreased expression of the downstream gene was expected by optimization of the distance and DNA sequence between the termination of the upstream gene and the start of the downstream one (Hui et al. 2020b; Hui et al. 2018c; Levin-Karp et al. 2013). Therefore, a cloning strategy employing a dicistronic *bpsA-pcpS* genetic cassette was used. As a substrate for PcpS, BpsA, which is expected to be expressed with a high level, is

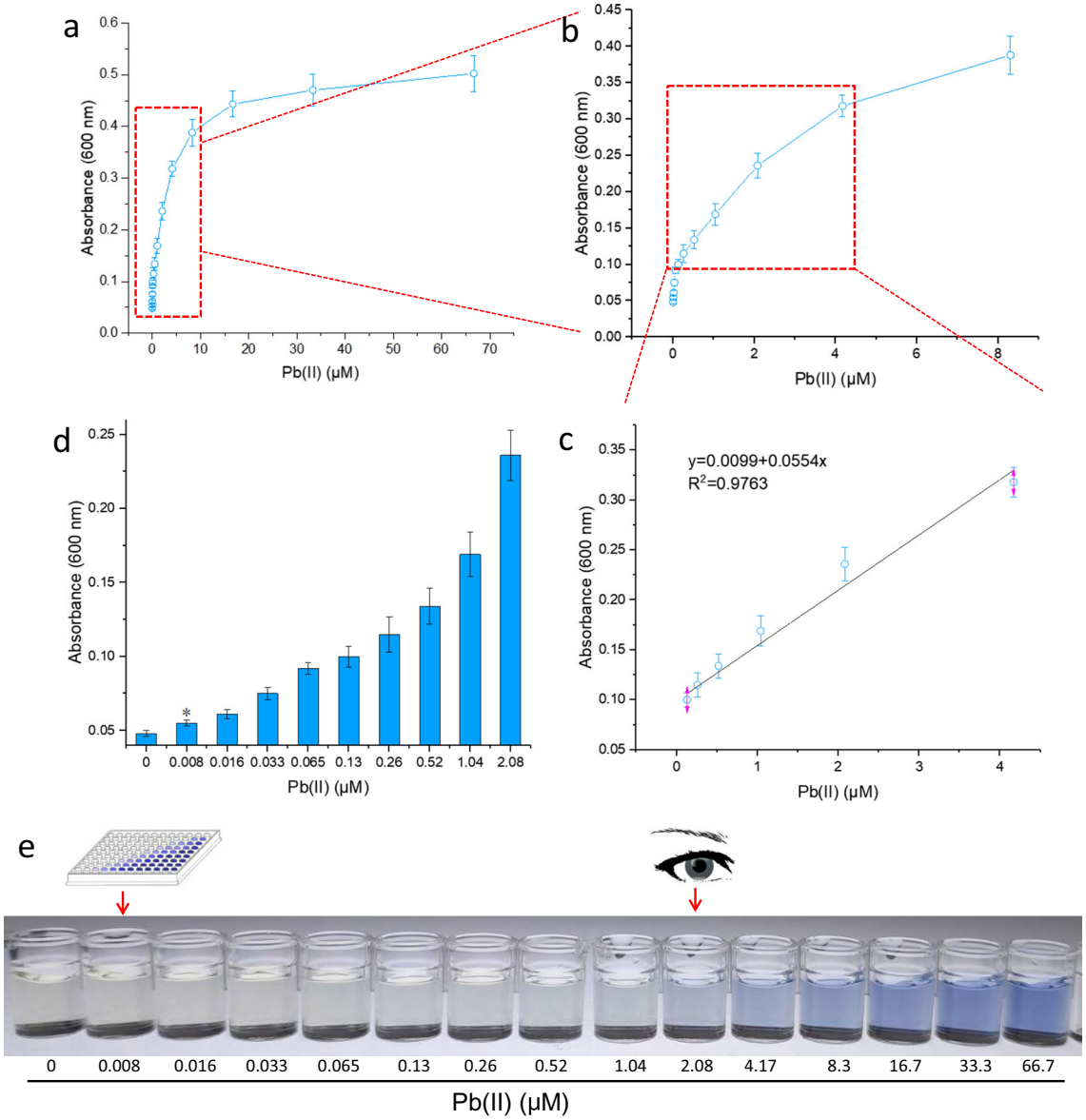
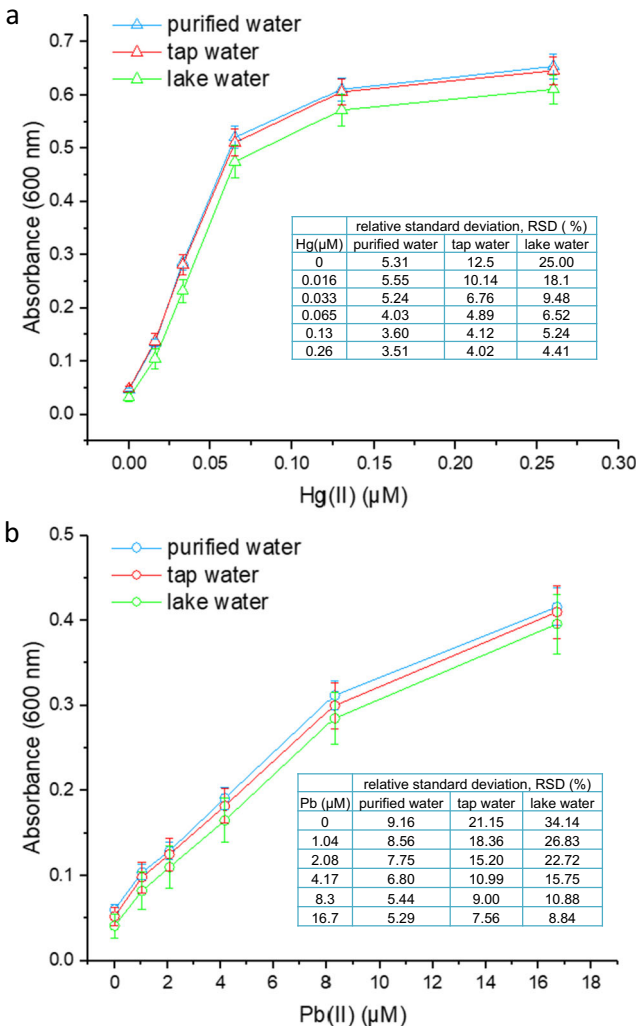


Fig. 9 The response of resting TOP10/pPpbr-ind exposed with increased concentrations of Pb(II). Recombinant *E. coli* TOP10 harboring the plasmid pPpbr-ind was inoculated in LB broth containing increased concentrations of Pb(II), and cultured at 37 °C for 7 h. Whole-cell biosensor dose–response curve with Pb(II) concentrations ranging from 0 to 66.7 μM (**a**), and dose–response curve with Pb(II) concentrations ranging from 0 to 8.3 μM (**b**). (**c**) The linear relationship was in the concentration range of 0.13–4.17 μM (R^2=0.9763). (**d**) The response sensitivity of TOP10/pPpbr-ind. The asterisk denotes a statistically significant difference (two-tailed *t* test, P<0.05) in absorbance at 600 nm compared with the 0 μM Pb(II) exposure group. Error bars represent the standard deviation from at least triplicate measurements. (**e**) A representative picture of an assay is shown here. The visible detection limit is about 2.08 μM, and the instrumental detection limit is nearly 0.008 μM

certainly located upstream of PcpS in this genetic construct (Fig. 1).

When inoculated in a liquid medium, engineered bacteria secrete indigoidine, which turns the culture supernatant into a ready-to-assay blue color (Fig. 2). In addition, the blue pigment reporter system does not require an extra substrate since the indigoidine is produced from endogenous and constitutively available L-glutamine. The designed indigoidine reporter system, as an ideal reporter system, is then demonstrated to be instrumental in heavy metal biosensing. Stable indigoidine output signal was obtained after a 3-h treatment with Hg(II) and with Pb(II) (Fig. 3). However, the maximum fluorescent signals were usually obtained after an overnight incubation in most studies (Guo et al. 2019b; Hansen and Sorensen 2000; Hui et al. 2019; Hui et al. 2018c; Zhang et al. 2021). It is hypothesized that indigoidine is first synthesized in the oxidized form as blue keto-indigoidine, and then subsequently reduced by 4-oxalocrotonate tautomerase to the reduced form as leuco-indigoidine (Reverchon et al. 2002; Takahashi et al. 2007). Possibly due to this reason, a slight decrease in



	relative standard deviation, RSD (%)		
Hg(μM)	purified water	tap water	lake water
0	5.31	12.5	25.00
0.016	5.55	10.14	18.1
0.033	5.24	6.76	9.48
0.065	4.03	4.89	6.52
0.13	3.60	4.12	5.24
0.26	3.51	4.02	4.41

	relative standard deviation, RSD (%)		
Pb (μM)	purified water	tap water	lake water
0	9.16	21.15	34.14
1.04	8.56	18.36	26.83
2.08	7.75	15.20	22.72
4.17	6.80	10.99	15.75
8.3	5.44	9.00	10.88
16.7	5.29	7.56	8.84

Fig. 10 The responses of biosensor cells toward target metal ions in culture medium prepared using environmental water. Exponential phase of TOP10/pPmer-ind (**a**) and TOP10/pPpbr-ind (**b**) grown in LB medium prepared with three different water samples was exposed to varying concentrations of Hg(II) and Pb(II), respectively. Purified water was used as a control. After culturing at 37 °C for 3 h, the pigment signals were measured. Error bars represent the standard deviation from at least triplicate measurements. Relative standard deviation (RSD) is shown in the table embodied in the figure

indigoidine signal was observed in both heavy metal biosensing systems above 4-h induction (Fig. 3). The blue color then completely faded after an overnight incubation (data not shown). Considering that the reduction of keto-indigoidine is biologically mediated, we subsequently investigated the influences of temperature and different solvents on this biological reaction. Low temperature and the introduction of organic solvents were demonstrated to facilitate the stability of blue keto-indigoidine (Fig. 4). It is difficult to prevent this reduction reaction by the direct supplement of oxidants into the culture medium during cultivation, and the culture must be harvested at the exact time point. However, the culture containing indigoidine can be kept stably at low temperatures for a long time by the introduction of some organic reagents to slow down this reduction reaction. Although the fluorescent leuco-isoform is colorless, it can also be detected by fluorescence microscopy (Muller et al. 2012). After keto-indigoidine was reduced completely by the reductants such as sodium dithionate, fluorescent quantification can be another alternative for the determination of the indigoidine-derived signal (Brown et al. 2017).

Many pigment secondary metabolites of microorganisms including indigoidine have been verified to have antimicriobial activity in natural environment (Cude et al. 2012). The reduced form of blue keto-indigoidine is predicted to be responsible for the antimicrobial activity (Cude et al. 2015). Furthermore, the low levels of expressed keto-indigoidine induced by heavy metal ions were mainly secreted into extracellular matrix. It is well understood that the bacterial cell densities of both biosensors were not decreased after induction within the range of Hg(II) and Pb(II) concentrations without significant cytotoxic effects (Fig. S3). An extra centrifugation step is currently necessary for the preparation of the culture supernatant containing indigoidine. Since the bacterial cell densities of both biosensors are consistent under induction of heavy metals in a wide concentration range, the absorbance of induced biosensor cell culture can be directly measured at 600 nm with or without normalization by the cell density of the control group without heavy metal exposure. The resultant absorbance can be considered to quantify the bioavailable heavy metal directly in a certain concentration range.

Due to the extremely high selectivity of MerR family regulators toward their cognate metal ions (Jung and Lee 2019), it is not surprising to see that two developed indigoidine-based biosensors specifically responded to target Hg(II) or Pb(II) in both LB culture medium (Fig. 5) and SLB culture medium (Fig. 6). The specific responses were not significantly jammed when various metal ions and target metal ions co-existed in the same LB culture system (Fig. 5b and 5d). The upward trends of both biosensors slowed down and approached steady state within the range of Hg(II) and Pb(II) concentrations without significant cytotoxic effects. It was currently hypothesized that the gradual saturation of metal-binding sites of basal expressed MerR-family metalloregulators might lead to the above phenomenon (Guo et al. 2021; Hui et al. 2020a; Yoon et al. 2016). Above all, both the indigoidine-based biosensors responded to their cognate heavy metals in a quantitative manner within a certain concentration range whose limits were preliminarily determined in this study.

The detection limit of whole-cell biosensor using MerR as the sensing element and the GFP as a output signal was 0.1 μM Hg(II) (Guo et al. 2019a). By combining quorum sensing-based positive feedback systems, the detection limit was enhanced to 0.02 μM Hg(II) (Cai et al. 2018). An improved Hg(II) sensitivity was observed in the exponential-

phase culture of indigoidine-based Hg(II) biosensor. Although a visible color change occurred with 0.033 μM Hg(II) exposure, the detection limit of spectrophotometry method was as low as 0.008 μM (Fig. 7e). The United States Environmental Protection Agency (USEPA) recommends the criteria maximum concentration (CMC) for mercury in freshwater of 1.4 μg/L (0.007 μM) (USEPA 1984). Engineered indigoidine-based biosensor is expected to detect Hg(II) using a colorimetry method at the USPEA's CMC level to prevent acute toxicity in aquatic organisms. Bacterial biosensors using PbrR as the sensing element have been developed previously. The detection limit was above 10 μM Pb(II) with GFP as an output signal (Bereza-Malcolm et al. 2016), and above 1 μM Pb(II) with lacZ as an output signal (Guo et al. 2019b; Hui et al. 2020b). Similarly, an improved Pb(II) sensitivity was also observed in the exponential-phase culture of indigoidine-based Pb(II) biosensor. The spectrophotometry method showed a detection limit of 0.065 μM Pb(II), and a visible color change occurred with above 4.17 μM Pb(II) exposure (Fig. 8e). The USEPA recommends the CMC for lead in freshwater to be 82 μg/L (0.396 μM) (USEPA 1984). The World Health Organization (WHO) guidelines recommend the guideline value for lead in drinking water to be 10 μg/L (0.048 μM) (WHO 2011). Engineered indigoidine-based biosensor could detect lead levels near the WHO criteria in drinking water using a colorimetry method. Both high cell density and multi-component medium have been demonstrated to decrease the amount of intracellular bioaccessible heavy metal fraction, which is essential for the determination of the detection limit (Rasmussen et al. 1997; Tao et al. 2013). As expected, our studies showed that non-growing biosensors with low cell concentration (Fig. 9) responded to lower concentration of Pb(II) than exponentially growing biosensor cells (Fig. 8). A good linear relationship was observed in a high concentration range of 0.26–8.3 μM Pb(II) exposure at the cells' exponential phase (Fig. 8c). However, a linear relation was observed in a low concentration range of 0.13–4.17 μM Pb(II) exposure during the cells' lag phase (Fig. 9c). Our preliminary studies suggest that optimization of culture conditions facilitates the improvement of the performance of whole-cell indigoidine-based biosensors.

Physical, chemical, and instrumental methods of analysis have been widely used for heavy metal element detection of environmental samples. As an alternative to these methods mentioned above, whole-cell biosensors selectively monitor and quantify the toxic fractions of heavy metal, which are generally more bioaccessible and bioavailable to living organisms (Gupta et al. 2019). The indigoidine-based biosensors developed in this study have been preliminarily demonstrated to have a potential to determine bioavailable heavy metals in drinking water and in environmental water samples (Fig. 10). Several impurities in environmental water have been demonstrated to influence the stability of developed indigoidine-based biosensors. However, the RSD values were greatly decreased with increased concentrations of spiked target Hg(II) and Pb(II), especially in lake water samples. This suggests that the adverse effects of unknown impurities were gradually weakened when the concentrations of spiked target heavy metals were elevated. The stability of heavy metal quantitative detection using this pigment signal output system is expected to be enhanced with increased concentrations of target heavy metal in aqueous environmental samples. In summary, precise metabolic engineering of indigoidine biosynthesis needs to be developed into a low-cost, fast-responding, minimal-equipment biosensor.

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Author contribution C-Y. H. and N-X. Z. designed the study; Y. G., L-M. L., Y-T. C., and J. Y. performed the experiments. C-Y. H. and L. L. analyzed the experimental data; C-Y. H. and L. L. wrote and revised the manuscript. All the authors reviewed the manuscript and approved the final version.

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Data availability All data generated or analyzed during this study are included in this published article (and its Supplementary information files).

Declarations

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

Conflict of interest The authors declare no competing interest.

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