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REVIEW

Synthetic biology for microbial heavy metal biosensors

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Abstract

Using recombinant DNA technology, various whole-cell biosensors have been developed for detection of environmental pollutants, including heavy metal ions. Whole-cell biosensors have several advantages: easy and inexpensive cultivation, multiple assays, and no requirement of any special techniques for analysis. In the era of synthetic biology, cutting-edge DNA sequencing and gene synthesis technologies have accelerated the development of cell-based biosensors. Here, we summarize current technological advances in whole-cell heavy metal biosensors, including the synthetic biological components (bioparts), sensing and reporter modules, genetic circuits, and chassis cells. We discuss several opportunities for improvement of synthetic cell-based biosensors. First, new functional modules must be discovered in genome databases, and this knowledge must be used to upgrade specific bioparts through molecular engineering. Second, modules must be assembled into functional biosystems in chassis cells. Third, heterogeneity of individual cells in the microbial population must be eliminated. In the perspectives, the development of whole-cell biosensors is also discussed in the aspects of cultivation methods and synthetic cells.

Keywords Synthetic biology · Microbial whole-cell biosensor · Heavy metals

Introduction

As industrialization and transportation develop, the resultant dissemination of toxic chemicals, environmental pollutants, and pathogens can endanger human health and threaten ecosystems. Dangerous substances such as harmful chemicals can react with biomolecules or accumulate in cells, causing DNA damage or severe oxidative stress [1]. Therefore, rapid and accurate detection and monitoring of hazardous materials is essential.

Broadly speaking, analytical methods have developed in two different directions. One approach, instrument-based analytical chemistry aimed at precisely monitoring harmful substances, is represented by gas chromatography (GC), high-performance liquid chromatography (HPLC), inductively coupled plasma/mass spectrometry (ICP/MS), atomic absorption spectrometry (AAS), and ion-selective electrodes (ISEs) [2–5]. However, instrument-based analytical methods have several disadvantages, including high prices, technical difficulties, time-consuming sample preparation, and an inability to perform multiplex analyses. The other approach involves biosensors, which combine sensing biomolecules (such as DNAs, aptamers, peptides, enzymes, or antibodies) with physicochemical transducers. These methods have several advantages relative to conventional approaches, including low cost, high speed, and the ability to perform real-time monitoring [6, 7].

Heavy metal biosensors can be categorized into cell-free and whole cell-based systems [8]. Cell-free systems use aptamers [9], nucleic acids [10, 11], peptides [12], enzymes [13–16], antibodies [17], or proteins [18, 19] as probes to sense heavy metals. Although purified enzymes, proteins, and antibodies can detect analytes quickly and specifically, the expression and purification conditions must be optimized, and the active agents must be immobilized [20, 21]. On the other hand, whole cell-based biosensors have several advantages: they are easy and inexpensive to cultivate, can be used in multiple assays, and do not require any special techniques for analysis. Furthermore, information about cellular toxicity

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of target analytes can be obtained using the whole cell-based biosensors, and desired biosensors can be further modulated through genetic manipulation [21–25].

For decades, cellular biosensors for detecting heavy metals have been developed based on classic recombinant DNA technology. These approaches take advantage of the fact that cells can respond specifically to hazardous heavy metals via metal-responsive transcription or two-component signal transduction. These biologically specific responses to heavy metals have evolved as a result of various interactions between microorganisms and metals in the environment (e.g., influx, efflux, and resistance) [26].

In the modern era, cutting-edge DNA sequencing and gene synthesis technologies have accelerated the creation of new synthetic biological systems, including whole-cell biosensors and cell factories [27] (Fig. 1). First, next-generation sequencing (NGS) technologies have generated an enormous amount of sequence information about cellular genomes and environmental metagenomes, from which we can select parts and modules. Standard bioparts (promoters, operators, regulators, pathways, etc.) are available from public websites such as iGEM (http://parts.igem.org) [22, 28–30]. Second, gene synthesis and genome editing technologies have facilitated the construction of tailor-made biosystems for the detection of specific molecules (i.e., biosensors) or the synthesis of biochemicals by synthetic cells (i.e., cell factories). Recently, synthetic biology has enabled the design of a wide range of whole-cell biosensors, based on the concept of modularity [31]. In general, a whole cell-based biosensor consists of a module that senses the input signal and a reporter module that converts the biosensor input into an output signal, all within a chassis cell that implements the designed devices [32, 33].

Here, we review the current status of microbial heavy metal biosensors, along with future perspectives, in terms of synthetic bioparts, sensing and reporter modules, genetic circuits, and chassis cells.

Sensing modules

Because many microorganisms exist and grow in diverse environments containing heavy metals, they have evolved gene regulation pathways involved in metabolic detoxification and homeostasis in the presence of these toxic elements. Resistance to heavy metals in bacteria can be achieved in three ways: removing harmful metals from the cell via efflux pump transporters, converting them into nontoxic or less toxic forms, and biosorption [26, 34]. The nucleotide sequences of various heavy metal-related bioparts are available from microbial genome or metagenome databases. Sensing modules can be constructed from elements such as transcription factors (activators/repressors) or sensor kinases of two-component systems, in conjunction with regulatory elements such as promoters, operators, and ribosomal-binding sites (RBSs).

In prokaryotes, when heavy metals are present in the environment, metalloregulatory proteins (metal-sensing

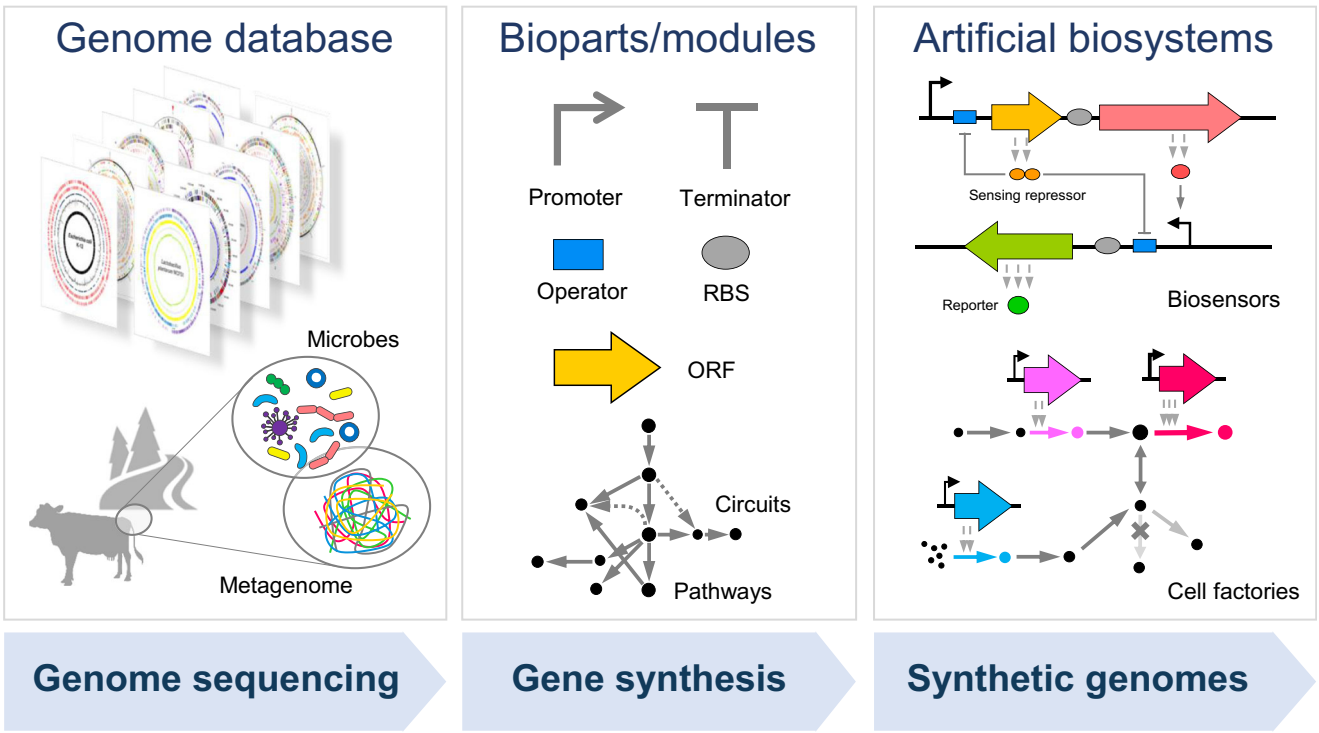

Fig. 1 Synthetic biology for biosensors and cell factories, driven by cutting-edge innovative DNA technologies

transcription factors) sense their presence and drive the expression of metal-resistance genes [35, 36]. Accordingly, diverse bioparts and modules for sensing heavy metals have been excavated from the genomes of various microbes (Table 1). Heavy metal-responsive transcriptional factors and promoters can be retrieved from genome databases by BLAST search [47] and transcriptome analysis [68], respectively. Whole-cell biosensors for heavy metals comprise two main sensing elements: (1) heavy metal–responsive transcription factors, and (2) sensor kinases of two-component systems.

Metal-sensing transcription factors can be classified into five distinct families depending on their structure and function: MerR, ArsR/SmtB, DtxR, Fur, and NikR. Among them, the MerR and ArsR/SmtB families control the expression of genes related to metal ion detoxification, efflux, and sequestration. Members of the MerR family, including MerR (Hg), CueR (Cu), ZntR (Zn), and PbrR (Pb), act mainly as activators [69]. By contrast, members of the ArsR/SmtB family, including ArsR (As, Sb), CadC (Cd, Pb), CzrR (Zn), and NmtR (Ni), act as repressors [70]. Both families include factors that act primarily as co-repressors in association with metal ion uptake [34, 71]. Many types of biosensors have been developed using various metal-responsive transcription factors and regulatory regions.

In the most common scheme, a reporter gene is expressed from regulatory DNA regions (promoter and operator) under the control of transcription factors (activator and/or repressor). In activator-type transcription factors, the regulatory region and reporter gene can be combined directly [36]. For example, a reporter gene was fused to the promoter of the cadmium transporter gene, which is regulated by the CadR transcriptional activator. When cadmium ions are introduced into the cell, the promoter of the reporter gene is activated, and the reporter gene is expressed [45, 62]. In the case of repressor-type transcription regulators, autoregulation of repressor genes is frequently observed; therefore, reporter genes can be transcriptionally fused to a repressor gene that is controlled by the regulatory machinery. For example, reporter genes can be located downstream of transcriptional repressor genes, such as arsenic-responsive *arsR* and cadmium-detecting *cadC*. When arsenic or cadmium ions are introduced into cellular sensors, both repressor and reporter genes can be transcriptionally derepressed and expressed at the same time [40, 42, 50]. In addition, insertion of multiple operators (i.e., DNA-binding sites of the ArsR transcriptional factor) between the P_{arsR} and the reporter gene, or upstream of the reporter gene, can reduce background expression of the reporter gene [41].

On the other hand, two-component systems consist of a sensor kinase (input domain) and a response regulator (receiver domain). A sensor kinase responds to a heavy metal signal by autophosphorylation at a conserved histidine residue. Subsequently, the phosphorylated sensor kinase transfers the phosphate to a conserved aspartate residue in the response regulator [72]. Using two-component systems, prokaryotic cells can detect a wide range of environmental signals, including light, oxygen, pH, temperature, heavy metals, and organic contaminants [73]. For example, in *Escherichia coli*, the ZraRS two-component system detects the concentrations of Zn^{2+} and Pb^{2+} and controls expression of the zinc efflux protein encoded by *zraP* [74]. Similarly, CusRS detects Cu^{2+} and controls expression of *pcoE* [75]. Various metal-responsive two-component systems exist in other species, including silver-detecting SilRS in *Salmonella enterica*, Ni^{2+}- and Co^{2+}-sensing NrsSR in *Synechocystis* sp. PCC6803 [76], and ferric iron-sensing PfeSR in *Pseudomonas aeruginosa* [77].

Over the course of evolution, the specificity of transcription factors for heavy metals has been altered by changes in certain amino acid residues. Comparison of structures of CadC and SmtB proteins, which belong to the ArsR/SmtB family, revealed that CadC transcription factors cannot respond to zinc ions because the presence of the Gly84 residue in CadC, corresponding to Arg87 in SmtB [78]. CadC transcriptional repressors with higher Cd^{2+} or Pb^{2+} specificity have been excavated from microbial genome sequence databases and evaluated experimentally [47, 79].

Recently, molecular engineering of bioparts has been attempted with the goal of modulating heavy metal specificity. FACS (fluorescence-activated cell sorting) was used to isolate a mutant MerR mutant protein that reacts to cadmium, but not mercury, from a *merR* mutant library [60]. Similarly, an error-prone PCR mutant library of the P_{asrR}-*arsR* operator from *E. coli* plasmid R773 was constructed and FACS-sorted to isolate a mutant that is 12-fold more sensitive to As(III) [40].

Reporter modules

In cell-based biosensors, biological responses are converted by the sensing module into a physiochemical signal [80]. Reporters such as color development, bioluminescence, and fluorescence are widely used in these systems [33]. The reporter gene should be selected based on considerations of sensitivity and convenience of measurement. Because the transcription of the reporter gene is under the control of the promoter/operator, the expression level of the reporter gene is designed to be proportional to the sensed signal. Two different reporting methods are currently available. In one method, the reporter gene encodes an enzyme that can catalyze formation of a measurable product. For example, β-galactosidase and luciferase catalyze the generation of a colored product and light, respectively. In the other method, the protein itself is the product that is measured (e.g., green fluorescent protein [GFP]). Enzymatic reactions that yield visible products can lead to considerable signal amplification in comparison with direct fluorescence. For example, luciferase performs better than fluorescence in terms of heavy metal sensitivity [43].

Table 1 Design and performance of various microbial heavy metal biosensors

Analytes	Bioparts and modules	Output signals	Chassis cells	Incubation time (h)	Limit of detection	Characteristics	Reference
Arsenite	P_{ars}-*arsR*(*E. coli*)-*crtA*(*R. sulficophilum*)	Colorimetric	*R. sulfidophilum* Δ*ctrA*	24	3 μg/l (naked eye)	Carotenoid as a reporter	[37]
Arsenite	P_{ars}-*arsR*-*lacZ*(*E. coli*) P_{ars}-*arsR*-ccp(*S. cerevisiae*)	Colorimetric	*E. coli* DH5a	4 (*lacZ*) 4 (*ccp*)	*lacZ*: 0.2-5 μg/L *ccp*:1–10 μg/L	Cytochrome *c* peroxidase gene (reporter) and guaiacol (substrates)	[38]
Arsenite	P_{ars}-*arsR*(*E. coli*)-*mtrB*(*Shewanella* spp.)	Bioelectrochemical	*S. oneidensis*	12	40 - 100 μM	Metal reduction pathway gene (*mtrB*) for bioelectrochemical output	[39]
Arsenite	P_{ars}-*arsR*(*E. coli*)-*gfp*	Fluorescence	*E. coli*	1	0.75 - 3 μg/L	Directed evolution of *arsR* opreon	[40]
Arsenite	P_{arsR}-*arsR*(*E. coli*), P_c-*gfp*-P_{hbpR}-*hbpR*(*P. azelaica* HBP1)	Fluorescence	*E. coli*	2	1 – 100 μg/L	Operator optimization and construction of reporter circuits	[41]
Arsenite, Arsenate	P_{arsR}-*arsR*(*E. coli*)-*phi*YFP(*Phialidium* sp.)	Fluorescence	*E. coli*	2	As^{3+}: 0.05-8 μmol/L As^{5+}: 0.1-25 μmol/L	*phi*YFP as a reporter gene	[42]
Arsenite, mercury	P_{arsR}-*arsR*(*S. aureus*)-*luxCDABE* P_{merR}-*merR*-*luxCDABE* P_{merR}-*merR*(*S. aureus*)-*gfp*	Luminescence Fluorescence	*E. coli*	2	*lux*: As(III) 0.1 μM, Hg(II) 0.025 μM *gfp*: As(III) 0.4 μM, Hg(II) 0.025 μM	Comparison of *luxCDABE*-based and *gfp*-based reporter system	[43]
Arsenite, mercury, copper	[P_{ars}-*hrpR* AND P_{merT}-*hrpS* ➔ P_{hrpL}-*luxI*] ➔ [P_{luxI}-*hrpR* AND P_{cusC}-*hrpS* ➔ P_{hrpL}-*rfp*]	Fluorescence	*E. coli*	6	As: 2.8 μM Hg: 2.2 μM Cu: 1.0 nM	Multiple input biosensor using AND logic gates	[44]
Cadmium	P_{tac}-*cadR*, P_{cadR}-*lacI*^{q}(*P. putida* 06909)-*gfp*	Fluorescence	*P. putida* 06909	4	0.01 μM (1.12 ppb)	Toggle switch	[45]
Cadmium	P_{DR_0659}-*crtI*(*D. radiodurans*)	Colorimetric	*D. radiodurans* strain R1 Δ*crtI*	24	50 nM - 1 mM (naked eye)	Carotenoid as a reporter	[46]
Cadmium, lead	P_{cadC}-*cadC*(*B. oceanisediminis* 2691)-*egfp*	Fluorescence	*E. coli* DH5α	2	Cd^{2+}: 5 – 50 μM Pb^{2+}: 5 – 25 μM	Six CadC homologs with different metal specificities	[47]
Cadmium, lead	P_{cadC}-*cadC*(*B. oceanisediminis* 2691)-T7 RNAP, P_{T7}-*egfp*	Fluorescence	*E. coli* BL21(DE3)	2	Cd: 3 - 30 μM Pb: 10 nM - 10 μM	Amplification module (T7 RNAP-T7 promoter)	[47]
Cadmium, lead	P_{T7}-*cadC*, P_{cadC}-*cadC*(*S. aureus*)-*lacZ* P_{T7}-*cadC*, P_{cadC}-*cadC*-*rsgfp*	Colorimetric Fluorescence	*E. coli* BL21(DE3) *zntA*::KmR	2	*lacZ*: Cd^{2+}, Pb^{2+} 10^{-11} mol/L *rsgfp*: Cd^{2+}, Pb^{2+} 10^{-8} mol/L	Dual-plasmid-based sensing system	[48]
Cadmium, lead, antimony	P_{cadC}-*cadC*(*S. aureus*)-*lucFF*	Luminescence	*Bacillus subtilis* BR151 *S. aureus* RN4220	2	Cd^{2+}:3.3 - 10 nM Pb^{2+}: 33 nM Sb^{3+}:1 nM – 1 μM	*B. subtilis* strains are available as freeze-drying sensor	[49]
Cadmium, lead, antimony	P_{cadC}-*cadC*(*S. aureus*)-*gfp*	Fluorescence	*E. coli* DH5α	2	Cd(II): 0.1 nmol/L Pb(II): 10 nmol/L	High sensitivity in contaminated sediment and soil samples	[50]

Table 1 (continued)

Analytes	Bioparts and modules	Output signals	Chassis cells	Incubation time (h)	Limit of detection	Characteristics	Reference
					Sb(III): 0.1 nmol/L		
Cadmium, lead, arsenite	P_{cadA}-*cadC*(*S. aureus*)-*luc* P_{nptII}-*zntR*/P_{zntA}(*E. coli*)-*luc* P_{arsR}-*arsR*(*E. coli*)-*luc*	Luminescence	*E. coli*	2	Cd^{2+}: 0.1 μM Pb^{2+}: 0.05 μM As^{3+}: 0.1 μM	Binary regression models in a co-polluted environment	[51]
Cadmium, lead, zinc	P_{czc1}(*P. putida* KT2440)-*lux*	Luminescence	*P. putida* KT2440.2431	3	Zn^{2+}: 0.05 μM, Cd^{2+}: 0.09 μM, Pb^{2+}: 0.02 μM	Disrupting metal efflux transporters	[52]
Chromate	P_{chrB}(*O. tritici* 5bvl1)-*gfp*	Fluorescence	*E. coli*, *O. tritici*	5	*E. coli*: 0.1-10 μM *O. tritici*: 1-100 μM	*Ochrobactrium* genus is able to survive in diverse environments	[53]
Copper	P_{cueR}(*P. putida*)-*gfp*	Fluorescence	*P. putida*	3	Cu^{2+}: 1–70 mg/L	Optimization of culture condition	[54]
Gold	*cupR*(*C. metallidurans* CH34)-$P_{cupA/R}$-P_{cupC}-*rfp*	Fluorescence	*C. metallidurans* *R. eutropha*,	24	0 - 1000 nM	Using heavy metal tolerant proteobacteria	[55]
Gold	P_{golB}(*S. enterica* serov. *typhimurium* 14028s)-*gfp*, P_{golTS}-*golS*	Fluorescence	*E. coli golTSB*$^{+}$	3	470 nM	*E. coli* chassis cells low background signal with sensing modules from *S. enterica*	[56]
Lead	P_{pbrR}-*pbrR*(*C. metallidurans* CH34), P_{pbrR}-*rfp*	Fluorescence	*E. coli*	overnight	0.001 μM	Lead biosensor and bio-adsorption	[57]
Lead	P_{pbrR}(*K. pneumoniae* CG43)-*gfp*	Fluorescence	*Enterobacter* sp. NCR3 *Enterobacter* sp. LCR17 *E. coli* *P. aeruginosa* PAO1 *S. oneidensis* MR-1	4	0.2–1 μg/mL	Lead biosensor construct introduced into several Gram-negative bacterial species	[58]
Mercury	P_{merR}(*E. coli*)-*luxCDABE*	Luminescence	*E. aerogenes* NTG-01 *P. putida* X4	4	100 pM – 100 nM	Highly specific mercury biosensor	[59]
Mercury	MerR/P_{mer}(*E. coli*)-*lucFF*	Luminescence	*E. coli*	2	-	Directed evolution of sensing module	[60]
Mercury, lead, gold	P_{golTS}-*golTgolS* (*S. enterica*), P_{golB}-*gfp*	Fluorescence	*E. coli*	3	Hg^{2+}: 4.4 nM Pb^{2+}: 39.6 nM Au^{+}: 42.7 nM	*golS* mutants based biosensor detected Au(I) with high sensitivity	[61]
Mercury, zinc, cadmium	P_{cadR}-*cadR*TC10(*P. putida*), P_{cadR}-*gfp*	Fluorescence	*E. coli*	4	Hg^{2+}:1.6 μM Zn^{2+}: 0.9 μM Cd^{2+}: 1.3 μM	Improved performance with CadR mutants	[62]
Nickel	P_{rcn}-*rcnR*(*E. coli*)-P_{rcnA}-*lux*/Ni transporter(*B. suis*)	Luminescence	*E. coli*	12-20	80 nM – 40 μM	Introducing uptake transporter	[63]
Nickel, cobalt	P_{cnr}-*cnrYXH*(*R. eutropha*)-*luxCDABE*	Luminescence	*R. eutropha*	16	Ni^{2+}: 0.1-60 μM Co^{2+}: 9-400 μM	Highly selective biosensor for nickel and cobalt	[64]
Nickel, cobalt	P_{coaT}-*coaR*(*Synechocystis* sp. PCC6803)-*luxAB* $P_{nrsBACD}$-*nrsR*-*luxAB*	Luminescence	*Synechocystis*. sp. PCC6803	3	Ni^{2+}: 0.2–6 μM Co^{2+}: 0.3–6 μM	*Synechocystis* expressing the *luxCDE*	[65]

Table 1 (continued)

Analytes	Bioparts and modules	Output signals	Chassis cells	Incubation time (h)	Limit of detection	Characteristics	Reference
Zinc	P_{czcR3}(*P. putida* X4)-*gfp*	Fluorescence	*P. putida* X4	4	5 - 55 mmol/L	Different *czcRS* operons with *lacZ* reporter	[66]
Zinc, cadmium mercury	P_{zntR}-*zntR-zntA*(*E. coli*)-*gfp*	Fluorescence	*E. coli*	16	Zn^{2+}: 3 - 800 ppm Cd^{2+}: 0.005 - 4 ppm Hg^{2+}: 0.001 -0.12 ppm	Correlation of sensitivities with toxicities	[67]

As an example of color development, β-galactosidase catalyzes the degradation of *o*-nitrophenyl-β-galactopyranoside (ONPG) to generate yellow *o*-nitrophenol, which can be detected by measuring absorbance at 420 nm on a spectrophotometer [48]. Alternatively, colorimetric reporter systems can exploit the carotenoid biosynthetic pathway. Carotenoids are mainly plant-derived pigments, but are also produced by several bacteria, fungi, plants, and algae. For use of these compounds as reporters in cellular biosensors, one gene in the carotenoid biosynthetic pathway is deleted, and the deleted gene is compensated by a reporter module. The purple photosynthetic bacterium *Rhodovulum sulfidophilum* can synthesize carotenoids through the spheroidene pathway. Specifically, spheroidene monooxygenase (encoded by *crtA*) catalyzes the formation of yellow-colored demethylspheroidenone, which is visible to the naked eye [81]. Fujimoto et al.. developed a cellular biosensor capable of naked-eye detection of 3 μg/L arsenic by placing the *crtA* gene under the control of P_{asrR}-*asrR* in *crtA*-deleted *R. sulfidophilum* cells [37]. Joe et al. constructed a cadmium biosensor in which the cadmium-responsive DR_0659 promoter was fused to the *crtI* gene; the resultant construct was subsequently transformed into *Deinococcus radiodurans* Δ*crtI* cells. Using this strain, it is possible to detect cadmium concentrations as low as 50 nM with the naked eye [46].

Bioluminescence reporters can be classified into two groups according to the type of luciferase. First, bacterial luciferase, encoded in the *lux* operon (*luxCDABE*), converts fatty aldehyde and $FMNH_2$ to fatty acid and FMN, emitting light that can be measured at 490 nm. In whole-cell biosensors, the *luxAB* genes, encoding the catalytic subunits, are combined with sensing modules [33]. Second, firefly luciferase catalyzes a two-step oxidation reaction in which luciferin is first converted to luciferyl-adenylate using ATP as a cofactor, and then oxidized to oxyluciferin; the latter step emits bioluminescence that can be measured at 550–570 nm on a luminometer [80]. Although luciferase is widely used as a reporter because of the convenience of measuring bioluminescence, it should be noted that bacterial luciferase is heat-labile, whereas firefly luciferase requires external substrates [49, 65].

Fluorescence is widely used as a reporter system because of its high stability and ease of measurement, without a requirement for exogenous substrates. Emission of light from fluorescent proteins can be measured by a fluorometer with excitation at specific shorter wavelengths. The GFP from the jellyfish *Aequorea victoria* has been used most frequently in cell-based biosensors [50, 54]. RFP (red fluorescent protein) [55] and phiYFP (yellow fluorescent protein from the jellyfish *Phialidium* sp.) [42] have also been used as reporters in whole-cell biosensors. However, fluorescent reporter proteins have some drawbacks, including the relatively long time required for expression of stable fluorescent proteins and the need to distinguish auto-fluorescence or background

fluorescence from the real fluorescent signal in cells during measurement.

The sensitivities of various reporters in whole-cell biosensors were compared. The limit of detection (LOD) of a β-galactosidase-based construct in a cadmium biosensor is 10 pM, whereas that of a *gfp*-based construct in the same biosensor is 10 nM [48]. Recently, GFP and Lux reporters were compared in a whole-cell arsenite biosensor. The LOD of a *gfp*-based construct was 0.4 μM 2 h after induction, whereas that of a *lux*-based construct was 0.1 μM [43]. Together, these reports indicate that enzyme-based reporters are more sensitive than fluorescence-based reporters in the same biosensor cells.

Several examples exist of arsenite biosensor cells that utilize different types of enzyme reporters. The *mtrB* gene in the metal reduction pathway in *Shewanella* sp. has been harnessed as a reporter gene in arsenite biosensor cells, in which reduction of iron(III) citrate by MtrB is detected using a bioelectrochemical system [39]. In an *E. coli*-based arsenite biosensor, the cytochrome *c* peroxidase gene from *Saccharomyces cerevisiae* is utilized as a reporter in the presence of its substrate, guaiacol [38].

Genetic circuits

Genetic circuits, including toggle switches, amplification modules, and AND gates, can be introduced into cellular biosensors that harbor sensing and reporter modules to improve both specificity and sensitivity (Fig. 2).

In one study, a toggle switch was constructed to improve heavy metal sensitivity [45]. In this system, a cadmium-inducible promoter P_{cadR} was fused to the *lacI*^{q} and *gfp* genes, and an IPTG-inducible promoter P_{tac} was placed upstream of the *cadR* gene. In the presence of IPTG, abundant CadR protein tightly represses the P_{cadR} promoter. In the presence of cadmium ions, the *lacI*^{q} and *gfp* genes are derepressed, resulting in expression of the LacI and GFP proteins. Subsequently, LacI represses the P_{tac} promoter and decreases expression of the *cadR* gene, leading to higher expression of the *gfp* gene. Using this toggle switch, background fluorescence was reduced, and the LOD was increased dramatically.

Amplification of intracellular signals is an important strategy for improving sensor cell performance. Transcriptional signals can be amplified via gene network cascades. Wang et al. showed that the transcriptional signal of *arsR* gene derepression could be amplified and transferred to the P_{hrpL} promoter using the fixed-gain *hrpRS* amplifier [82]. Alternatively, signal amplification can be achieved by a robust T7 transcription system. The T7 promoter is recognized with high specificity by T7 RNA polymerase (T7 RNAP) [83], but not by *E. coli* RNA polymerase (i.e., the promoter has high biological orthogonality). Recently, Kim et al. reported cadmium- or lead ion-detecting CadC-T7 whole-cell biosensors with improved specificity and sensitivity. In their system, a sensing module (P_{cadC}-*cadC*) was combined with the T7 RNAP structural gene in the chromosome, and the T7 promoter was fused to the *egfp* reporter gene in the plasmid. In the presence of cadmium or lead ions, T7 RNAP is expressed, immediately turning on the T7 promoter. The CadC-T7 amplification system improves the sensitivities of heavy metal

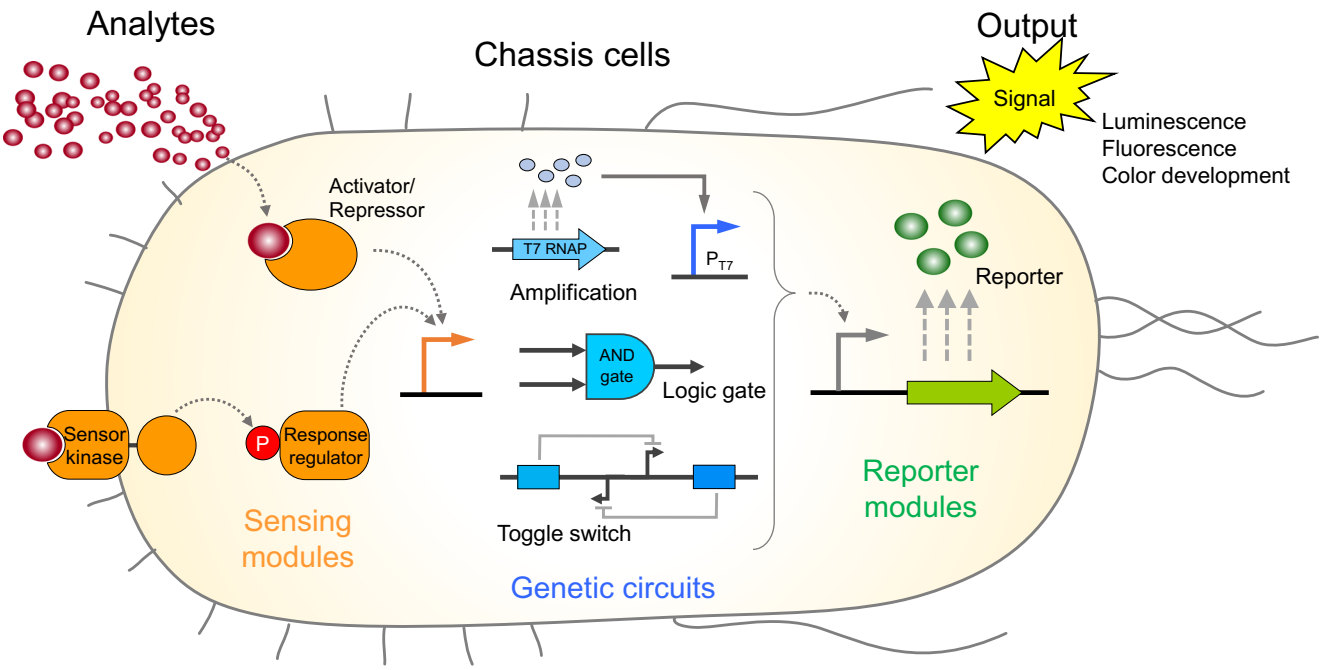

Fig. 2 Transfer of sensing signals to reporters by genetic circuits in whole-cell biosensors

biosensors in comparison with prototype biosensors in which the sensing module (P_{cadC}-*cadC*) is directly fused with the *egfp* reporter gene [47].

An AND logic gate can integrate two input signals to make a single output with a higher signal-to-background ratio. Because T7 RNAP can function even when its N-terminal and C-terminal domains are expressed separately (split T7 RNAP), it can be used in transcriptional AND gates [84, 85]. Pu et al. reported that split RNAP components can be optimized by phage-assisted continuous evolution (PACE), and therefore represent a versatile biosensor platform [86].

A multi-input whole-cell biosensor was developed to detect heavy metals using a combination of various sensing modules, AND gates, and cell-to-cell communication [44]. First, it was confirmed that various heavy metal-sensing modules such as arsenite-responsive P_{arsR}, copper-reactive P_{cusC}, and cadmium-sensing P_{zntA} could be combined to construct AND logic gates. Next, transcription of the sensor kinase *hrpS* and response regulator *hrpR* was placed under the control of heavy metal- and quorum-sensing AND gates. The results showed that a multi-input logic gate could be used as a biological signal filter or amplifier to increase the sensitivity and selectivity of cell-based biosensors.

Chassis cells

Chassis cells for device implementation in whole cell-based biosensors can be categorized as eukaryotic microorganisms or prokaryotic cells.

Eukaryotic microorganisms

The primary advantage of whole-cell biosensors based on eukaryotic microbial cells, including yeasts, ciliated protozoa, and microalgae, is that their cellular organization and metabolic pathways are more similar to those of other eukaryotic cells, including humans, than those of bacteria [80].

Yeasts are well-known eukaryotic model organisms that have been used in both basic research and industrial applications. Among them, *Saccharomyces cerevisiae* is the most widely used in biotechnology and bioengineering [87]. A whole-cell biosensor using *S. cerevisiae* as a chassis cell and GFP as a reporter was designed to detect copper ions [88, 89]. Specifically, the cadmium-inducible *SEO1* promoter found in the genome of *Hansenula polymorpha* was recombined with the *gfp* gene to develop a biosensor that could detect cadmium with the range from 1 to 900 μM [68].

Because ciliated protozoa do not have cell walls in the vegetative phase, and their metabolic pathways are more similar to those of human cells than yeast or bacterial cells, they can be used in exotoxicological applications involving toxic compounds such as heavy metals [90]. Ciliated protozoa-based biosensors have been constructed by fusing the *Tetrahymena thermophila* MTT1 and MTT5 metallothionein promoters and the eukaryotic firefly luciferase gene (*lucFF*), yielding systems capable of detecting heavy metals such as Cd, Pb, As, Hg, Zn, and Cu [91–93]. In addition, microalgae-based biosensors are very important for marine applications [94], and an electrochemical biosensing system using the flagellate microalga *Chlamydomonas reinhardtii* to detect toxic chemicals (toluene, Cu^{2+}, or Ni^{2+}) has also been developed [95]. However, eukaryotic cell-based biosensors have two main limitations: only a few heavy metal-sensing bioparts are available, and successfully developed sensor cells are quite rare.

Prokaryotic cells

Bacterial biosensors have several advantages over eukaryotic microbial chassis cells. Because of their long evolutionary history of adaptation to diverse environments, bacterial cells carrying the desired bioparts can be obtained easily, and a wide range of tools for genetic manipulation have been developed. Moreover, due to their simple growth conditions and high growth rates, bacterial cells can be used easily as biosensors. *E. coli* cells, which are suitable for the heterologous expression of bioparts found in other bacteria, have been widely used as chassis cells for whole-cell biosensors [48, 50].

In many cases, the bacteria used as chassis cells are the species in which the sensing or reporter modules were originally found. For example, to use the *crtA* gene of *Rhodovulum sulfidophilum* as a reporter, a *crtA*-deleted strain of *R. sulfidophilum* was used as the chassis cell [37]. In another system, the photosynthetic bacterium *Rhodopseudomonas palustris* was harnessed as a chassis cell, and the *crtI* gene (involved in carotenoid biosynthetic pathway of *R. palustris*) was used as a reporter [96]. A metal reduction pathway gene was excavated from *Shewanella oneidensis* for use as a reporter gene, and *S. oneidensis* was used as the chassis cell [39]. *Ochrobactrum tritici* strain 5bvl1, in which the chromate-responsive *chrB* promoter was identified, has also been used as a chassis cell [53]. *Pseudomonas putida* strains have been employed as chassis cells because of the abundance of various heavy metal-responsive transcription factors in this species, including CadR, CzcR, and CueR [45, 52, 54, 66].

Significant effort has been devoted to identifying chassis cells that grow well in specific conditions or harsh environments. For example, *Deinococcus*, a desiccation- and radiation-tolerant bacterium isolated from the environment, has been used as a chassis cell for a heavy metal biosensor [46, 97]. Heavy metal-tolerant proteobacteria *Cupriavidus metallidurans* and *Ralstonia eutropha* have also been used as robust analytical hosts [55, 64].

Many studies have been carried out to find out which microorganisms perform best as chassis cells. The reactivity of P_{cadC}-*cadC-lucFF* to cadmium and lead, achieved using the

cadC gene derived from plasmid pI258 in *S. aureus*, was confirmed using the Gram-positive bacteria *S. aureus* and *B. subtilis* as the chassis cells. Although the heavy metal sensitivities were similar in both strains, the induction coefficient (the ratio of luminescence between induced and uninduced cells) was higher in *S. aureus* than in *B. subtilis* [49]. Recently, the sensitivities of a lead-responsive *pbrR* construct were tested in several different Gram-negative bacterial chassis cells, including *E. coli*, *Enterobacter* sp., *Pseudomonas aeruginosa*, and *Shewanella oneidensis*. The most sensitive was *P. aeruginosa*, which was capable of detecting 0.1 μg/mL lead [58].

Heavy metal uptake and release in chassis cells can also be modified to improve the sensitivities of heavy metal cellular biosensors. It has been known that cadmium and lead ions can be transported into cells via intrinsic manganese and zinc uptake pathways [98, 99]. In one case, introduction of a metal uptake transporter increased the intracellular nickel concentration [63]. In another, a metal efflux pump gene was disrupted to prevent leakage of heavy metals from the sensor cells [52]. Alternatively, the ligand-responsive receptor concentration in the chassis cells represents another factor that could be tuned to dramatically improve sensitivity [100].

Proper cultivation of microbial cells is indispensable for the operation of cell-based biosensors. Microfluidic systems are thought to be suitable for application to microbial sensors because they can be used to grow small cultures in a short time, and can be used in multiple assays with high resolution and repeatability [101]. ArsR-based whole-cell biosensors have been applied to micro-centrifugal microfluidic platforms to achieve miniaturization of biosensing systems and rapid detection of arsenite [102]. In addition, cellular biosensors have been encapsulated in agarose beads, which were subsequently immobilized in a microfluidic system; one such system is capable of detecting 10 μg/L arsenite [103]. Additionally, a chemostat-like microfluidic platform in conjunction with microbial biosensors has been developed to facilitate the sensitive detection of lead and cadmium ions [104]. These microfluidic systems will help to overcome some of the weaknesses of cell-based biosensors, such as low sensitivity and inconvenient portability.

Synthetic biology

In order to develop cell-based biosensors, it is essential to design, manufacture, and analyze biosystems from the perspective of synthetic biology. Systematic creation and optimization of a biosystem requires a design–build–test process [105].

In the design step, bioparts that play pivotal roles in standardization and modularity should be identified from genome databases. A chassis cell should be also chosen in which the bioparts can work properly without host interference. Bioparts or genes with the potential for cross-reactivity should be removed from the genome of chassis cells. In addition to improving chassis cells, molecular engineering of bioparts should be continued to artificially upgrade sensing modules, without depending on the discovery of sensing modules in nature (Fig. 3A).

During the build process, DNA synthesis is required to assemble the excavated bioparts, enabling them to operate functionally. Artificial biosystems composed of parts and modules from diverse microorganisms must work properly in specific chassis cells. For example, codon optimization is required to properly drive eukaryotic bioparts in prokaryotic chassis cells (Fig. 3B). Although replacing rare codons with frequent codons during gene synthesis represents an attractive strategy for achieving higher expression of proteins, it must be noted that proteins with altered conformation or substrate specificities could be generated by differential folding due to ribosome stalling at synonymous codons (i.e., translation speed) [106–108].

In the testing step, the specificity and sensitivity of constructed biosensors should be verified by various experimental methods. Although biosensors are carefully designed to achieve higher sensitivity and specificity, it is often impossible to obtain reproducible results because of signal interference or cellular heterogeneity in the population. Accordingly, genetic and environmental aspects should be considered to improve the biosystems or synthetic cells.

Measurement of total bioluminescence and fluorescence of cultured microorganisms can be used to estimate the responses of whole-cell biosensors to analytes. Although this method is simple and convenient, it can overlook heterogeneity of the cells in the population, potentially causing reproducibility problems (Fig. 3C). To eliminate the heterogeneity of microbial populations, synthetic genetic circuits can be used to drive individual biosensor cells in the desired direction.

Perspectives

To develop whole-cell biosensors, we must consider several aspects of cultivation methods and synthetic cells. Self-replication is a major advantage of whole-cell biosensors because it is not necessary to repeatedly produce the sensors. Whole-cell biosensors are living cells, and can monitor the toxicity and bioavailability of analytes, representing a fundamental advantage relative to analytical instruments. However, cell-based biosensors can be affected by environmental variables or growth-inhibitory factors in real environmental samples. Because cellular responses may also depend on physiochemical conditions such as medium, temperature, pH, etc., cell culture and assay methods must be standardized and optimized. It is possible to develop effective cell-based biosensors by combining them with new cultivation technologies such as microfluidics or nanotechnology [101]. In addition,

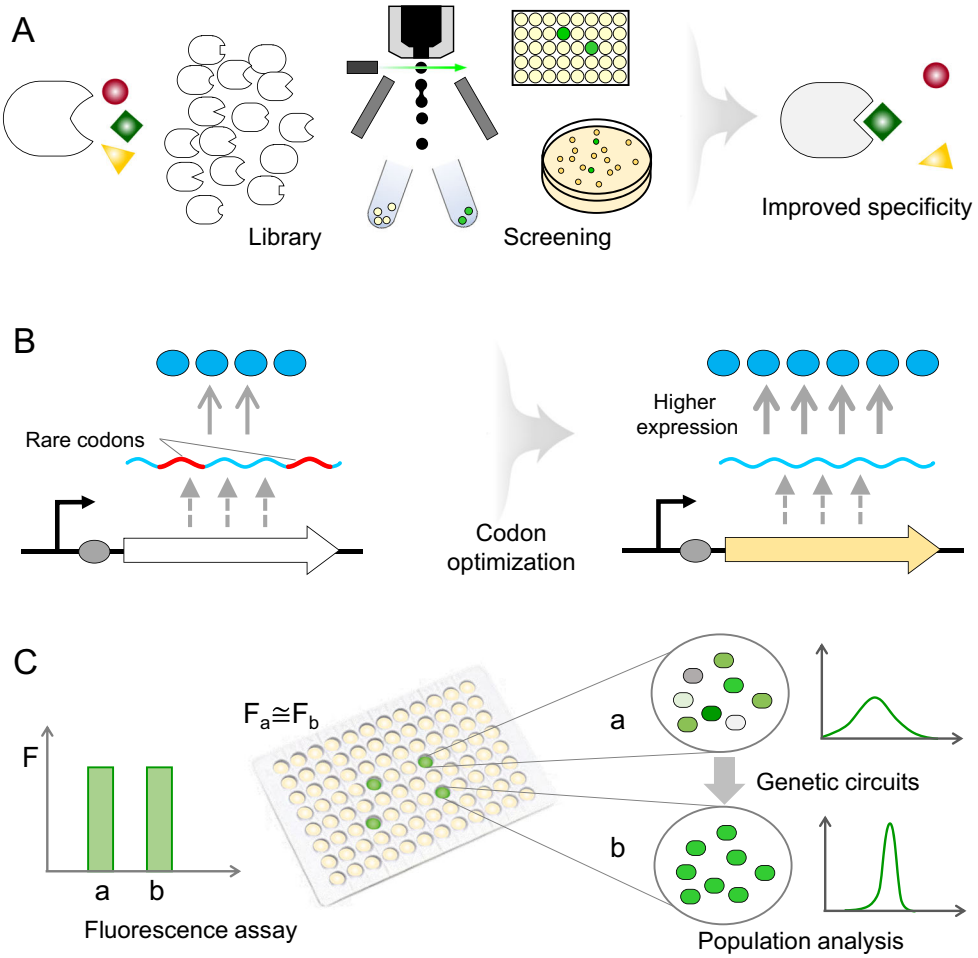

Fig. 3 **(A)** Improvement of cell-based biosensors by molecular engineering of bioparts. **(B)** Proper assembly to achieve functional expression and codon optimization in chassis cells. **(C)** Elimination of cellular heterogeneity in the microbial population

the development of appropriate technology should be taken into account. For example, naked-eye detection of color generated by pollutants in cell culture could be more cost-effective than detection methods that require expensive instruments.

The sensitivity and specificity of cell-based biosensors have been successfully improved by engineering of sensing modules such as transcription factors [40, 60] and operators [41], increasing metal ion uptake into chassis cells [52, 63], and introducing artificial genetic circuits or gates [47, 109, 110]. Synthetic cells can grow and respond to environmental signals based on the programming of their genomes and genetic circuits. The creation of new genetic logic gates has the potential to efficiently modulate and transfer cellular signals. Such approaches could also accelerate our understanding of regulatory networks in biological systems. Synthetic biology has great potential to be applied in cell-based biosensors to ultimately achieve programmed/customized sensitivity, specificity, and dynamic ranges of sensors to meet their real world detection requirement. The amount of available genome information, from which we draw bioparts and modules, is increasing rapidly, and these resources can be combined and reassembled in new synthetic cells. It is therefore doubtless that the development of synthetic whole-cell biosensors will continue in the future.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest regarding the publication of this article.

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