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REVIEW ARTICLE

Modular tuning engineering and versatile applications of genetically encoded biosensors

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ABSTRACT

Genetically encoded biosensors have a diverse range of detectable signals and potential applications in many fields, including metabolism control and high-throughput screening. Their ability to be used *in situ* with minimal interference to the bioprocess of interest could revolutionize synthetic biology and microbial cell factories. The performance and functions of these biosensors have been extensively studied and have been rapidly improved. We review here current biosensor tuning strategies and attempt to unravel how to obtain ideal biosensor functions through experimental adjustments. Strategies for expanding the biosensor input signals that increases the number of detectable compounds have also been summarized. Finally, different output signals and their practical requirements for biotechnology and biomedical applications and environmental safety concerns have been analyzed. This in-depth review of the responses and regulation mechanisms of genetically encoded biosensors will assist to improve their design and optimization in various application scenarios.

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Genetically encoded biosensors; synthetic biology; function fine-tuning; response curve; input signal extending; output signal

Introduction

After the first biosensor, an electroenzymatic glucose sensor based on an immobilized glucose oxidase electrode, was created in 1962 [1], various biosensors have been developed. Genetically encoded biosensors can recognize and respond to specific intracellular and extracellular stimuli (such as changes in a metabolite concentration, O_2, light, or pH) and transduce them into easily detectable output signals (such as fluorescence, color, or cell survival) by designing gene circuits to control the reporter's expression [2,3]. Given the wide variety of biosensors, this review is limited to genetically encoded biosensors that are cell based.

Genetically encoded biosensors have many advantages over other detection methods, such as the noninvasive detection of a broad spectrum of metabolites [4–6], online monitoring and control of *in vivo* signals in real-time, and high-throughput screening at the single-cell level with advanced selection systems [7]. Therefore, these biosensors have gained much interest in the biomanufacturing field for the optimization of microbial cell factories, being regarded as instrument gauges for microbes. They can be used for the dynamic control of metabolic pathways, fast high-throughput screening, and multiplexed evaluation of phenotypes and novel biocatalysts. Besides, their advantages have increased their application in many fields, including: biomedicine, food safety, and environmental monitoring [8]. Several cell-based genetically encoded biosensors have already progressed to clinical trials [9,10]. However, there are still very few commercial examples. Despite the great potential of the genetically encoded biosensors, the lack of versatility and function tuning of these biosensors limited their applications. This review focuses on the current efforts and future concepts of functional design principles of various genetically encoded biosensors based on the modularity. We tried to characterize the interdependency between tunable parameters and the dose-response curves of the biosensors, exposed how engineered sensing modules can facilitate the tuning of biosensors for new ligands, and summarized the signal output of biosensor that can interface the genetically encoded biosensors with various application platforms.

Architecture and classification of genetically encoded biosensors

Paralleling the principles of electronic circuit design, the construction of genetically encoded biosensors in

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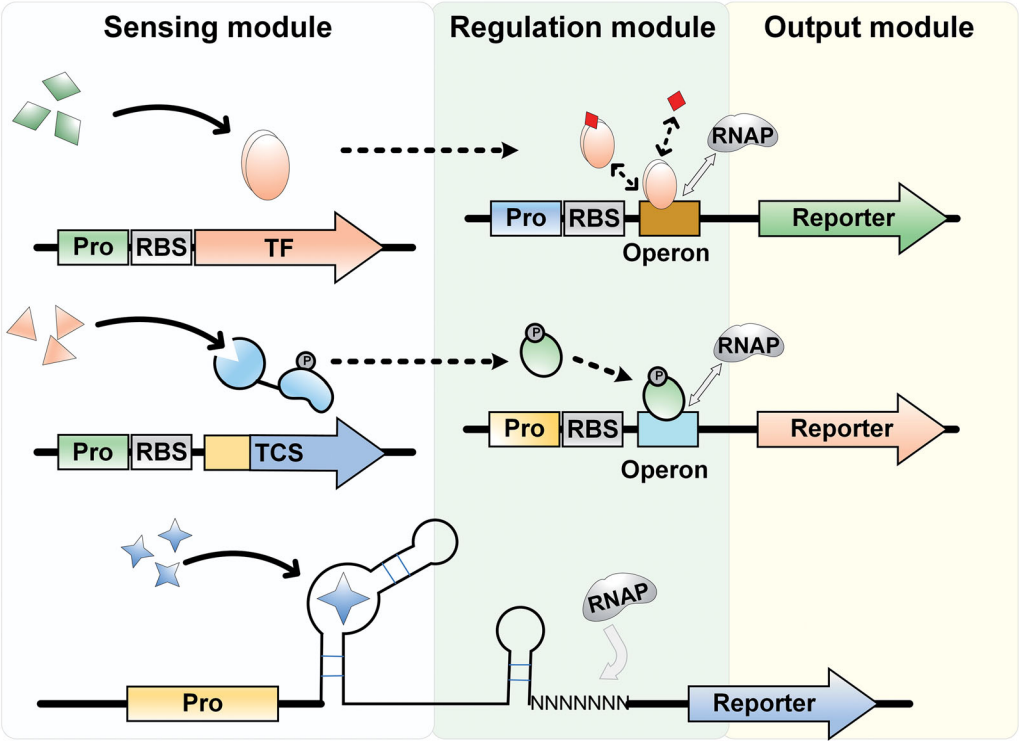

Figure 1. Architecture and classification of genetically encoded biosensors: From top to bottom: Transcriptional factor-based biosensor: It contains the transcriptional factor (TF), the promoter, the regulatory sequence and the regulatory gene (RNA polymerase is an important component and considered as a general configuration in these biosensors). TF promotes or prevents the binding of RNA polymerase to specific gene sequences by altering the transcription rate of the output genes through their interaction with ligands. Two component system-based biosensor. It contains the histidine kinase (HK), the response regulator (RR), the promoter, the regulatory sequence and regulatory gene. The extracellular sensing part of HK (indicated in blue) senses the ligand and causes autophosphorylation, and then transfers the phosphate group to the RR (indicated in yellow), and the phosphorylated RR binds to the regulatory sequence (indicated in red) to activate the transcription of the target gene. RNA-based biosensor. It contains the promoter, the riboswitch sequence, an expression platform and an output gene. The specific region in the riboswitch binds to mRNA (indicated in yellow), which hinders translation. When the ligand is present, the ligand binds to the ligand binding region to change the conformation of the riboswitches that terminates transcription or initiates translation.

synthetic biology needs to be standardized and modularized. A genetically encoded biosensor can be conceptualized as a processor containing a sensing module, a regulation module, and an output module that can also be reduced to rewiring a target-responding input to an observable output. Practically, implementing genetically encoded biosensors requires the selection of the chemical compound to be analyzed, identification of the correct genetic elements responding to this analyte, and the correlation of the responding gene's expression with the given genetic elements. Biosensors can be mainly categorized by transcription factors (TFs), RNA, or two-component systems (TCSs), based on their mode of action (Figure 1). These categories are widely found in nature as regulatory elements that respond to specific metabolites or other signals concentration and alter their own structures to regulate the output of downstream gene elements.

TF-based biosensors are developed mainly on transcription regulator proteins that promote or prevent the binding of RNA polymerase to specific gene sequences by altering the transcription rate through their interaction with ligands. TFs usually contain a DNA-binding domain (DBD) responsible for the recognition and binding of the operator DNA, and a ligand-binding domain (LBD) responsible for the binding of the effector and transmission of the effector signal to the DBD. TFs are widely present in nature; for example, *Escherichia coli* contains more than 230 TFs [11]. The design and construction of TF-based biosensors can be achieved by using TFs and its synthetic metabolite-responsive promoters to regulate bioreporters with fold-change outputs. Numerous TF-based biosensors have been created that respond to various metabolites, such as xylose, amino acids [11–14], organic acids [15–17], aromatic compounds [18–20], flavonoids [21], macrolides [22], fatty acyl-coenzyme A [23–25], butanol [26], alkane [27], and NADPH [28] biosensor. Koch (2018) *et al* established a small-molecule library containing more than 4000 small molecules that can be sensed using known genetically encoded biosensors [29].

RNA-based biosensors mainly include riboswitches and their variations, known as aptazymes, which are RNA regulatory elements comprising a ligand-sensing aptamer and an expression platform [30,31]. The aptamers respond to intracellular ligand concentrations to generate a conformational change, leading to premature transcription termination, inhibition of translation initiation, or mRNA degradation [32,33]. Therefore, RNA-based biosensors usually have a lower metabolic burden [34] and tighter gene regulation [35] than other biosensors. Many natural riboswitches have been developed into biosensors, responding to metabolites including: amino acids, vitamins [36], coenzymes [37], nucleobases or their derivatives [38], and other small-molecule ligands [39,40]. The natural lysine riboswitch has been used to construct lysine biosensors to monitor changes in the intracellular concentration of lysine [41,42] and improve lysine production in *Corynebacterium glutamicum* [43]. As a riboswitch component, ribozymes cleave themselves in a highly sequence-specific manner to control gene expression [44–46]. Ribozymes have been developed as *in vivo* biosensors for molecules including: N-acetylneuraminic acid [47], glucosamine-6-phosphate [32], and flavin mononucleotide [36].

TCSs are often designed as genetically encoded biosensors to detect extracellular signals [48]. Complementary to TF-based biosensors, they can sense extracellular signals such as: light, O_2, or osmolarity changes [49] and transmit them to intracellular transcriptional changes. TCSs contain a histidine kinase (HK) and a response regulator (RR). The membrane-bound HK has an N-terminal stimulus-sensing domain localized in the extracytoplasmic compartment and a C-terminal autokinase domain, usually in the cytosol. HK catalyzes its autophosphorylation and then transfers the phosphoryl group to an RR, which regulates gene expression [50]. The HK domain of native TCSs with various signal recognition capabilities has been used to construct a series of TCS biosensors for quantifying: methanol [51], fumarate [52], malate [53], and acidic amino acid [54]. Because of the homology of the HK domains, chimeric TCS systems can also be created by swapping these domains in HK and their cognate regulators [55].

Tuning the functions of genetically encoded biosensors

Dose – response curve parameters for genetically encoded biosensors

Recent work has focused on developing new biosensors, but their characteristics often cannot meet the specific requirements of practical applications. The metabolite concentration must lie within the detection range of these biosensors to allow precise dynamic regulation and high-throughput screening. It is still a challenge for function tuning of the genetically encoded biosensors. Early experimental reports usually valued at the extremes of a dose-response curve (uninduced and fully saturated induction) or in some instances just the ratios of the saturated levels in the form of "fold increases", while, great progress has been made so far. The calculation and determination of multiple parameters of the biosensor's dose response curve is a beginning of the biosensor engineering toward precision and quantification.

The dose–response curve relates chemical concentration (input or the dose) to enzyme expression (output or the response), which can truly reflect biosensor's function. Here, we summarize and characterize the shape of the curve using four parameters: (1) basal leakage, (2) dynamic range, (3) response threshold, and (4) response sensitivity [56,57] (Figure 2(A)). Basal leakage refers to the output leakage level, which represents gene basal expression before activation or repression. Dynamic range is the vertical change parameter of the response curve. It represents the maximal expression increase/decrease relative to the basal leakage and is also called the "induction fold-change". Response threshold (θ) is the concentration required for 50% maximal output expression and is also called the "half-maximal effective concentration" (EC_{50}). Response sensitivity is defined as the slope of the dose–response curve at the threshold.

The shape of the response curve (and parameter values) can be altered experimentally by modifying genetic properties or tuning their specific arrangement in the biosensor [57]. Here, we classified the experimentally tuning strategies of biosensors into four categories according to the four parameters of the dose–response curve. For TF/TCS -based biosensors, their function and characteristics mainly depend on the physical interactions between ligands and TF/TCS, TF/TCS and DNA (operator site)/RNA Polymerase (RNAP), and DNA and RNAP. Many studies have attempted to elucidate the relationships between experimental tuning and biosensor properties. Because of the different mechanisms of action, the tuning of TCS -based biosensors may be more complicated than TF -based biosensors and the tuning of the dose–response curve of the riboswitch includes modulating its dynamics through RNA folding and its responsiveness to ligand binding. The folding rate constant and the ligand dissociation constant for

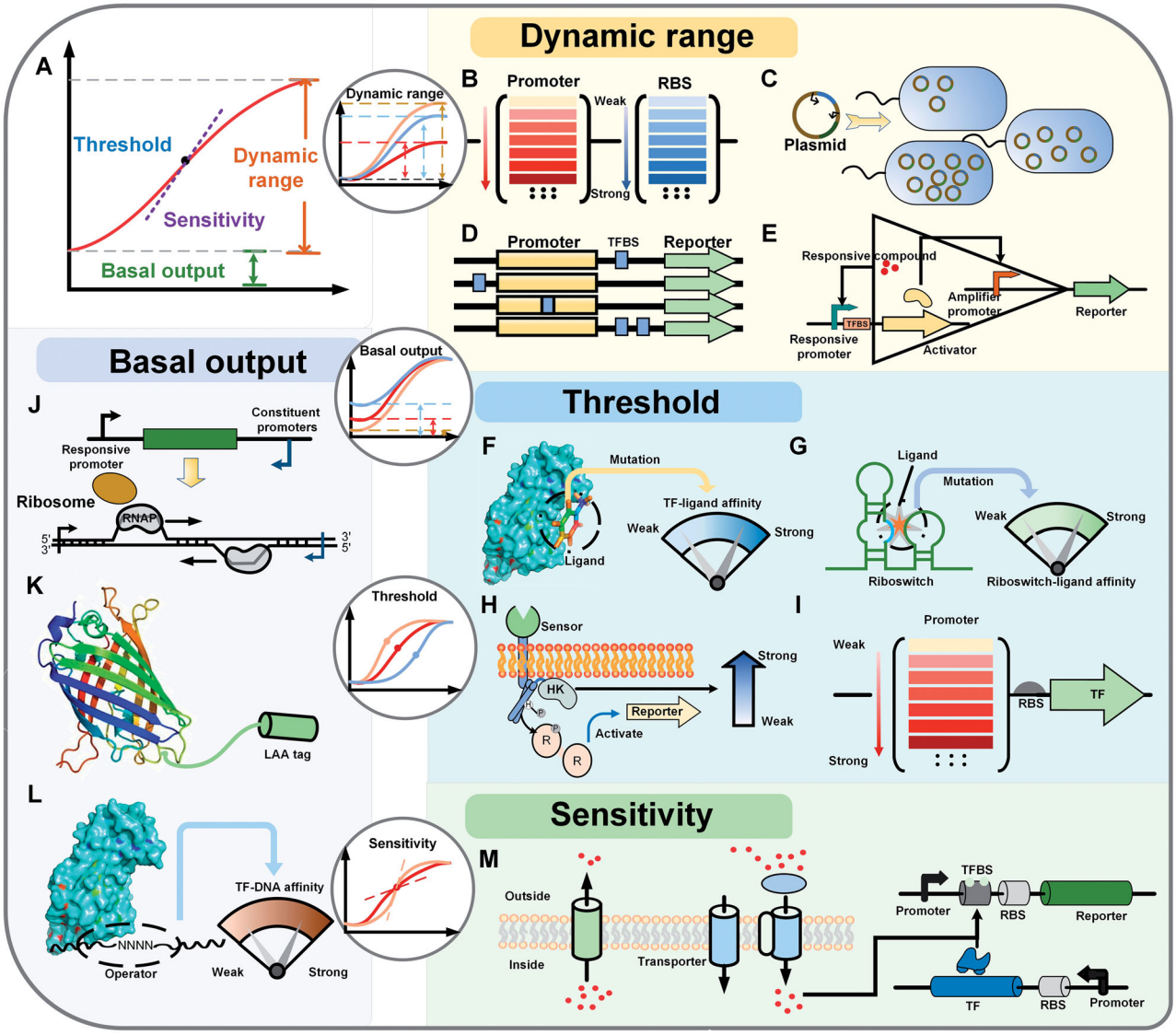

Figure 2. (A) Function tuning strategies of the genetically encoded biosensors toward dynamic range B-E; threshold F-I; basal leakage J-L and sensitivity M. Strategies for changing the dynamic range of biosensors. (B) Change the promoter and RBS strength of reporter gene; (C) Change the copy number of the plasmid containing the regulatory gene; (D) Change the position and number of transcriptional factors binding sites; (E) Using genetic amplifiers, the ligand activates the expression of the activator, and the activator activates the expression of the reporter. Strategies for changing the threshold of biosensors. (F) Mutate key sites of transcriptional factors to change ligand affinity; (G) Mutate key sites of riboswitch to change ligand affinity; (H) Change the enzyme activity in the two-component system to change the activation level of RR on the reporter; (I) Change the promoter strength of transcriptional factors. Strategies for changing the basal leakage of biosensors. (J) Adding a reverse promoter sequence to reduce the expression of the reporter gene. (K) Adding degradation tag to reporter gene; (L) Mutate key sites of transcriptional factors to change DNA affinity. Strategies for changing the sensitivity of biosensors. (M) Adding a specific transporter of target small molecule.

the aptamer can provide critical references for the tuning of these biosensors.

Dynamic range

The dynamic range represents the vertical operational range of the dose–response curve, it reflects how strong or distinguishable are signals between uninduced and induced cells. It can be shifted or scaled by tuning the promoter strength and by changing the plasmid copy number encoding the output gene or altering the integrating times of the gene into chromosomes. To optimize a naringenin biosensor, a combinatorial library of different plasmid copy numbers of the reporter gene was generated to test the impact of plasmid copy number on the dynamic range using model

based on the Hill equation [58]. The dynamic range can scale up proportionally within a certain plasmid copy number range (Figure 2(C)).

Studies have also indicated that the dynamic range can be tuned by changing the plasmid copy numbers of the encoding TFs [59,60]. Tuning the dynamic range can also be accomplished by tuning the transcriptional activities or translation rates of the regulated genes through promoter engineering, RBS (ribosomal binding site) engineering, 5′ untranslated region engineering, or changes of transcriptional factor binding sites (Figure 2(B,D)).

Because the regulation modules of genetically encoded biosensors are ligand-inducible promoters, the increased protein production rates will improve both ON (in the presence of an inducer) and OFF states (in the absence of an inducer), which causes the dynamic range to shift or scale unpredictably. To make this tuning more precise and cost effective, core promoter strengths of the regulated gene have been modified using various −10 and −35 sites. For example, promoter strength modulation by substituting the −10 and −35 regions of the weak P_{lacUV5} with those of the promoter PA1 (phage T7) produced a four-fold increase in the dynamic range. Employing the strong phage lambda promoter (P_L), P_{T7} promoter, and the FadR operator site to replace the natural FadR-responsive promoters, the dynamic range of a fatty acid biosensor exhibited a significant 60-fold increase [56].

To have a spectrum of dynamic ranges, a modular approach was used to modulate promoter strength by assembling promoter libraries of various −10 and −35 regions. The dynamic range of a series of promoters was elaborately tuned because the library covered the entire range of possible input and output relationships. A thermodynamic model for the transcriptional rate of the AraC- and LasR-regulated promoters was developed by predicting the contribution of σ^{70} binding-free energy to the overall transcriptional initiation rate. It effectively identifies the ideal promoters with user-defined dynamic ranges [61].

In *Saccharomyces cerevisiae*, different positions of FapR-binding sites in the core promoters were evaluated to enlarge the dynamic range of a FapR-based biosensor. Six locations and their combinations generated a high maximum dynamic range with minor leaky expression under the OFF state. To simulate the yeast native system, a fuzing FapR with a yeast native repressor can further increase the maximum dynamic range because of the strong repression effects [62]. Meyer et al. (2018) engineered 12 biosensors to facilitate the precise regulation of different biosynthetic pathways, thereby making these biosensors suitable for different scenarios [63]. Mutations in the regulating promoters that occur in the −10 or −35 regions affect absolute expression.

In addition to modulating core promoter strength, genetic amplifiers were used to boost the dynamic range by using additional regulators (Figure 2(E)). For example, adding transcriptional activators or repressors, to amplify the tunable gain to the amplifier's promoter [64]. Because the genetic amplifier may also amplify any basal expression, methods to reduce leakage with different combinations of genetic amplifiers were used to maximize the amplification of the dynamic range [65]. The dynamic range of the riboswitch biosensors can also be increased by increasing the copy number of the plasmid and modulating the strength of the promoters of the riboswitch and the regulated gene. An artificial L-tryptophan riboswitch was optimized by modulating its constitutive expression level [66].

Basal leakage

Basal leakage reflects the basal level of transcription activity, especially in the activation expression case. A high basal leakage usually indicates elevated gene leaky expression, which is highly disadvantageous for metabolic control applications. Leaky expression is usually decreased by acting on the transcription and translation of the reporter gene.

It is essential to tune this basal level without affecting the maximal output level (the ON state). Altering the affinity between the TF and its DNA operator sites was found to effectively reduce basal leakage (Figure 2(L)). A series of mutations at the LacI binding site in lacUV5-based promoters caused significant differences in the resulting dose–response curves. A TF-based biosensor with a low TF–operator affinity produces an almost constitutive output, independent of the metabolite. As the TF–operator affinity increases, the basal fluorescence decreases. The TF–operator affinity change has a relatively minor impact on the maximal output because this affinity may not affect the transcription activity of the promoter [56]. Changing the basal leakage of a downregulated gene amounts to varying the repression strength of each bound TF protein. The basal leakage changes can also cause a fold-change in the dynamic range and threshold because these parameters are usually related to each other.

Basal leakage can also be reduced by acting on translation of the regulated reporter gene, which can be achieved using degradation tags and antisense transcription, accelerating the reporter protein turnover to

minimize basal leakage (Figure 2(K)). Changing the strength of the degradation tag can help optimize this effect [65]. Antisense RNA can be used to reduce leaky expression post-transcriptionally by generating double-stranded RNA, thus triggering RNA degradation, or preventing translation (Figure 2(J)) [67]. The strength of the promoter used to generate the antisense RNA can alter how much of the mRNA has bound complementary RNA, thereby interfering with its translation. Antisense transcription can also directly interfere with mRNA transcription. Although adding degradation tags and antisense RNA can also affect the maximum output, their expressions must be tuned to eliminate this side effect. Like all gene regulatory systems, TCSs can exhibit substantial leakiness in the OFF state. These performance features can be improved by redesigning the output promoter sequence and optimizing HK and RR expression levels [68–70].

Response threshold

In practical applications, the molecules of interest detected with genetically encoded biosensors are sometimes present in very low concentrations. Conversely, chemical products are often present at relatively high concentrations. Therefore, the relevant concentration range of these biosensors must be lower than the minimum concentration (also known as the limit of detection, LOD) or higher than the maximum concentration of the molecule to be detected. The threshold in the dose–response curve represents half the maximal effective concentration that can intuitively reflect the detection concentration range. The response threshold can be tuned by modifying the affinity between metabolites and the LBD of the TF/RNA/TCS (Figure 2(F,G,H)), although this threshold change is usually accompanied by a variation in sensitivity and detection concentration range.

In the case of TFs, to tune the binding affinity of a heme biosensor HrtR, the key amino acid sites involved in heme binding and stabilization on HrtR were selected for saturated mutations [71]. The hrtR-mutated biosensors showed an eight-fold EC_{50} (response threshold) change and over a two-fold sensitivity change. Yu et al. (2019) expanded the detection range of an isobutanol biosensor BmoR. They confirmed the isobutanol binding sites by modeling the BmoR–isobutanol complex and site-directed mutagenesis. Some BmoR mutations have wider detection ranges ($0-100$ mM) of isobutanol and relatively high dynamic ranges. Mutant BmoR biosensors used for screening isobutanol overproduction strains, demonstrated a clear linear relationship between response value and isobutanol titer [72].

In addition to metabolite-binding affinity, changing the TF amount can also affect the response threshold. By changing the promoter strength of the TF genes (*tetR*, *luxR* and *arsR*), the amount of TF expression increases, which can amplify the response to the target ligand [73]. However, a very high TF density is not recommended because it may cause growth retardation because of nonspecific interactions with the genome [74]. Altering the TF concentration can also optimize the response threshold as well as LOD. Depending on whether the TF is an activator or a repressor, its intracellular concentration can be increased or decreased for the optimal response threshold [73,75,76].

A detailed and comprehensive mechanism model of an ArsR arsenic biosensor was developed based on the molecular mechanism and affinities of ArsR binding to DNA and to arsenic. It predicts the fluorescence output as a function of arsenic concentration. Using this model, high arsenic–ArsR binding affinity and low ArsR–DNA binding affinity resulted in ArsR dissociation from the promoter at lower arsenite concentrations where the GFP output was higher, indicating a decreased threshold [77]. When designing a biosensor based on the lac system, Mannan et al. found theoretical constraints between the dynamic range and response threshold with changes in TF–operator affinity [56]. This revealed tunable parameters that facilitate the precise control of the biosensor's function [56]. A mathematical model based on Michaelis–Menten kinetics was also used to tune a vanillin biosensor to select lignin transforming enzymes, elucidating the influence of TF concentrations on response parameters (Figure 2(I)) [78].

In the case of riboswitches, introducing mutations into the ligand-binding region may alter ligand-binding capabilities [79]. Two loops (approximately 18 nts) of an N-acetylneuraminic acid (NeuAc) riboswitch aptamer involved in NeuAc binding were adopted for random mutagenesis. Based on a growth-coupled positive-negative screening, four mutations were screened out from the riboswitch library. The riboswitch threshold increased from 9.77 g/L to 21.97 g/L, and a dynamic range greater than 3 was obtained [80]. A variant library of an L-tryptophan riboswitch was constructed using a tetA-mediated dual selection process. The optimized riboswitches with different dissociation constants were employed to alter their operational range. The dose–response curve of those aptamers with a higher dissociation constant was inclined to have a higher L-tryptophan concentration threshold [66].

In the case of TCSs, a general strategy is to alter the sensor HK activity to tune the response threshold. Enzyme mutations with enhanced kinase activity or

reduced phosphatase activity of NarX in *E. coli* NarXL TCS resulted in a reduced detection threshold from 1138 to 12 μM when applied to a nitrate biosensor. These lower thresholds indicate an increase in response sensitivity. Mutations of widely conserved residues that are crucial for the phosphatase activity of HK can be extended to similar TCSs for sensing chemicals [81].

Combined with experimental approaches, mathematical modeling using the Hill equation revealed that the enhancement of RR protein expression can regulate the response threshold for TCS biosensors. A TCS HydHG-based zinc biosensor was constructed by enhancing RR protein HydG expression in the manner of self-activation. As RR protein expression increased (vector expression), the threshold level decreased from 200 to 10 μM. Conversely, when the self-activated HydG was integrated into the chromosome, the detection threshold increased to 500 μM [82]. This provided useful strategies for creating a TCS biosensor with varying thresholds and sensitivities. Meyer et al. [63] found that mutations in TFs may affect the affinity and specificity to the ligand, as well as its ability to bind to DNA and interact with the host's transcription machinery. For example, mutations that limit the ligand-binding pocket reduce ligand analog binding, which reduces sensitivity and increases specificity.

Response sensitivity

If a dynamic range reflects the vertical output of the dose–response curve and the threshold and substrate detection range reflect its horizontal input, then the sensitivity reflects the ratio of output and input signals of the sensor. Thus, changes to any one module of the biosensor may affect dose–response curve sensitivity, such as modifying target–ligand binding or TF activity. The intracellular concentration of the target chemicals is another factor that can influence sensitivity. Because some target compounds cannot be, or are rarely, transported into cells, many targets become unavailable for the *in vivo* detection of these biosensors. Accordingly, increasing the intracellular concentration of the target chemicals by introducing or increasing import machinery [83], or knocking out cognate exporters to prevent target export [84] can improve sensitivity.

Strategies for extending the input signals of genetically encoded biosensors

Function tuning of the genetically encoded biosensors provide timely, precise response of the molecule signal and actionable monitoring and control. Rapid advances in synthetic biology are enabling us to exploit new biosensors for more and more signal sensing and detection. Although the development of genetically encoded biosensors is increasing, the number of the ligand-inducible TFs, riboswitches, and TCSs are still relatively small. Strategies to expand the libraries of genetically encoded biosensors that identify appropriate gene or RNA candidates have been developed. One strategy is the "Analog Generation toward Catabolizable Chemicals" (AGTC) method developed by Zhang et al. This approach first searches for a list of target chemical analogs, once an analog is identified its catabolic gene cluster and inducible candidates can be found and developed into new biosensors [85]. Searching for new natural response elements of compounds of interest necessitates an increase in the number of compounds that can be detected with existing biosensors.

A related method called enzyme-coupled biosensors can use enzymatic ways to transform undetectable molecules of interest into detectable molecules using existing biosensors [86]. This requires a systematic knowledge of biochemical reaction pools and metabolic pathways to design tailor-made pathways from thousands of individual enzymatic modules. Usually, enzyme-coupled biosensors can be developed using one-step or multiple-step reactions with the assistance of computer-aided design (CAD) tools [87]. This review concentrates on the creation of extensible and custom ligand specificities of genetically encoded biosensors themselves.

Random and site-specific mutagenesis

Mutagenesis is remarkably effective in changing the ligand specificity of biosensors when responding to a novel ligand. When the biosensor structural information is unknown, random mutagenesis is the general approach. Error-prone PCR and DNA shuffling are usually used to create the whole length or the LBD sequence of the TF. It is challenging to shift the specificity from its natural ligand to the new selected ligand because random mutagenesis produces variants with a wider range of specificities [88]. For example, engineering a quorum-sensing TF LuxR can alter its acyl-homoserine lactone (acyl-HSL) specificity to a broader range specificity toward acyl-HSLs [89]. These mutations can be further modified toward the target ligand specificity by employing more comprehensive mutation strategies such as DNA shuffling, iteration mutation, and combination of mutations [90–92]. The response specificity range of LuxR to target compounds was narrowed down using N-terminal LBD mutagenesis and a dual

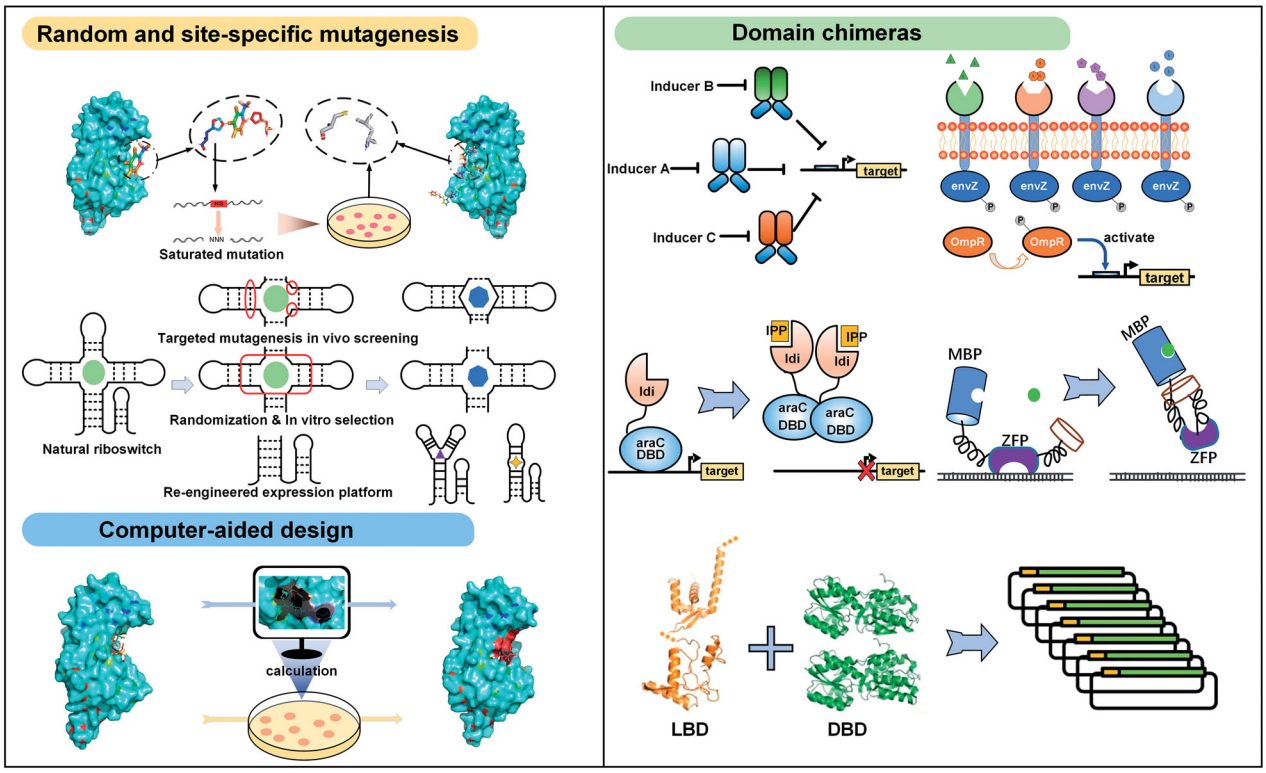

Figure 3. Extending the signal input of genetically encoded biosensors. Random and site-specific mutagenesis. Saturation mutant key sites of transcriptional regulatory factors or riboswitch ligand binding domain. Mutants were used to regulate reporter, and verify whether the mutants respond to the new ligands by detecting the expression of the reporter; Computer-aided design; Domain chimeras. Use linker to connect different ligand binding domains and DNA binding domains to construct a library and screen the biosensors that respond to the target ligand.

positive/negative screening method [93]. The random mutation variants of C-terminal LBD of NahR generated altered specificity for different aromatics, particularly benzoate. Analysis of these mutations indicated the new specificity changes were mostly because of single amino acid substitutions involved in ligand recognition of NahR [94]. Mutations targeting the whole TF can reveal unexpected mutation hotspots [91], whereas mutations specific to only LBDs tend to retain the natural DNA-binding ability, resulting in a higher mutation success rate.

Site-directed and saturation mutagenesis can be used to mutate ligand-interaction residues directly when the crystal structure of a TF, riboswitch, TCS, and its ligand-binding pocket is available (Figure 3). An L-arabinose-responsive AraC TF was used for site-saturation mutagenesis at five specific residues in the ligand-binding pocket. Using the fluorescence-activated cell sorting (FACS) mediated positive/negative screening method, the same AraC TF was designed and successfully selected for D-arabinose, mevalonate, triacetic acid lactone, and ectoine responsiveness, respectively [95–98]. Site-saturation mutagenesis was also used to target specific residues in the ligand-binding pocket to reduce the sensitivity for other ligands and increase genuine specificity for the selected ligand, such as in the engineering of TetR [99], and TraR [100].

Computer-aided design

CAD can be used for *de novo* design of ligand-binding pockets for new ligand specificity from a larger *in silico* mutagenic space [101]. It can also execute more efficient screening of workflows and give more valuable mutational data sets than random and structure-based site-directed mutagenesis. Combining computational design with experimental mutation and screening gives a promising approach for designing TF proteins that bind to a given ligand [102]. Tinberg et al. developed a general computational method by pre-organizing proteins to generate complementary small-molecule-binding sites upon steroid digoxigenin (DIG) [103]. Using the computational protein design method Phoenix Match, the TF QacR was predicted with the potential ligand-binding sites of vanillin, which is dissimilar in structure to the natural ligands. Critical amino acid positions were selected and specific amino acid substitutions were created for *in vitro* and *in vivo* screening, the

isolated mutants showed an increased sensitivity for vanillin [104,105].

Using homology modeling and ligand docking for binding pocket identification, and assisted by conservative mutations in the pocket, a novel specificity in an Acinetobacter TF PobR was engineered [106]. Combined strategies of computational design, protein-wide single-amino-acid saturation mutagenesis, and error-prone PCR, the TF LacI was synthesized in three mutant segments and this captured a full-length design with mutations through overlapping PCR. Screening with fluorescence-activated cell sorting and modifying LacI variants can sense molecules that are dissimilar from the native inducers: fucose, gentiobiose, lactitol, sucralose [90], or lactulose [107]. Many TFs, such as MphR, PobR, and QacR, have also been modified with this combined strategy to change TF selectivity toward various macrolides [22], such as: 3,4-dihydroxybenzoate [108], and vanillin [105].

Many natural RNA regulatory elements can be engineered to alter the ligand specificity of RNA-based biosensors [109]. Alternatively, artificial modules can be generated by an *in vitro* selection method called systematic evolution of ligands by exponential enrichment (SELEX) or computational design for ligand binding [110]. Nucleotides in the binding pockets of natural aptamers were randomized to produce potential aptamer libraries, the library can then be incubated with the target ligand and ideal aptamers will bind to the ligand and avoid shedding so that suitable mutants can be screened *in vitro*.

Domain chimeras

Through the fusion of different domains (DBDs and LBDs) from homologous or heterologous proteins, biosensors can be given new functions and new ligand specificity. Although this cannot change each domain's function, it can increase the predictability of the design of novel biosensors [111]. Shuffling different LBDs from homologous XylR, DmpR, and TbuT proteins, created a XylR chimeric mutant library to sense a set of non-natural aromatic ligands [112]. Site mutagenesis combined with chimeragenesis was also used to modulate the binding pocket to acquire novel ligand specificities. Similarly, a chimeric LacI/GalR library was constructed by fuzing nine different LBDs that bind sugar or purine molecules from the same TF family [113]. The chimeric LacI/GalR TFs successfully reserved the ligand specificities of each LBD and even the mode of transcription regulation.

Some TCS biosensors fused with the transmembrane-sensing domain of another species with the cytoplasmic phosphorylation domain of *E. coli* employed to detect different chemicals, such as methanol [114]. These chimeric biosensors are limited to fusions within families of structurally related TFs or TCSs, such as the LacI/GalR family, to preserve the mechanisms of ligand responsiveness that arise from allosteric regulation [113,115].

Fuzing a known metabolite binding protein with a unique functional domain can create a synthetic TF with induced activity changes toward the metabolite of interest. Furthermore, fuzing a zinc-finger DNA-binding motif with a maltose-binding protein resulted in the construction of a novel maltose biosensor [116]. Using an *in vitro* transposon insertion method, a random fusion and insertion of a DBD into a maltose-binding protein was achieved. The domain fuzing strategy has also been applied to benzoate [117] by using various linkers and systematically switching DBDs.

Recently, broader concepts of chimeric modularity were created to fuze a metabolite-binding protein with a natural TF. The generated synthetic TFs can change the activity of the functional domain [116,118,119]. For example, an isopentenyl diphosphate (IPP) biosensor was generated by fuzing the N-terminal DBD of AraC with an IPP isomerase Idi [120]. Upon binding to IPP, Idi can dimerize and cause chimeric TFs to repress transcription. By fuzing the MetJ repressor with a transcriptional activation domain B42, a chimeric fusion activator MetJ-B42 was created to successfully obtain an S-adenosylmethionine biosensor [119]. Moreover, a chimeric biosensor platform was constructed to engineer fused transmembrane and cytosolic synthetic modular receptors by using single-domain antibodies as LBDs and monomeric split DNA-binding domains of transcriptional regulators as DBDs. This chimeric receptor can detect caffeine and has been used in connection with downstream synthetic gene circuits [121].

The output signal of genetically encoded biosensors coupled with various applications

Fluorescent reporters

The output actuators of genetically encoded biosensors were first created using fluorescent reporters that can be detected with a fluorescent reader. As bioreporter, fluorescent proteins (FPs) can easily provide a quantifiable link between detected chemicals (genotype) and fluorescent intensity (phenotype) (Figure 4(A)). FPs have no requirement for substrate or additional cofactors to exhibit fluorescence, and they are relatively stable

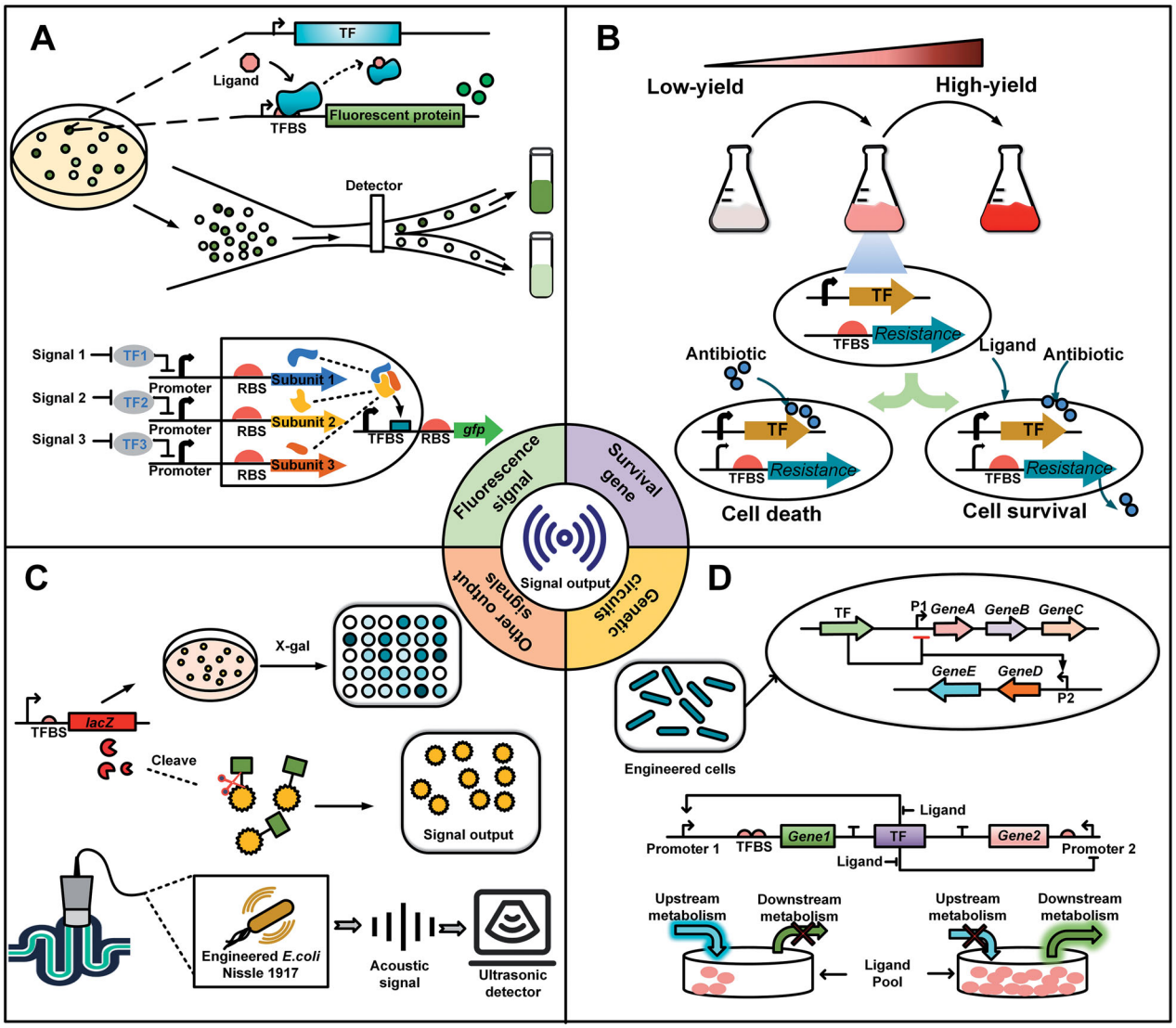

Figure 4. A summarized signal output of genetically encoded biosensors for various applications. (A) The output signal is the fluorescent signal. These high fluorescent output cells can be measured and sorted by flow cytometry. (B) The output signal is for cell growth and survival. The biosensor can be outputted using a resistance gene to combat antibiotics toxicity. The target small molecule deactivates the TF to derepress resistance gene expression, resulting in survival in the antibiotics medium. (C) The other output signal of biosensors. Using biosensors to regulate specific genes can detect signals under specific conditions. For example, regulating the lacZ gene can be detected by blue-white selection. The modified E. coli Nissle 1917 can detect small molecules in the intestine and convert them into acoustic signals. (D) The output signal is wired to genetic circuits. These biosensors can dynamically control the expression of related genes to achieve the steady state of the target ligand pool, which is beneficial to the production of the target compound.

under exterior stresses. Thus, they are usually used for high-throughput screening of mutant libraries to select the desired phenotypes in microbial cell factories [122], and can become rapid analysis tools when accompanied by FACS and a microfluidics system without the need for labor-intensive analysis techniques, such as GC and HPLC.

An FMN-responsive ribozyme is an illustrative example. In the presence of FMN, the ribozyme cleaves itself, thereby activating GFP expression. Next, *B. subtilis* variants with improved vitamin B_2 production capacity could be screened out under high fluorescence intensity [36]. The *E. coli* FMN biosensors, which can convert vitamin B_2 to FMN, were co-confined with *B. subtilis* variants of a genome-shuffling library. FACS allowed effective isolation of higher vitamin B_2 producers from cellobiose. Furthermore, genetically encoded biosensors combined with FACS were successfully used for high-throughput screening and the identification of improved mutant enzymes for lysine and histidine biosynthesis [11,123] and lignin degradation [20]. Moreover, using fluorescence reporters, these

biosensors also facilitated the real-time monitoring of 3-hydroxypropionate (3HP), acrylate, glucarate, and muconate [124,125] for the optimization of fermentation process parameters.

Bioluminescent reporters

As FPs, bioluminescent luciferase (LUX) is also a useful output signal due to their feasibility for rapid, sensitive, and quantitative visualization and detection [126–128]. Although bioluminescent luciferase requires exogenous substrate, coenzymes ($FMNH_2$, NADH), oxidoreductase and H^+, it does not rely on excitation light [129]. It has been extensively used in the environmental monitoring area. These bioreporters can detect pollutants to verify their impact on the environment and bioavailability [130]. For example, many organic toxins were measured using these bioreporters under complex conditions, such as 1,3-dinitrobenzene (1,3-DNB), 2,4,6-trinitrotoluene (2,4,6-TNT), and 2,4-dinitrotoluene (2,4-DNT)[131]. Two screened strong response promoters, P_{yqjF} and P_{ybiJ}, were fused with the bioluminescent gene *luxCDABE* and could effectively detect toxin ligands on damp garden soil. Additionally, these biosensors also allow remote testing for dangerous pollutants such as explosive residues from mines [132]. A naphthalene biosensor was created to evaluate the naphthalene in natural water and soil samples. Cells could effectively metabolize naphthalene into salicylate, which can induce the expression of *luxCDABE* and *salAR* operons. This biosensor demonstrated a positive response in the presence of 0.01 μM naphthalene, showing high specificity and sensitivity [130].

Cell growth and survival

When the output of a genetically encoded biosensor is a gene necessary for growth or survival under selective conditions, it can be used to enrich mutant libraries with a high-performing population or a high producer (Figure 4(B)). For example, a fatty acid–responsive biosensor FadR was outputted using a tetracycline efflux pump (TetA) to combat tetracycline toxicity [133]. FadR, believed to repress *tetA* expression, can be deactivated by fatty acyl-CoA when fatty acid is overproduced, resulting in consistent expression of *tetA* and its survival in the tetracycline medium. The high fatty acid producing population would be selected using this PopQC design. Moreover: 1-butanol-responsive BmoR biosensor [26], theophylline riboswitch [134], and NeuAc-responsive ribozyme [47] were used to control *tetA* expression to effectively select combinatorial libraries comprising promoters, RBS, and enzymes for improved chemical production in *E. coli*. To eliminate evolutionary escapees, this selection workflow was implemented using a toggled negative–positive iteration selection method [135]. A similar approach was also applied to increase the microbial production of naringenin and glucaric acid by 36 and 22 times, respectively.

Other output signals

The fluorescent output of a biosensor must be accompanied by a fluorescent reader, which makes employing these biosensors inconvenient. Various reporters or actuators have been developed and standardized, which can reduce readout operating costs and expand their application in biological systems (Figure 4(D)). For example, colorimetric reporters such as chromoproteins and enzymes that catalyze chromogenic substrates or produce pigments are often used for direct observations of the output with the naked eye [136,137].

Furthermore, some output modules can convert detection signals into electrical currents that can be measured through electrodes [138]. Typically, the microbial fuel cell (MFC) biosensors compose of biological recognition elements and physical transducers translating the biologic response into an electrical signal correlated to the analyte concentration [139]. Similarly, a genetically encoded biosensor outputted a direct electric signal for remote toxic metal monitoring was created. An *E. coli* strain was engineered by controlling an essential component of the metal reduction (Mtr) pathway of *Shewanella oneidensis* using an arsenic-sensitive promoter, which can produce increased current in response to arsenic when inoculated into a bioelectrochemical system. Similar sensing systems may be developed to detect other analytes by the swap of a single genetic part.

Another interesting report is based on intracellular gas vesicles emitting ultrasound waves that penetrate living tissues noninvasively, leading to imaging contrast. Thus, acoustic reporter genes can be used to sense intracellular gas vesicles in *E. coli* [140]. Notably, an *E. coli* biosensor entrapped in an ingestible microbio-electronic device can detect analytes in the gastrointestinal tract. The luminescence output of the cells can be converted into digital signals through photodetectors. The signal can then be further relayed to computers *via* Wi-Fi [141]. Moreover, the luciferase reporter is self-luminous, which makes it a preferred reporter in cancer diagnosis and cell biology when combined with bioluminescence imaging. Oral delivery of the probiotic *E. coli* Nissle 1917 (EcN), which specifically grows within

tumors, can provide a platform for cancer diagnosis. Injection of LuGal and oral delivery of engineered EcN that expresses LacZ causes the cleavage of LuGal to produce luciferin in urine. This signal can be used in a biosensor and is a convenient early identification tool for liver cancer [142]. When accompanied by magnetic resonance imaging, optoacoustic deep tissue imaging (MSOT), and powerful visualization tools [143], biosensors offer noninvasive, rapid, and accurate diagnoses compared with traditional methods, leading to better patient treatments [144].

For the development of health and environment monitoring, the measurable output data of biosensors should be ultimately transmitted to an external device. Thus, the development of equipment using biosensors is necessary. Now it has entered a proof-of-concept stage such as the ingestible microbial electronic device for blood detection, and hydrogel–elastomer hybrids hosting programmed cells for cognate inducers detection [145], etc. In the future, more and more genetically encoded biosensors will be developed into devices for biomedical and environmental monitoring etc.

A Potential use: genetic circuits

As the important applications of synthetic biology, genetically encoded biosensors can be wired to genetic circuits that can dynamically control and switch the metabolic flux of biosynthetic pathways; or even to layer logic circuits that can control more complex genetic networks [122,146]. Dynamic control based on biosensors could balance cell growth and the production of target molecules (Figure 4(C)) [147] in metabolic engineering. These biosensors can provide negative feedback control on growth by sensing quorum signal molecules (AHLs) [148,149] or intracellular metabolites that reflect growth status [150]. Furthermore, they can also reduce the accumulation of toxic intermediates, end-products, and enzymes by inhibiting upstream synthesis pathways and/or activating downstream pathways that consume them. The classic examples are the acyl-CoA biosensor FadR [23] and the malonyl-CoA biosensor FapR, which were used to dynamically regulate the biosynthesis of fatty acid ethyl esters and fatty acids [24,25,151] by maintaining the level of CoA intermediates.

To regulate multiple layered pathways, bifunctional, or more than two kinds of genetically encoded biosensors were interconnected into layered genetic controllers or logic gates for dynamic regulation strategies. Dinh et al. combined lux and esa components of the QS systems to dynamically up- and downregulate the expression of two sets of genes and successfully addressed the bottleneck in both the naringenin and salicylic acid synthesis pathways [152]. A pyruvate biosensor PdhR was assembled to form bifunctional genetic circuits through gene upregulation and an antisense transcription-transduced gene downregulation. The fluxes were then redirected toward bioproduction by inhibiting *zwf* in glycolysis and *pgi* in the pentose phosphate pathway and activating *ino*1 in glucaric acid biosynthesis [17]. Lo et al. designed a two-layered circuit containing a nutrient-sensing module and substrate-sensing module to decouple growth and production. The substrate-sensing module can be activated upon nutrient depletion at high cell density, resulting in biomass accumulation and enzyme expression delays in the conversion of hydroxycinnamic acid and oleic acid into value-added compounds [153].

Genetic circuits assembled in biosensors have been widely used in different host cells (from *E. coli* to mammalian cells) for numerous infectious diseases and cancer therapies. The QS biosensor switch that controls a self-motility gene enables *E. coli* to be a pathogen seeker and activates pathogen killing when the pathogenic *P. aeruginosa* releases QS signaling ($3OC_{12}HSL$) [154]. A tumor-colonizing *Salmonella* sp. equipped with a lux-QS system can be designed as a tumor destroyer to avoid damage to healthy tissue [155]. FlaB can activate the immune response *via* the Toll-like receptor 5 (TLR5) signaling pathways and exhibit strong tumor-suppressive effects, making it an effective cancer immunotherapy. Zheng et al. designed an L-arabinose switch in a strain of *Salmonella typhimurium* that controls the secretion of *Vibrio vulnificus* flagellin B (FlaB) to cure cancer [156].

Genetically encoded biosensors integrated with genetic circuits that can sense and treat metabolic diseases and provide novel theranostic strategies. Urate is a tumor marker that usually fluctuates in disorders such as tumor lysis syndrome and gout. A mammalian urate biosensor HucR was designed to control urate levels in blood [157]. Ye et al. constructed a green tea-triggered genetic control system in a protocatechin (PCA) biosensor using a secondary metabolite of green tea. A transcription factor PcaV and its operon DNA sequence O_{PcaV} in response to PCA was used to create a PCA switch that regulates insulin (for treating type 1 diabetes) or GLP-1 (glucagon-like peptide-1, for treating type 2 diabetes) expression in the human HEK-293 cell line. Microcapsules were used as a precise release delivery system for PCA-regulated hypoglycemic drugs for diabetes treatment [158]. They also designed a far-red

light to control insulin expression devices in order to maintain blood glucose homeostasis [159].

Concluding remarks and prospects

The rapid development of synthetic biology has facilitated the design of novel cell biosensors using numerous genetic switches based on TF–promoter pairs, RNA regulators, and TCS systems. The development of: genetically encoded biosensors has permitted easier quantitatively detection of various metabolites, environmental toxins, and disease biomarkers and provided strategies to fight infectious diseases and for cancer therapy.

To improve the properties of natural genetic switch-based synthetic biosensors, it is necessary to characterize and fine-tune the main characteristics of the biosensor. To this end, various techniques ranging from mutagenesis to computer-aided design have successfully been applied to alter the dynamic range, basal leakage, threshold sensitivity, and ligand selectivity of biosensors. Computer-assisted fine-tuning of biosensors is a promising strategy that allows the main features of biosensors to be redesigned.

Stability are important issues that should be taken into the account in the optimization of biosensors. Notably the copy number of plasmids is sometimes unstable in cells and results in different burdens. One of the resolutions is to insert the biosensor circuit in the chromosome [160,161], making it more stable and eliminating the need for antibiotics to maintain plasmids. Another example used an incoherent feedforward loop (iFFL) to regulate promoters to have a near-identical expression in different genome locations and plasmids [162]. Besides, bacterial cells survival and tolerance in real world scenario is another key issue for biosensor's long-term stability. Storing and extending the shelf life of these biosensors such as freeze drying the cells to maintain their activity at 4 °C or 20 °C [163], or using: all-aqueous sol-gels, nanoparticles, soil particle fixed chromium(VI) etc are feasible strategies to store the cells for a longer period of time while maintaining its activity [164–166]. Synthetic biology and multidisciplinary integrative technology are urgent to overcome the challenges and improve the stability.

The development of an overall computing framework that considers various interactions of different properties can simplify and accelerate the development of new biosensors. Advanced high-throughput analysis and quantitative technologies, such as microfluidic systems, FACS, and electronic chips, are effective tools for massive parallelization monitoring and can accelerate the construction of advanced biosensors. Moreover, efforts in biosecurity and the stability improvement of these biosensors should be continued to make them safer and more competitive with current commercial sensors. In the future, biosensors can be transplanted in tissues for real-time monitoring and targeted drug delivery, as well as being used in microrobots responding to environmental signals, leading to more applications of biosensors.

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