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REVIEW ARTICLE

Genetically encoded ATP and NAD(P)H biosensors: potential tools in metabolic engineering

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ABSTRACT

To date, many metabolic engineering tools and strategies have been developed, including tools for cofactor engineering, which is a common strategy for bioproduct synthesis. Cofactor engineering is used for the regulation of pyridine nucleotides, including NADH/NAD^+ and NADPH/$NADP^+$, and adenosine triphosphate/adenosine diphosphate (ATP/ADP), which is crucial for maintaining redox and energy balance. However, the intracellular levels of NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP cannot be monitored in real time using traditional methods. Recently, many biosensors for detecting, monitoring, and regulating the intracellular levels of NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP have been developed. Although cofactor biosensors have been mainly developed for use in mammalian cells, the potential application of cofactor biosensors in metabolic engineering in bacterial and yeast cells has received recent attention. Coupling cofactor biosensors with genetic circuits is a promising strategy in metabolic engineering for optimizing the production of biochemicals. In this review, we focus on the development of biosensors for NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP and the potential application of these biosensors in metabolic engineering. We also provide critical perspectives, identify current research challenges, and provide guidance for future research in this promising field.

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Introduction

Metabolic engineering, which is the science of altering cellular metabolism, was developed in the early 1990s [1]. In microorganisms, more than 1500 reactions depend on the cofactors NADH/NAD^+NADPH/$NADP^+$, including 740 reactions dependent on NAD(H) and 887 reactions dependent on NADP(H) [2,3]. NAD(P)$^+$ as an electron acceptor and NAD(P)H as an electron donor play crucial roles in the regulation of redox balance [2,3]. ATP as an energy supplier plays a central role in the growth and reproduction of living organisms in various environments. NADP(H), NAD(H), and ATP also have other functions, including: protein modification, DNA repair, reducing oxidative stress, transport, and as adenosine donors for metabolite synthesis (Figure 1) [4–10]. NADH is primarily generated from glycolysis and the tricarboxylic acid cycle (TCA), whereas the oxidative pentose phosphate pathway (PPP) and the NADPH-dependent isocitrate dehydrogenase of the TCA cycle are responsible for the generation of NADPH. The transhydrogenase system also participates in NADPH generation in many eukaryotes and prokaryotes [11]. ATP is produced through substrate-level phosphorylation that occurs in glycolysis or by proton-gradient driven ATPases (Figure 1) [12].

Cofactor balance is fundamental to cell survival in varying environments. Therefore, the cellular synthesis and consumption rates of cofactors are tightly regulated. Generally, reconstructing host cells disturbs the cofactor balance, especially the balance of NADH/NAD^+, NADPH/$NADP^+$, and ATP. Although numerous cofactor regulation strategies can regulate the intracellular NAD(P)H and ATP pool [13–15], such regulation is generally temporary in engineered cells. Thus, developing strategies for the dynamic regulation of NAD(P)H and ATP *in vivo* is desirable. Biosensors are tools to record inputted information that is transmitted into an output signal, through sensing changes in micromolecules and cell physiological markers [16]. Several genetically encoded biosensors (e.g., transcription factor- and riboswitch-based biosensors) have been developed that respond to intracellular and extracellular stimuli [17], which are increasingly being used in metabolic engineering. We have previously reviewed the application of

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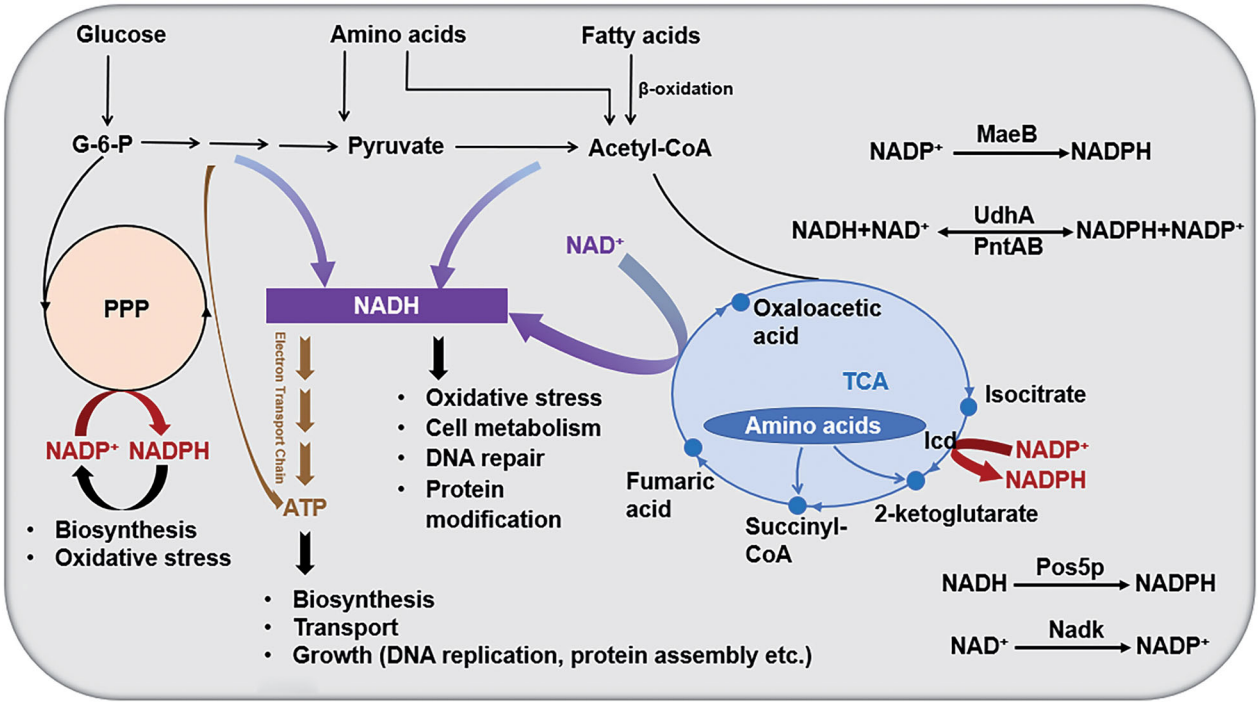

Figure 1. The pathways and functions of ADP/ATP, NADH/NAD$^+$, and NADH/NAD$^+$ in cell metabolism. NADH is generated from glycolysis and the TCA cycle, and NADPH is mainly generated from the oxidative PPP and ICD catalytic reaction. ATP is produced from substrate-level phosphorylation that occurs in glycolysis or the electron transport chain. G-6-P, glucose-6-phosphate; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; MaeB, NADP$^+$-dependent malic enzyme; ICD, isocitrate dehydrogenase; MaeB, NADP$^+$-dependent malic enzyme; UdhA, soluble transhydrogenase; PntAB, membrane-bound transhydrogenase; Nadk, NAD$^+$ kinase; Pos5p, NADH kinase.

light-sensitive biosensors in tuning synthetic circuits and metabolic engineering, and the design principles of genetically encoded biosensors [17,18]. Recently, our group has designed *N*-acetylneuraminate (NeuAc) biosensors to screen high-yielding strains [19,20] and developed a heme regulatory system containing the heme biosensors HrtR and CRISPRi to regulate the intracellular heme balance and improve the production of 5-aminolevulinic acid [21]. In the past few years, several ATP and NAD(P)H biosensors have been designed to regulate gene expression, monitor dynamic changes in real time, quantify intracellular concentrations, and image spatial distribution *in vivo*, and these sensors have been applied in high-throughput screening in prokaryote and eukaryote cells. However, while genetically encoded cofactor sensors are useful tools, their application has been limited to date due to the deficiency [22–25]. Developing different types of cofactor biosensor kits will be conducive in reducing the time for the construction of highly efficient engineered microbial cell factories. The development of cofactor biosensors with: high sensitivity, high specificity, and a broad threshold range is currently a key challenge in metabolic engineering. Cofactor-dependent conformation changes and transcription regulation are two major response mechanisms. Biosensors with different performances can be designed by mutating the binding pockets of enzymes. Transcription regulation is carried out through promoting or preventing the binding of RNA polymerase to promoter under the interaction of between transcription factor or riboswitch and cofactors for gene regulation. Because the optimal intracellular pH and temperature values are inconsistent in varying microorganisms, maintaining the stability of a biosensor is also a challenge. It is desirable to review the existing cofactor biosensors and describe their potential applications in metabolic engineering. This review focuses on the current efforts and future directions in the development of ATP and NAD(P)H biosensors, and the application of such biosensors in metabolic engineering. In addition, we discuss the potential use and challenges in the development of ATP and NAD(P)H biosensors as tools in metabolic engineering.

Development of ATP sensors

Many types of ATP sensors with multifunction have been designed. ATP sensors have been categorized into four types according to their characteristics: small

organic fluorescent chemosensors with a rapid response but that are cytotoxic toward cells; fluorescent nanosensors based on ATP-dependent luciferase, which have good sensitivity and photostability but require ATP consumption; aptamers conjugated with nanomaterials, which have the potential for flexible synthesis and high affinity but that lack a specific DNA aptamer; and genetically encoded fluorescent reporters that are nontoxic and have high specificity and good biological compatibility [26]. Although the operating steps to develop genetically encoded biosensors are relatively complicated, it can be generated autonomously as cells divide, which makes genetically encoded biosensors powerful tools for metabolic engineering.

The key factor in the design of a genetically encoded ATP biosensor is screening for effectors, such as: transcription factors, riboswitches, and promoters. Factors, including: the response threshold, substrate specificity, sensitivity, and biosensor stability, should be investigated. Typically, an ATP biosensor consists of an ATP response element and a reporter. ATP interacts with the response element as an input signal to change the conformation and converts the response element to the output signal of the reporter protein for the detection and monitoring of ATP levels. Gene regulation can be performed only after the regulatory sequence of the ATP response element. In the next section, we focus on the design and application of genetically encoded fluorescent biosensors (Table 1), classified into ATP biosensors and ATP/ADP biosensors.

ATP biosensors

ATP sensors can be categorized into native and artificial biosensors. Native biosensors are unmodified response elements, including natural transcription regulators and RNA. Artificial biosensors are types of recombinant response elements, such as QUEEN, which is the fusion protein of green fluorescent protein (cpEGFP) with the bacterial FoF1-ATP synthase ε subunit [34]. Some native ATP biosensors, including: the mTOR regulator, *ydaO* and *mgtCBR* leader, which consist of a messenger RNA sequence that can respond to ATP, have been identified by studying the natural self-regulation mechanism of cellular energy (Table 1). A representative example is the *Bacillus subtilis* derived riboswitch *ydaO* [32]. The *ydaO* riboswitch was the first native ATP riboswitch to be discovered and can specifically bind to ATP with an apparent dissociation constant (*Kd*) value of 0.6 ± 0.1 mM. The A69, A70, and U82 nucleotides in the *ydaO* backbone are important sites for binding ATP. Mutations at A69, A70, and U82 can abolish the ATP binding to *ydaO*. Similarly, Eun-Jin Lee and Eduardo A. Groisman found that the *mgtCBR* leader of *Salmonella* can respond to intracellular ATP levels to regulate *mgtCBR* operon expression [33]. In addition, Patrick B. Dennis et al. found that mTOR activity can be directly regulated by intracellular concentrations of ATP, which indicated that mTOR can act as an ATP sensor [28].

Over the past few decades, many sensitive biosensors have been assembled, including ATP detection biosensors and ATP imaging biosensors [12,27,29,30,35] (Table 1). These recombinant biosensors were created by linking fluorescent proteins with ATP responsive proteins.

ATP detection biosensors include: ATP-sensitive K^+ channels (ECFP-Kir6.2-EYFP), ATeams, Rho-MatB, yAT1.03, and QUEEN. ATP-sensitive K^+ channels are octamers comprising two subunits of Kir6.12 [39]. The binding of ATP can inhibit the channel activity by altering the conformation and the interaction between the *C*-terminus and *N*-terminus of Kir6.12. To investigate the structural changes that occur when ATP binds to Kir6.12, Takashi Tsuboi et al. designed the fluorescence resonance energy transfer (FRET)-based Kir6.12 indicator, ECFP-Kir6.2-EYFP, to study the interaction between ATP and Kir6.12 [27]. ECFP-Kir6.2-EYFP was constructed by fuzing the *N*-terminus and *C*-terminus of Kir6.12 with enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), respectively. The distance between the *N*-terminal and *C*-terminal domains changed in response to the ATP concentration. However, this indicator can only be used in the plasma membrane, thereby limiting its application in the cytoplasm and organelles.

To overcome this limitation, Imamura and coworkers have developed FRET-based ATP biosensors, called ATeams, to visualize ATP levels in subcellular compartments in real time [29]. The ATeams were assembled by genetically linking two fluorescent proteins, a variant of cyan fluorescent protein (mseCFP) and yellow fluorescent protein (YFP), with the ε subunit of FoF1-ATP synthase. The ε subunit consists of an *N*-terminal and two *C*-terminal domains. The ε subunit is a good scaffold for ATeams because this subunit has higher affinity toward ATP than toward other nucleotides, such as: uridine triphosphate, guanosine triphosphate, adenosine diphosphate (ADP), and cytidine triphosphate. Binding with ATP leads to large conformational changes in the ε subunit, which allows the detection of a wide range of FRET signals. The conformation of the ε subunit changes from an open to a closed form when ATP is present. The affinity of ATeams to ATP can be regulated by altering the ε subunit structure to accommodate a range of ATP levels (2 μM to 8 mM) [29].

Table 1. Genetically encoded sensors for ATP and their application in metabolic engineering.

Sensor	Description	Mechanism	Regulation	Application	Reference
ECFP-Kir6.2-EYFP	The N- and C- terminal of Kir6.2 were fused with ECFP and EYFP, respectively.	ATP-dependent conformation changes	+	Monitoring ATP changes in the subplasmalemmal space of living cells	[27]
mTOR	mTOR is a member of phosphatidylinositide kinase-related protein kinase family	ATP-dependent conformation changes	+	Regulation of gene expression	[28]
ATeams	ATeams were generated by linking two fluorescent proteins, mseCFP and YFP, with the ε subunit of bacterial FoF1-ATP synthase.	ATP-dependent conformation changes	+	Visualizing ATP levels in single living cells	[29]
Rho-MatB	Combination of the ATP-dependent conformational changed RpMatB with two tetramethylrhodamines.	ATP-dependent conformation changes	+	Measuring ATP changes *in vitro*	[30]
Mutated Rho-MatB	Combination of the mutated RpMatB with two tetramethylrhodamines increased the selectivity of ATP over ADP by > 200-fold.	ATP-dependent conformation changes	+	Detecting ATP formation and depletion *in vitro* in real time	[31]
Riboswitch *ydaO*	The *ydaO* motif is a type of natural RNA that can specifically bind to ATP to generate feedback loops.	ATP-dependent transcription termination	–	Regulation of gene expression	[22,32]
mgtCBR leader	A long messenger RNA of the *mgtCBR* leader	ATP-dependent transcription activation	+	Regulation of gene expression	[33]
yAT1.03	yAT1.03 is adjusted by AT1.03, a pH-insensitive ATP sensor.	ATP-dependent conformation changes	+	Monitoring ATP levels in yeast	[12]
QUEEN	QUEEN was created by inserting the green fluorescent protein (cpEGFP) into the two α-helices of the ε subunit of FoF1-ATP synthase.	ATP-dependent conformation changes	+	Monitoring and quantifying ATP levels in bacterial and yeast	[4,34]
iATPSnFRs	iATPSnFRs is generated from linker remolding and replacement of GFP with a circularly permuted superfolder GFP36 (cpSFGFP) in QUEEN.	ATP-dependent conformation changes	+	Imaging ATP levels *in vivo* and *in vitro*	[35]
Perceval	Integration of cpmVenus into the T loop of GlnK1 yielded Perceval.	ATP/ADP- dependent conformation changes	+	Reporting the ratio of ATP/ADP levels in living cells	[5]
PercevalHR	PercevalHR is a super Perceval obtained by mutating Glnk1, which can sense higher range of ATP: ADP ratios.	ATP/ADP- dependent conformation changes	+	Monitoring ATP/ADP ratio changes in cells	[36]
AMPK	AMPK, AMP activated protein kinase, is a heterotrimeric protein complex comprising a catalytic domain (α-subunit) and regulatory domain (β- and γ-subunits).	AMP, ADP- dependent conformation changes	AMP and ADP: + ATP: –	Regulation of gene expression	[37]
AMPfret	AMPfret was generated by fuzing cyan FP (CFP) and yellow FP (YFP) with two subunits of AMPK.	AMP, ADP- dependent conformation changes	AMP and ADP: + ATP: unknow	Monitoring intracellular energy state	[38]

+, activation; –, repression.

Although ATeams are promising tools for sensing ATP, their function may be disrupted under varying pH conditions. The intracellular pH values change dynamically during the growth process and in response to signals (such as light, temperature, and small molecules). Therefore, creating ATP biosensors that are not sensitive to pH changes to reliably monitor ATP levels in real time is important. Botman and coworkers have developed a biosensor, yAT1.03, that is not sensitive to the pH to study ATP dynamics during glycolytic startup in yeast [12]. yAT1.03, the second generation of AT1.03, was obtained by replacing mseCFP and cp173-mVenus with ymTq2Δ11 and yAT1.03, respectively. To quantify the ATP concentration in bacterial cells, Yaginuma and coworkers have designed a single fluorescence protein biosensor, QUEEN, by inserting an enhanced GFP (cpEGFP) into the two α-helices of FoF1-ATP synthase in the ε subunit [34]. The ATP-free and ATP-bound forms of QUEEN have different emission intensities of 513 nm at 400 nm and 490 nm excitation wavelength. Unlike with FRET-based double fluorescence protein biosensors, the quantification of the intracellular ATP concentration is not disturbed by the cell growth rate with a QUEEN biosensor. More recently, Pelosse and coworkers have developed an energy biosensor, AMPfret, by fuzing cyan FP (CFP) and yellow FP (YFP) with two subunits of AMP kinase (AMPK) to elucidate the activation mechanism [38]. AMPfret was used to dynamically monitor and detect conformational changes in AMPK when AMP or ADP was bound to AMPK γ-CBS sites. In addition to measuring AMP and ADP levels, AMPfret might be a useful tool for monitoring cellular ATP.

Reagentless fluorescent biosensors have also recently been developed. Vancraenenbroeck and Webb have designed a biosensor, Rho-MatB, by covalently coupling malonyl-coenzyme A synthetase from *Rhodopseudomonas palustris* (RpMatB) with two tetramethylrhodamines (reporter fluorophores); this biosensor reflects the ATP concentration through changes in the fluorescence [30]. The mechanism of this sensor is similar to that of the ε subunit of ATeams; open and closed conformations are formed when ATP is absent and present, respectively. To expand the applications of Rho-MatB, Vancraenenbroeck et al. have designed the mutated Rho-MatB with the ability to sense a wide range of ATP concentrations [31]. This ability to design Rho-MatB sensors indicates that reagentless fluorescent biosensors might be promising ATP biosensors.

Recently, another promising ATP biosensor group, iATPSnFRs, has been used to image cell surface and cytosolic ATP [35]. iATPSnFRs include two types of biosensors, iATPSnFR$^{1.0}$ and iATPSnFR$^{1.1}$, which were developed from QUEEN. iATPSnFR$^{1.0}$ was designed by optimizing L1 linkers (from Thr-Arg to Val-Leu) and L2 linkers (from Leu-Gly to Gly-Leu-His) with an ATP response range from 30 μM to 3 mM. For iATPSnFR$^{1.1}$, two mutations—Ala95Lys and Ala119Ser—were introduced into iATPSnFR$^{1.0}$. iATPSnFRs are not sensitive to ADP, AMP, or adenosine at concentrations similar to the ATP concentration. iATPSnFR$^{1.1}$ is more sensitive to ATP than iATPSnFR$^{1.0}$ but has weaker expression.

ATP/ADP biosensors

Perceval and PercevalHR were designed for sensing ATP/ADP levels. Berg and coworkers constructed the genetically encoded fluorescent indicator, Perceval, by inserting cpmVenus, a yellow circularly permuted fluorescent protein, into the T loop of GlnK1 [5]. ATP and ADP competitively bind to the same site of GlnK1with discrepant affinities (ATP: ∼0.04 μM; ADP: ∼0.2 μM). Only ATP binding results in the closure of the T-loop and a change in the fluorescence intensity at 490 and 405 nm. Although Perceval is a useful biosensor for sensing the ATP/ADP ratio, it is unsuitable for use in mammalian cells. The ATP/ADP ratios are between 1 and 100 under normal conditions in mammalian cells, whereas the ATP/ADP ratio detection limit of Perceval is ≤ 5. To overcome this limitation, Tantama et al. created a modified Perceval, PercevalHR, by mutating amino acids near the ATP-binding pocket of GlnK1 [36]. PercevalHR has more than eightfold of maximum signal change, which is approximately fourfold that of Perceval. The half-maximal signal change (K_R) value of PercevalHR to ATP/ADP is ∼3.5, which is approximately sevenfold times that of Perceval. The ability of PercevalHR to sense ATP/ADP ratios of 40 (92% sensor saturation) enables the reporting of the ATP/ADP ratio instantly in a single mammalian cell. However, the performance of both these biosensors is influenced by the pH.

Development of NAD(H) sensors

To analyze the relationship between NAD(H) levels and physiological changes in various environments, NAD(H) biosensors have been designed to track and detect NAD(H) concentrations in cells in real time, including: NADH biosensors (Frex) [40], NADH/NAD$^+$ biosensors (Peredox, RexYFP, SoNar, and GPD2p-yEGFP3) [41–43], and NAD$^+$ biosensors (LigA-cpVenus) [44] (Table 2).

NADH biosensors

Rex, an effector from *Streptomyces coelicolor*, can be used to respond to NADH/NAD$^+$ levels to regulate

Table 2. Genetically encoded sensors for NAD(H) and NADP(H) and their application in metabolic engineering.

Sensor	Description	Mechanism	Regulation	Application	Reference
Frex	cpYFP was inserted between two subunits of Rex from *Bacillus subtilis* to generate Frex.	NADH-dependent conformation changes	+	Monitoring NADH levels in living cells	[40]
LigA-cpVenus	LigA-cpVenus was assembled by fuzing the bacterial DNA ligase (LigA) with cpVenus.	NAD^+-dependent conformation changes	–	Monitoring NAD^+ levels in subcellular compartments	[44]
Rex	Rex is a homodimer comprising the N-terminal and C-terminal domains.	NADH-dependent conformation changes	+	Regulation of gene expression and high-throughput screening	[45–47]
Peredox	cpFP was inserted between two subunits of Rex from *Thermus aquaticus* to generate the pH-insensitive Peredox biosensor.	NADH/NAD^+-dependent conformation changes	+	Monitoring cytosolic NADH/NAD^+ ratios	[41]
RexYFP	RexYFP was constructed by inserting cpYFP into Rex from *T. aquaticus*.	NADH/NAD^+-dependent conformation changes	+	Monitoring NADH/NAD^+ redox state in cellular compartments	[48]
SoNar	SoNar was generated by inserting cpYFP between residues 189 and 190 of the T-Rex monomer.	NADH/NAD^+-dependent conformation changes	+	Monitoring changes of cytosolic NADH/NAD^+ redox state and high-throughput screening	[42]
GPD2p-yEGFP3	Glycerol-3-phosphate dehydrogenase (GPD) 2p-based biosensor was obtained by fuzing GDP2p with yEGFP3.	NADH/NAD^+-dependent promoter activation	+	Monitoring the NADH/NAD^+ ratio levels in *Saccharomyces cerevisiae*	[43]
Ndh-Rex	Rex regulates Ndh transcription by sensing NADH/NAD^+ levels.	NADH/NAD^+-dependent conformation changes	+	Regulation of gene expression	[49]
iNap sensors	These are mutated SoNar sensors including iNap1–iNap4 that specifically responds to NADPH.	NADPH-dependent conformation changes	+	Monitoring NADPH levels dynamically in living cells	[50]
mBFP	mBFP is an NADPH-dependent fluorescent protein.	NADPH-dependent conformation changes	+	Real-time monitoring of intracellular NADPH levels in *Escherichia coli* and *Corynebacterium glutamicum*	[51]
NADPsor	NADPsor is a FRET-based biosensor obtained by inserting ketopantoate reductase (KPR) between CFP and YFP.	$NADP^+$-dependent conformation changes	–	Monitoring $NADP^+$ levels *in vivo* or *in vitro*	[52]
Apollo-$NADP^+$	Apollo-$NADP^+$ is a homoFRET-based biosensor designed by fuzing signal fluorescent protein with inactivated G6PD.	$NADP^+$-dependent homodimerization	–	Monitoring and quantification of $NADP^+$ levels *in vivo* and *in vitro*	[53]
SoxR	SoxR is a homodimer containing a redox sensitive [2Fe-2S] cluster. Only oxidized SoxR can activate gene transcription.	$NADP^+$-dependent conformation changes	+	Regulation of gene expression	[24,54]
Yap1p	Yap1p is an oxidative stress responsive transcription factor in yeast.	NADPH/$NADP^+$-dependent conformation changes	–	Regulation of gene expression and high-throughput screening	[25]
pSenSox	SoxR can regulate the expression of reporter genes *in vivo* by sensing NADPH/$NADP^+$.	$NADP^+$-dependent conformation changes	+	Monitoring, quantification and high-throughput screening	[24]
Shikimate-based biosensor	This is a novel metabolite sensor constructed using the catalytic reaction with shikimate dehydrogenase.	NADPH/$NADP^+$-dependent metabolites changes	NADPH: +	Monitoring and quantification of NADH/$NADP^+$ ratio	[55]

+, activation; –, repression.

cellular respiratory pathways [45,46] in most gram-positive bacteria [45]. Rex is a homodimer and each subunit of Rex consists of an *N*-terminal DNA-binding domain and a *C*-terminal NADH-binding domain. The Rex homodimer is stabilized by altering the α-helix at the *C*-terminal region to inhibit gene transcription [56]. McLaughlin and coworkers have revealed that Rex is released from the DNA-binding site when NADH is combined with Rex through subunit rotation. NAD^+ binding does not alter the DNA binding of Rex [46]. Rex preferentially binds to NADH over NAD^+. However, NADH binding can be affected by NAD^+ competition. A series of fluorescent biosensors based on the Rex biosensor have been constructed. The biosensor, Frex, was constructed by fuzing two subunits of Rex from *Bacillus subtilis* (B-Rex) with circularly permuted yellow fluorescent protein (cpYFP) [40]. This biosensor has two excitation peaks at 421 and 500 nm and an emission peak at 518 nm on NADH binding. The K_d of Frex binding to NADH was approximately 3.7 μM and 11 μM at pH 7.4

and pH 8.0, respectively. There was no interference from other NADH analogs, such as: NAD^+, $NADP^+$, and NADPH. The response threshold was 0.15 to 90 μM, indicating that this system can potentially be used in *Escherichia coli*, which has a minimum NADH level of 0.039 mM *in vivo* [57]. Although Frex has high specificity for NADH, it has some limitations, including pH sensitivity and low fluorescence intensity *in vivo*.

NADH/NAD^+ biosensors

For sensing NADH/NAD^+ ratios, a biosensor similar to Frex, Peredox, was constructed by combining the circularly permuted GFP T-Sapphire with Rex from *Thermus aquaticus* [41]. Compared with Frex, Peredox can be used to measure NADH/NAD^+ ratios within a pH range of 4.0–8.4 using a pH-insensitive fluorescent protein (cpFP) and a mutated Rex. The mechanism of the NADH/NAD^+ sensing with Peredox involves NADH binding to Peredox, which enhances the fluorescence intensity of Peredox. NAD^+ binding cannot directly change the fluorescence intensity but reduces the fluorescence intensity of NADH-Peredox by the competitive binding of NAD^+ to Peredox. Considering that Peredox is nonfunctional in mitochondria, Bilan and coworkers designed a biosensor, RexYFP, which has lower NADH affinity than Peredox, and can be used to sense NADH/NAD^+ levels in mitochondria [48]. RexYFP was generated by inserting cpYFP into Rex from *T. aquaticus*. However, the accuracy of RexYFP was affected by the pH and the levels of NADPH. To overcome the disadvantages of NAD(H) sensors, including: the low fluorescence, pH sensitivity, and dynamic range limitations, Yang's group has developed a second-generation NADH/NAD^+ biosensor, SoNar, by inserting cpYFP into a Rex monomer from *T. aquaticus* [42]. SoNar shows a rapid response, and a response range (1500% fluorescence change) of NADH/NAD^+ levels, which is higher than those of Peredox and Frex by approximately 10-fold and 2-fold, respectively. The K_d value of SoNar for NAD^+ is ~5.0 μM and for NADH is ~0.2 μM, which are lower than the NAD^+ and NADH levels *in vivo*. In addition to the Rex-based biosensor, NADH/NAD^+ responsive promoters have been screened and designed as biosensors for single cell analysis. GPD2p-yEGFP3 was constructed in yeast by fuzing the glycerol-3-phosphate dehydrogenase 2 promoter (GPD2p) with a reporter yeast-enhanced green fluorescent protein (yEGFP3) [43]. This promoter-based biosensor may be used for distinguishing subpopulations that have different NADH/NAD^+ levels because of strain degradation in industrial production.

NAD^+ biosensors

Cambronne et al. have developed a genetically encoded biosensor, LigA-cpVenus, by linking a mutated bipartite NAD^+-binding domain from bacterial DNA ligase (LigA) with circularly permuted Venus fluorescent protein (cpVenus) to sense subcellular NAD^+ levels [44]. The subcellular availability of LigA-cpVenus indicates that it might be a useful tool in eukaryotic microorganisms, such as yeasts for the production of chemicals. The *Kd* value of LigA-cpVenus toward NAD^+ is ~65 μM and the dynamic range is 30 μM–1 mM. Another important property of LigA-cpVenus is the wide temperature stability from 20 to 37 °C, which indicates that this biosensor might be potentially used in microorganisms, including *E. coli*, the most commonly used species in metabolic engineering. Although LigA-cpVenus is highly specific for NAD^+ and has wide temperature adaptability, the low dynamic range of LigA-cpVenus might limit its application.

Development of NADP(H) sensors

Several biosensors have been developed for the real-time analysis of intracellular NADP(H) levels. Here, we describe NADP(H) biosensors for the detection of: NADPH (iNap sensors and mBFP), NADPH/$NADP^+$ ratios (SoxR, Yap1p, and shikimate-based biosensors), and $NADP^+$ (NADPsor and Apollo-$NADP^+$) (Table 2).

NADPH biosensors

To date, few biosensors have been developed for sensing NADPH *in vivo* [50,51]. Considering the similarity in the molecular structures of NADPH and NADH, Yang's group developed iNap biosensors (iNap1–iNap4) by mutating the binding pockets of SoNar [50]. Polar residues in the adenine-binding pocket are necessary for NADPH affinity because electrostatic interactions are required for binding. Mutations at positions 90, 112–116, and 130 resulted in iNap1-iNap4 biosensors with varied NADPH affinities. The iNaps show a response to NADPH with two excitation peaks at 420 and 500 nm, and one emission peak at 515 nm. The iNap library contains molecules with a wide range of affinities for NADPH, with K_d values from approximately 2.0 to 120 μM; therefore, increasing the potential applications of iNaps at cellular and subcellular levels. Because iNap responds only to NADPH, $NADP^+$ interference can be avoided *in vivo*. Moreover, iNap possesses some of the favorable properties of SoNar, including: a large dynamic range (500%–1000%), high fluorescence intensity, pH resistance, and rapid response to NADPH.

These properties have enabled the use of iNap to dynamically monitor NADPH levels in the cytoplasm and mitochondria. This biosensor has been shown to strongly and specifically respond to NADPH in *E. coli*. Therefore, iNap biosensors can be used in both prokaryotic and eukaryotic microorganisms for gene regulation and high-throughput screening. Recently, a blue fluorescent protein, mBFP, has been used to monitor intracellular NADPH levels because of its: NADPH specificity, rapid response, and high affinity to NADPH (K_d = ~0.64 mM) [51]. mBFP as an NADPH sensor has an excitation wavelength at 395 nm and an emission wavelength at 451 nm [58]. Because the maturation of this protein is oxygen independent, it can be used to monitor intracellular NADPH levels in real time in microorganisms undergoing anaerobic or aerobic fermentation. However, the low quantum yield ~ 18% of GFPuv and poor photostability may limit its application in light-controlled metabolic engineering.

NADPH/NADP$^+$ ratio biosensors

SoxR, Yap1p, and shikimate-based biosensors have been developed to respond NADPH/NADP$^+$ ratios. SoxR is a homodimer, and each subunit consists of: a DNA-binding domain, a dimerization helix, and an Fe-S cluster–binding domain [24,54]. Oxidized SoxR has been shown to change conformation to activate gene transcription, while reduced SoxR binds to the DNA binding site to prevent gene transcription. Therefore, oxidized NADP$^+$, and not reduced NADPH, specifically combines with SoxR to activate target gene expression. Similar to SoxR, on the basis of the oxidative stress responsive transcription factor Yap1p, Keasling's group have developed an NADPH/NADP$^+$ biosensor in yeast through linking promoters of TRX2 to green fluorescent protein [25]. Promoters with diverse output strengths were generated through adding upstream activating sequences and fuzing tandem repeats. Thus, various levels of gene expression could be achieved used in metabolic engineering. In addition to these transcription factor–based biosensors, Zhang and coworkers have designed a shikimate-based biosensor to respond the cytosolic NADPH/NADP$^+$ ratios in *S. cerevisiae* by detecting shikimate and dehydroshikimate concentrations [55]. This NADP specific biosensor was selected from *E. coli* derived shikimate dehydrogenase (EC 1.1.1.25) with high activity (19.9 units/mg). There was no appreciable effect of this biosensor on the central metabolism. The design of this metabolite sensor provides a new strategy for the development of biosensors.

NADP$^+$ biosensors

Biosensors, such as NADPsor and Apollo-NADP$^+$, have been developed that respond to NADP$^+$ in living cells [52,53]. NADPsor was assembled in *E. coli* with the CFP-KPR-YFP model to detect NADP$^+$ levels according to changes in FRET efficiency [52]. It has an excitation wavelength at 440 nm and two emission peaks at 478 and 526 nm for YFP and CFP, respectively. NADP$^+$ binding reduces the FRET efficiency by changing the conformation of NADPsor to an extended form. To improve the sensitivity of NADPsor, the affinity of NADPsor to NADP$^+$ was optimized through computerized redesign of the NADP$^+$ binding pocket. This biosensor has high specificity for NADP$^+$ with a response range from 1 μM to 10 mM. However, the dynamic changes (30%) and NADP$^+$ affinity (K_d = ~2 mM) of NADPsor are still very low. Cameron et al. have reported a series of biosensors (Apollo-NADP$^+$) for multiparametric imaging, which is different from most FRET-based genetically encoded biosensors. Apollo-NADP$^+$ is a homoFRET-based biosensor comprising a fluorescent signal protein and inactivated glucose-6-phosphate dehydrogenase (G6PD) [53]. The sensing mechanism of Apollo-NADP$^+$ is based on the decrease in steady state fluorescence anisotropy through NADP$^+$ induced homodimerization, rather than changes in fluorescence intensity. This biosensor has a response sensitivity to NADP$^+$ (*Kd* values from 0.1 to 20 μM), which is similar to wild-type G6PD. The biosensor is resistant to pH values from 7.25 to 8.0. However, the narrow dynamic range (15%–20%) caused by small changes in the conformation of Apollo-NADP$^+$ limits its application.

Although several NADH/NAD$^+$, NADPH/NADP$^+$, and ATP/ADP biosensors have been designed, some problems, such as: pH interference, the narrow dynamic range, low fluorescence intensity, and poor specificity remain, and these factors should be considered in the development of next-generation biosensors.

Potential application of ATP and NAD(P)H biosensors in metabolic engineering

In metabolic engineering, an imbalance in the levels of intracellular cofactors can disturb the physiological functions of living organisms. Therefore, regulating intracellular cofactor levels is desirable for improving the production of biochemicals, such as succinate and amino acids [59–61]. It is also important to screen for efficient cofactor-dependent enzymes and analyze intracellular cofactor levels in real time for the construction of efficient microbial cell factories. Nicotine adenine dinucleotides and ATP sensors have been shown to

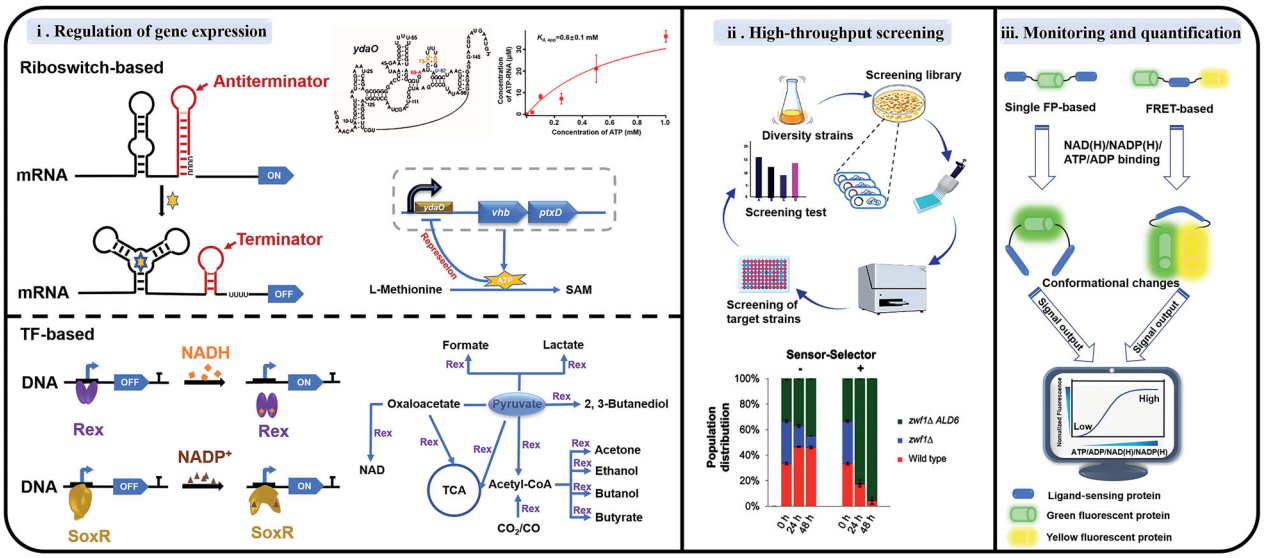

Figure 2. Applications of NAD(H), NADP(H), and ATP sensors in living cells. (i) Model of gene regulation *in vivo* with a transcription factor (such as Rex and SoxR) or riboswitch (such as *ydaO*) [24,49], and [22,25,32,63]. (ii) Model of high-throughput screening for enzymes (such as NADPH-dependent enzymes) or strains (such as strains with high levels of NADPH) using biosensors (such as SoxR and Yap1p) [24] and [25]. (iii) Model of monitoring and quantification of cofactor levels with single FP- or FRET-based biosensors.

be useful tools to investigate such processes [24,29,35,44,45,62]. Here, we review the current applications of cofactor biosensors in metabolic engineering (Figure 2). First, biosensors can be used to regulate the redox and energy balance during cell growth and chemical production [12,29,64]. Second, the biosensors can provide a high-throughput system to screen for cofactor-dependent enzymes with high activities and for high levels of cofactors in cells [24]. Moreover, these sensors can be conveniently used for the real-time monitoring of the ratios or levels of NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP *in vivo* during cell cultivation [55,62]. In this section, we outline three cofactor biosensors as potential tools in metabolic engineering.

Regulation of gene expression

To construct a robust microbial cell factory, the metabolic pathway or metabolic network should be carefully regulated not only by the deletion or overexpression of target genes using traditional methods but also using dynamic regulation strategies. In this section, we discuss the application of cofactor dynamic regulation using genetically encoded biosensors (Figure 2(i)) [22,45,49].

Several metabolic pathways have been regulated using Rex in *Clostridium acetobutylicum,* including: central metabolic (the TCA cycle), chemical fermentation (including acetone, butanol, formate, and lactate production), NAD biosynthesis, and CO_2/CO fixation [23,65] pathways [63,66,67]. For strict anaerobes and many facultative anaerobes, such as *C. acetobutylicum* and *Thermoanaerobacter ethanolicus,* ATP is formed from glycolysis instead of cellular respiration [68]. Accumulated NADH is consumed by oxidation into NAD^+ during fermentation. Thus, intracellular NADH levels in anaerobes should be precisely regulated to improve metabolite production. For example, Zhang and coworkers have demonstrated that natural Rex can be used to regulate the expression of NADH-consuming enzymes, such as aldehyde/alcohol dehydrogenase and other redox balance–related enzymes, through the response to oxidative stress to maintain redox homeostasis *in vivo* [23]. The *adhE2* gene was directly regulated by Rex in response to the NADH/NAD^+ concentrations. Consequently, the ethanol and butanol yields were improved in *C. acetobutylicum,* and the expression of: *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* was upregulated when Rex was used to respond to increases in the NADH/NAD^+ ratio. This research provided insights into biochemical synthesis in metabolic engineering in which redox-dependent gene expression is regulated by redox biosensors. In addition to increasing the production of chemicals, Rex also plays an important role in regulating oxidative stress in anaerobes [63,65,66]. In aerobic bacteria, such as *Bacillus subtilis*, the expression of NADH dehydrogenase (*ndh*), the key component of the electron transport chain, was up- or down-regulated to maintain a constant NADH/NAD^+ ratio under aerobic conditions [49]. When the NAD^+ concentration was higher than that of NADH *in vivo*, the repressor Rex was bound upstream of *ndh* to repress *ndh*

transcription, resulting in NADH accumulation. In contrast, when the intracellular NADH concentration was higher than that of NAD^+, Rex was released from the DNA-binding site to activate *ndh* transcription, leading to the oxidization of NADH to form NAD^+. Such a regulation model can be used to improve chemical production through preventing a large fluctuation in the NADH/NAD^+ ratio during the fermentation process.

In addition to the transcriptional regulator based biosensor, Tianwei Tan's group have used an ATP-sensing riboswitch, the *ydaO* motif, to balance the requirement of ATP in *S*-adenosylmethionine (SAM) biosynthesis [22]. ATP not only is a vital precursor of SAM but also is the energy carrier in cells. An increased ATP concentration is beneficial for SAM synthesis, whereas an excessive ATP concentration is harmful to cell growth. The *ydaO* motif was used to regulate ATP levels dynamically during SAM production in *E. coli*. Compared with the control strain, the SAM titer ($1.23\ mg{\cdot}L^{-1}$) was increased by up to 55% in the engineered strain through the dynamic regulation of ATP synthesis–related gene expression (*vhb* and *ptxD*) with the *ydaO* motif.

High-throughput screening

Generally, large amounts of NADH, NADPH, and ATP are consumed during biochemical synthesis. Cofactor-dependent enzymes with high activity increase the catalytic efficiency. Traditional screening and detection methods are time consuming and inconvenient for screening mutated cells and enzymes. Fluorescence can be used to indicate intracellular cofactor levels in single cells. Therefore, the use of NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP biosensors might be a promising strategy for high-throughput screening in metabolic engineering (Figure 2(ii)).

The genetically encoded biosensor SoxR has been designed as a high-throughput screening system to rapidly screen for NADPH-dependent alcohol dehydrogenases from *Lactobacillus brevis* (*Lb*Adh-A93M) with high activity [24]. Many catalytic reactions depend on NADPH as a cofactor to synthesize high-value products, such as: terpenoids, amino acids, and biofuels [3,69]. Screening for NADPH-dependent enzymes with high activity can improve the production of chemicals. A fluorescence-activated cell sorting (FACS) biosensor is a potential tool to screen variant enzymes efficiently and rapidly. In addition to the enzyme screening system, Keasling's group have reported a Yap1p-based NADPH/$NADP^+$ biosensor selector to screen for yeast populations with diverse NADPH/$NADP^+$ levels [25]. Using this sensor/dosage-sensitive gene (DSG)-based screening method, the overexpression of *ALD6* was shown to generate more NADPH than *ZWF1* in yeast. This DSG-based sensor-selector strategy provides a method for screening phenotypes of interest from large libraries and is promising for use in regulating the redox balance in metabolic engineering.

In metabolic engineering, the turbulent cofactor ratio is considered a major bottleneck because an optimal redox state can provide the thermodynamic driving force for a less favorable reaction [47,70]. A transcription factor-based FACS was constructed to screen bacterial strains containing high levels of NADH [47]. To verify the feasibility of using this redox biosensor reporter, wild-type *E. coli* was mixed with either Δ*ndh* or Δ*cydB* and Δ*appB* mutants at varying proportions (1:1, 10:1, 100:1, 1000:1, and 10 000:1). Approximately 80% of the screened strains contained high NADH/NAD^+ levels even at the 10 000:1 ratio. Therefore, this screening model can be used for selecting variants from medium-sized strain libraries (10^4 variants).

Monitoring and quantification

The types and availability of cofactors are crucial in metabolic engineering because of their essential role in many biochemical reactions [71]. However, the relationship between the levels of NAD(P)H and ATP, and cell growth and biochemical production is poorly understood. Therefore, the real-time monitoring and detection of NAD(P)H and ATP levels *in vivo* are desirable to better understand these relationships. In this section, we discuss the monitoring and quantification of ATP, NAD(H), and NADP(H) levels using genetically encoded biosensors (Figure 2(iii)).

To date, many cofactor biosensors have been designed for metabolism analysis [4,5,12,34,40,44,50,51]. *E. coli* is the most commonly used bacteria in metabolic engineering. Many chemicals are produced using engineered *E. coli*, including amino acids and polyhydroxyalkanoates [61,72]. Yaginuma et al. have determined intracellular ATP concentrations in *E. coli* [34]. Differences in ATP concentrations were found to occur even in engineered *E. coli* cell populations. These non-uniform ATP concentrations indicated there was metabolic diversity in single cells, which might have benefits for cell survival under severe conditions. In addition, intracellular ATP levels can be dynamically monitored in living yeast. Takaine et al. found that the ATP concentrations were consistent using diverse carbon sources and at various cell cycle stages in wild-type yeasts [4]. During glycolytic startup, the dynamics of ATP levels

have been studied in single cells by continuously measuring the ATP concentrations. Changes in the ATP levels mainly depended on previous growth conditions and transitional carbon sources [12].

In addition to monitoring the cofactors, some biosensors can be specifically used for quantification of cofactors *in vivo* and *in vitro* [30,31,55]. Rho-MatB and mutated Rho-MatB not only are suitable for real-time monitoring of ATP levels but also can be used to quantify ATP concentrations *in vitro* [30,31]. For cell-free metabolic engineering, the regeneration, regulation, and recycling of high-energy cofactors, including ATP and NADH, are key considerations [73]. Rapidly and accurately measuring ATP concentrations in cell-free metabolic systems using Rho-MatB will promote extracellular synthesis. In addition, a shikimate-based biosensor has been designed to determine the cytosolic NADPH/$NADP^+$ ratio in *S. cerevisiae* [55]. Because NADP(H) is compartmentalized in *S. cerevisiae*, the measurement of whole-cell amounts of NADP(H) is unsuitable for studying compartmentalized reactions (such as amino acid and farnesene biosynthesis). Using this biosensor, changes in the cytosolic NADPH/$NADP^+$ ratio and that of the whole cell were shown not to be synchronized under the glucose pulse condition. The determination of the cytosolic NADPH/$NADP^+$ ratio provides new insights into NADP(H)-dependent reactions. The developed biosensors enable convenient and rapid measurement, monitoring, and quantification of the levels of cofactors in real time *in vivo* and *in vitro*. In addition, the relationship between energy or redox changes and stimulants can be elucidated to enable effective metabolic regulation through metabolic engineering [55].

In summary, NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP biosensors provide guidance for biosynthesizing high-value chemicals through regulating metabolic pathways or screening highly catalytic enzymes by monitoring the dynamic levels of cofactors in the cytoplasm or organelles.

Challenges and perspectives

Cofactor biosensors have been skillfully designed in the past few decades. However, most of these sensors focus on monitoring and imaging in cells. To accurately regulate the levels of intracellular cofactors in real time, more diverse types of biosensors should be explored.

Although various design strategies have been developed and biosensors have been designed in response to metabolites and signals *in vivo* and *in vitro* [19–21,74–76], some intractable challenges remain in biosensor design and application [76]. First, the low response range and low sensitivity are major challenges in the rational design and application of biosensors [76]. Second, the nonspecificity of biosensors to substrates greatly affects their application in metabolic regulation [76,77]. For example, *ydaO*, a riboswitch, was first developed as an ATP biosensor [32]; however, studies later found that *ydaO* also responds to the second messenger c-di-AMP [78–80]. To overcome these challenges in the design process, random mutagenesis and saturation mutagenesis of the substrate-binding pocket of transcription factors or riboswitches have been performed to develop next-generation biosensors [77]. For example, our group has designed a high-threshold NeuAc riboswitch, with a response range of 0–35 g/L NeuAc, through random mutation of the aptamer of the NeuAc riboswitch [20]. Computer and *de novo* protein design have been proposed to redesign proteins for catalyzing bioreactions or for switching protein degradation [81–86]. For example, Langan et al. have designed a switchable protein, degronLOCKR, which can be induced to control gene expression and protein degradation [85,87]. Thus, computer and *de novo* protein design are promising and time-saving methods for developing genetic biosensors. The complex environmental influences *in vivo* and *in vitro* should also be considered while designing new biosensors. The genetic stability of biosensors *in vivo* is another key factor in the application of biosensors [88]. Overall, despite the challenges, the successful biosensor designs to date can provide guidance for future designs. The types of cofactor biosensors may be increased with the development of the artificial cofactors nicotinamide cytosine dinucleotide (NCD) and the reduced form of NCD (NCDH) [89,90]. The complex cellular reactions of native cofactor involved lead to the difficult of fine-tuning the cofactor dependent specific reaction. The bioorthogonality of the NCD/NCDH system with the native redox reactions makes it an attractive cofactor system for biosynthesis. Proteins, including NAD-dependent D-lactate dehydrogenase (*Lh* DLDH) and formaldehyde dehydrogenase (FalDH 9B2), can be engineered as effectors for NCD/NCDH response through coupling with a reporter gene.

The NAD(H), NADP(H), and ATP levels *in vivo* can be regulated individually with Rex, SoxR/Yap1p, or *ydaO*. However, redox and energy metabolism is correlated with cell survival and bioproduction, especially the NAD(H) and ATP levels [91]. To construct an improved microbial cell factory, a strategy that combines regulation of the intracellular redox balance and the energy state is desirable. Over the past two decades, some genetic circuits that play a vital role in synthetic biology

have been investigated [92]. Coupling biosensors with genetic circuits has become the ideal method for dynamic metabolic regulation through metabolic engineering [93–97]. Thus, the combination of cofactor biosensors and genetic circuits is a feasible strategy for simultaneously regulating redox and energy metabolism. Furthermore, this strategy has the potential for the high-throughput screening of strains with superior production of chemicals. As well as coupling cofactor biosensors with genetic circuits, we hope that cofactor biosensors will be coupled with other genetic tools (such as light sensory proteins) for metabolic regulation in the future.

Conclusions

The present review describes the development of genetically encoded cofactor biosensors and their application as potential tools in metabolic engineering. Cofactor engineering is a key strategy for cell reconstruction in metabolic engineering. However, randomly regulating the intracellular levels of NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP may cause an imbalance, which can affect the cell metabolism during fermentation. Thus, systematically understanding the fluctuations in NADH/NAD^+, NADPH/$NADP^+$, and ATP/ADP levels under stimulations is vital to engineering efficient strains by dynamically regulating intracellular cofactor levels or gene expression in biosynthesis pathways. Several biosensors have been designed and the affinity for cofactors, response concentrations, and pH stability have been optimized. Some biosensors have been shown to be feasible tools for regulating metabolic pathways and screening for efficient catalytic enzymes. Although most cofactor sensors are mainly used for imaging and monitoring *in vivo* to analyze intracellular levels of cofactors under changed cultivation conditions or growth stages, genetically encoded biosensors are still promising tools for the development and yield maximization of industrial metabolic engineering products. In recent years, the application of cofactor biosensors in metabolic engineering has been a subject of interest; for example, regulating the balance between the PPP and production pathways to maintain sufficient NADPH supply. Such regulation of metabolic networks can also reduce carbon loss. In summary, the application of cofactor biosensors in metabolic engineering shows promise and should be further explored.

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