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Advances in Cell-Free Biosensors: Principle, Mechanism, and Applications

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Synthetic biology has promoted the development of biosensors as tools for detecting trace substances. In the past, biosensors based on synthetic biology have been designed on living cells, but the development of cell biosensors has been greatly limited by defects such as genetically modified organism problem and the obstruction of cell membrane. However, the advent of cell-free synthetic biology addresses these limitations. Biosensors based on the cell-free protein synthesis system have the advantages of higher safety, higher sensitivity, and faster response time over cell biosensors, which make cell-free biosensors have a broader application prospect. This review summarizes the workflow of various cell-free biosensors, including the identification of analytes and signal output. The detection range of cell-free biosensors is greatly enlarged by different recognition mechanisms and output methods. In addition, the review also discusses the applications of cell-free biosensors in environmental monitoring and health diagnosis, as well as existing deficiencies and aspects that should be improved. In the future, through continuous improvement and optimization, the potential of cell-free biosensors will be stimulated, and their application fields will be expanded.

1. Introduction

Biosensors are analysis tools that take biological components as the main functional elements and used for rapid detection of various trace-level analytes. They generally consist of sensitive biometric elements, transducers, and signal analysis systems.[1] The sensitive element (enzyme, antibody, cell, etc.) identifies and binds the analytes. Then the transducer captures the interaction between the sensitive material and the analyte and converts the interaction into an identifiable signal (e.g., electricity signal). The quantity of the analyte is further calculated through the signal analysis system. Compared with traditional instrument-based analytical chemistry such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), biosensors are less costly, more specific, faster, easier to operate, capable of online analysis, real-time identification, and simultaneous output of results.[2–4] So their usage is more efficient and more convenient. With the advantages of biosensors, they have been widely used in environmental detection, food safety inspection, disease diagnosis, biochemical analysis, and other fields.[4–6]

Meanwhile, synthetic biology has propelled the development of biological sensors. Synthetic biology has great potential in the application of biosensors by designing genetic circuits and logic operations for biological components, resulting in a variety of genetic devices and biological modules.[7–9] It has promoted the construction of customized biological function system, which can finally realize sensors with customized programming sensitivity, specificity and dynamic range, and expand the scope of sensor detection target,[10,11] providing transformative tools for improving the performance of biosensors.

The design of synthetic biology is mainly dependent on living cells in recent decades, which leads to the rapid development of cell biosensors. Cell biosensors can use animal cells, plant cells, and living microbial cells as basal cells (sensitive biometric element). Cells can metabolize a variety of compounds using intracellular enzymes with high stability and activity. Cell biosensors have a long service life and high reproducibility of experimental results.[12] Moreover, the sensitivity and specificity of biosensors can be improved by the introduction of corresponding genetic modules.[9,13] Heavy metals, pesticides, vitamins, and some macromolecules can be detected using cell biosensors. For example, Wang et al.[14] constructed an *Escherichia coli* consortium-based biosensor that can detect and integrate three environmental signals (arsenic, mercury, and copper ion levels) via either its native two-component signal transduction pathways or synthetic signaling sensors derived from other bacteria in combination with a cell–cell communication module. Nevertheless, there are some shortcomings in cell biosensors that need to be addressed. Since cell biosensors use live genetically modified organisms (GMOs), if they are released into the environment, there are certain unsafe factors.[15] In order to prevent the occurrence of this situation, it is usually impossible to apply these GMO

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biosensors.[16,17] Furthermore, the response time of biosensors is prolonged because some analytes are hard to cross over the cell membrane, and some analytes may be toxic to living cells. Therefore, some cell-based biosensors are not suitable for detecting cytotoxic or impermeable membrane analytes such as organophosphates. They may also be influenced by actual environmental samples (e.g., toxic wastewater), as well as their own physiological and chemical conditions (e.g., temperature and salt concentration).[18] The above disadvantages lead to significant limitations for cell biosensors in applications.

However, the emergence of cell-free synthetic biology not only promotes the construction of cell-free biosensors but also solves the limitations described above. And the biosensor platform based on cell-free protein synthesis (CFPS) system has been developed.[19] The CFPS system is a mixed system of DNA templates, transcriptional machinery, translational machinery, the necessary substrates (including amino acids, energy substrates, cofactors, and salts), and water.[20,21] CFPS system can be divided into crude extract system and PURE (protein synthesis using recombinant elements) system.[20] The main difference between the two systems is the source of the translational machinery. As the name implies, one is from the crude extract, and the other one is from purified components. Compared with the crude extract system, the PURE system is more stable; however, the cost is higher, and the yield is also lower.[22,23]

The CFPS system and DNA template can be assembled into a mature cell-free biosensor. CFPS systems perform biological transcription and translation (TX-TL) in an open environment, exhibiting a variety of unique functions superior to cellular systems and laying the foundation for biosensors to introduce more complex genetic circuits. Due to the uniqueness of cell-free systems, the cell-free biosensors have the following significant advantages. 1) Security: GMOs will not be released, and there is no biosafety problem.[24,25] 2) Toxicity/chemical tolerance: The system can operate in the presence of toxins.[26] 3) Stability: Cell-free systems have high long-term storage stability by the freeze-drying technology.[27] 4) Sensitivity: The limit of detection can reach the nanomolar level.[28,29] 5) Response time: The tested materials can react directly with the sensors in a cell-free environment to generate signals more quickly.[29] 6) Robustness: Cell-free system can keep its structure and function stable when it is disturbed by uncertain factors.[30] 7) Selectivity: Interference of other signals can be attenuated by the biosensor element selectivity.[29] The following contents focus on the design principle and workflow of cell-free biosensors, as well as the development of cell-free biosensors in environmental monitoring and biomedical applications.

2. Principle and Workflow of Cell-Free Biosensors

The design principle of most cell-free biosensors is to select the appropriate recognition mechanism and reporter gene based on the analytes, and then clone the encoded gene into the cell-free expression vector. Furthermore, the mature cell-free biosensors were constructed by using appropriate cell-free protein synthesis system and the coding template DNA. Cell-free biosensors can detect a variety of analytes, including metal ions, quorum-sensing molecules, antibiotics, viruses, and other substances such as amino acids (**Table 1**).

The workflow of cell-free biosensor (**Figure 1**) can be divided into two parts, the recognition and response of analytes by specific identification mechanisms, and the output of readable signals through reporter gene expression. First, accurate recognition of analytes is the key to cell-free biosensors. When analytes are added to the cell-free system, the sensor's recognition mechanism is regulated by specific molecular structure and binding.[57,58] Second, the downstream reporter gene is activated to be transcribed and translated into reporter proteins in a cell-free system. According to the presentation mode of different reporting proteins, besides the widely used optical signal, the detection results also can be output by electrochemical signals. For example, nanoscale microelectrodes can be used in cell-free solutions to recognize specific DNA sequences and output electrochemical signals.[34,47,52]

2.1. Recognition-Response Mechanisms

With the emergence of various analytes, the application of traditional sensor recognition mechanisms has been limited, such as the lack of high selectivity of enzymes and antibodies, which has led to developing diversified molecular structures as recognition elements. The following contents mainly introduce the recognition mechanism of cell-free biosensors based on different molecular structures, including transcription factors, CRISPR-Cas (clustered regularly interspaced short palindromic repeats associated), toehold switches, and adaptors.[59]

2.1.1. Transcription Factor Activation/Repression

A transcription factor (TF) is a protein molecule with a specific structure that binds to a specific gene and regulates gene expression at a specific intensity at a specific time and place. Therefore, the sensitivity and specificity of TFs can be used to design the sensor as a recognition element. TF-based cell-free biosensors have been used to identify specific small molecules, such as inorganic ions and organic molecules (bacterial quorum sensing molecules), and they are also used in high-throughput screening and metabolic engineering.[48,60,61] Specific TFs or natural conformational TFs are selected by different analytes, such as TF in response to aromatic compounds (XylS-AraC, XylR-NtrC, and LysR), metal ions (MerR, ArsR, DtxR, Fur, and NikR), or antibiotics (TetR and MarR).[62] When analytes (ligands) are present in a cell-free environment, the response of TFs is immediately obtained, and binding of ligands to their activated TFs or the release of ligand-bound inhibitory TFs can lead to the reporter gene expression (**Figure 2**A). For unknown ligands, they can be converted into detectable ligands by using metabolic enzymes.[18,48]

To avoid the reaction of TFs to substances other than detection targets, TFs with enhanced specificity and sensitivity should be further expanded. Alternatively, a directed evolutionary strategy can be used to screen and select specific TFs.[63] Not all analytes can be identified due to the known limitations of TFs. There are other recognition mechanisms that can be used to identify.[64,65]

Table 1. The summary of existing cell-free biosensors.

Cell-free biosensors(Analytes)		Sensing elements	Output elements	Limit of detection (LOD)	Optimization methods	Institution	Reference
Metal ions	Mercury	MerR	sfGFP	6 μg L^{-1}	Temperature 5'-UTR RraA inhibitor protein	Bielefeld University	[31]
	Mercury	MerR	LucFF EmGFP	1 ppb	pH Chelating agent	Jawaharlal Nehru University	[32]
	Mercury	MerR	LucFF	—	Regulatory component concentrations	University of Turku	[33]
	Mercury	MerR	GFP mScarlet-I LacZ NanoLuc	1.0 nM	Reporter gene	University of Edinburgh	[34]
	Mercury Arsenic	MerR ArsR	GFP	1078 nM 661 nM	Cell extract Mg^{2+} concentration Promoter RBS	Tsinghua University	[29]
	Zinc Copper Cadmium Lead	SmtB CsoR CadC	sfGFP	10 μM 10 μM 10 μM 51.8 μM	Transcription factors	Northwestern University	[35]
Quorum sensing molecules	3-oxo-C_6-HSL	LuxR	NanoLuc mScarlet-I GFP LacZ	4.0 nM	Reporter gene	University of Edinburgh	[34]
	C_6-HSL C_8-HSL 3-oxo-C_8-HSL VB-C SCB1	ScrbR	eGFP	4–80 μM	Protein concentration	Seoul National University	[36]
	C_8-HSL	TraR	LacZ	100 nM	Beta-Glo substrate Incubation time pH	University of South Carolina	[37]
	3-oxo-C_{12}-HSL pC-HSL	LuxR RpaR	GFP	54.9 nM 8.9 nM	Cell extract Mg^{2+} concentration Promoter RBS	Tsinghua University	[29]
	3-oxo-C_{12}-HSL	LasRV	sfGFP	4.9 nM	Cell-free system Sample extraction	Imperial College London	[38, 39]
	3-oxo-C_{12}-HSL	LasR	GFP	—	Droplet microfluidics	University of California at San Francisco and California	[40]
Antibiotics	Tetracycline	OtrR CtcS MphR	sfGFP	—	Transcription factors	Northwestern University	[35]
	Aminoglycosides Tetracycline Chloramphenicol Macrolides	β-GAL	LacZ	0.5 mg mL^{-1} 2.1 mg mL^{-1} 0.8 mg mL^{-1} 6.1 mg mL^{-1}	Colorimetric substrate CPRG Temperature pH	Osaka University	[41]
	Aminoglycosides	T3 RNAP	NanoLuc	—	Recombinant RNase inhibitor	Osaka University	[42]
	Technetium	TetR	LucFF	10 ng mL^{-1}	Regulatory protein TF concentration	University of Turku	[33]
Nucleic acid	Zika virus	Toehold switch	LacZ	1 fM	NASBA	Cambridge	[43]
	Norovirus	Toehold switch	LacZ	270 zM	α-complementary	Arizona State University	[44]

(*Continued*)

Table 1. Continued.

Cell-free biosensors (Analytes)		Sensing elements	Output elements	Limit of detection (LOD)	Optimization methods	Institution	Reference
	Ebola virus	Toehold switch	LacZ	30 nM	Short RNA triggers	Harvard University	[45]
	microRNAs	Spinach RNA aptamer	4-hydroxy benzlidene imidazolinone	3 pM	T7 promoter	Hunan University	[46]
	Antibiotic resistance genes	TetO Toehold switch	Electrochemical signal	1 fM	NASBA	University of Toronto	[47]
Other environmental contaminants	Atrazine	AtzR	sfGFP	20 μM	Extract ratio Modularization	Northwestern University	[48]
	Fluoride	crcB riboswitch	sfGFP Catechol (2,3)-dioxygenase	50 μM	Mg^{2+} DNA concentration Reporter genes	Northwestern University	[49]
	Diacetyl	Nematode olfactory receptor ODR-10	Electrolyte – insulator semiconductor (EIS) sensors	0.01 nM	Detergent, Brij58t	Zhejiang University	[28]
	Cyanuric acid (CYA)	AtzR	sfGFP	1 μM	Promoter Plasmid concentration	University of Wisconsin	[50]
Metabolite/ Biproduct	BPA E2 DPN	Human estrogen receptor β (hERβ)	GFP	330 nM 26 nM 9 nM	RNAse inhibitors	Brigham Young University	[51]
	Benzoic acid	BenR	GFP	21 702.8 nM	Cell extract Mg^{2+} concentration Promoter RBS	Tsinghua University	[29]
	Benzoic acid Hippuric acid Cocaine	BenR	sfGFP	1 μM	Synthetic metabolic cascades DNA concentration	University of Montpellier	[52]
	Salicylate Benzalkonium chloride Uric acid	SAR2349 QacR HucR	sfGFP	≈μM ≈μM 300 μM	Transcription factors	Northwestern University	[35]
	Amino acids	CFPS component	sfGFP	100 nM	Gene knockout PURE system	Chungnam National University	[53]
	Amino acids	CFPS component	Electrons (PGM)	0.1–1.5 μM	Heat-treated FBS	Chungnam National University	[54]
	D-psicose	psiR	sfGFP	—	Addition sequence Dosage	Universite Paris-Saclay	[19]
	Thrombin	DNA aptamer	GFP	—	Aptamers position	University of Tennessee	[55]
	Alpha-fetoprotein	Antibody (ELISA)	FLuc	7 fM	Cell extracts	Chungnam National University	[56]

2.1.2. Toehold Switch and CRISPR-Cas Recognition

Cell-free biosensors based on toehold switch and CRISPR-Cas are mainly designed to detect genomic DNA or viral RNA of some pathogens. The toehold switch system is a programmable ribosome regulator, and it consists of two strands of RNA, called a switch or a trigger. The toehold switch sensor controls the translation of genes by triggering the binding of RNA through a trans effect. The switch contains a salient loop structure-forming in the upstream of the gene, which blocks gene translation in cis by blocking the ribosome binding site (RBS).[66] After binding to the complementary trigger RNA, the sequestration is released,

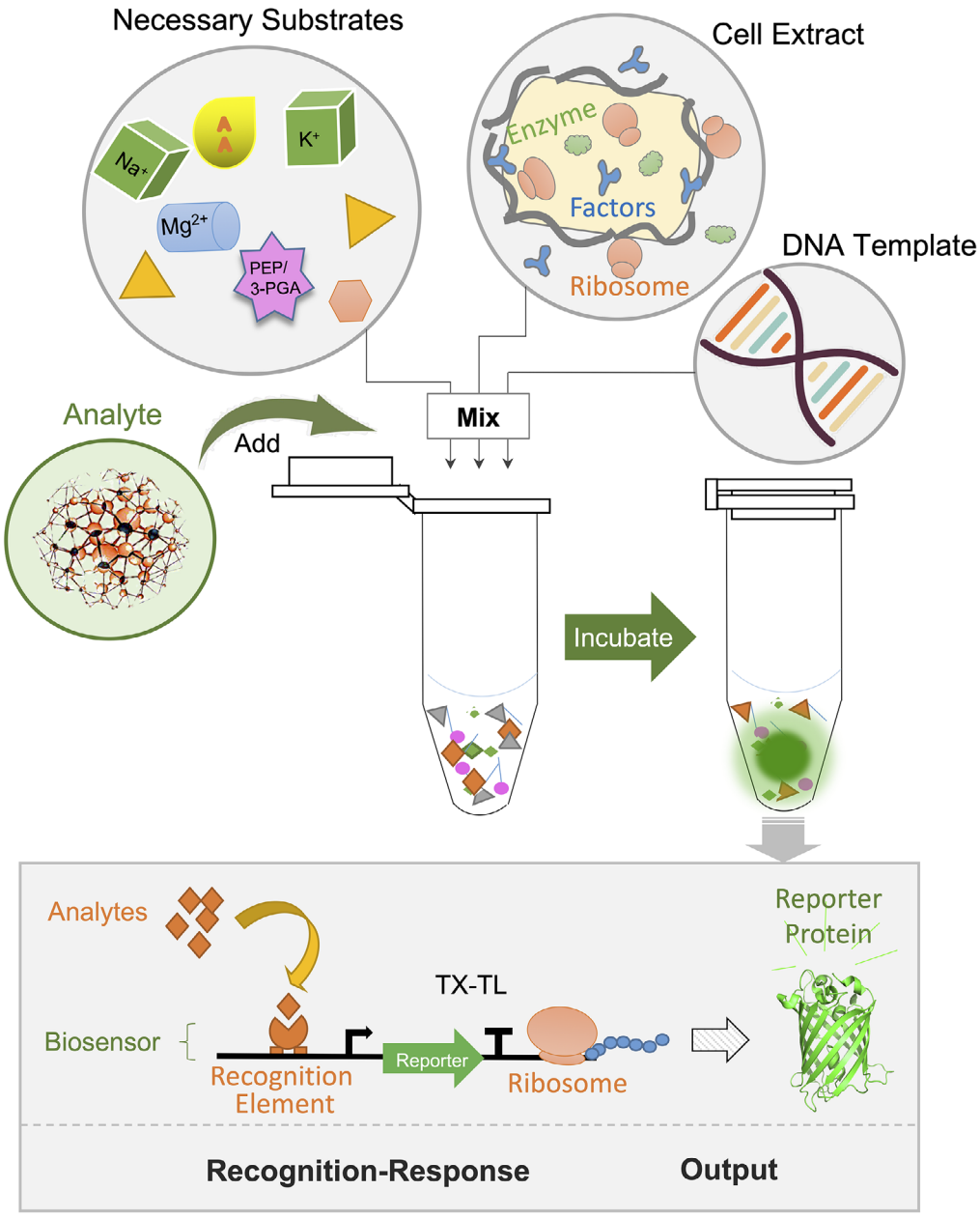

Figure 1. The workflow of cell-free biosensors. The analyte is identified by the specific recognition mechanism of the cell-free biosensor. Then, the downstream reporter gene is activated to be transcribed and translated into reporter proteins in a cell-free system.

and the gene translation is activated.[66] The target nucleic acid is used as a trigger RNA to activate the expression of the reporter gene in cell-free system.[45] Finally, the detection of the target can be determined directly by the expression of the reporter gene.

The cell-free biosensors based on CRISPR-Cas mainly rely on the cut principle of CRISPR-Cas to identify targets. CFPS system can express many kinds of active CRISPR machinery from plasmids or linear DNAs, and it can further output quantitative dynamics of gene cleavage or repression without the protein purification.[67] According to different Cas effectors (Cas9, Cas12a, and Cas13a), CRISPR biosensor systems have different shear cleavage principles.[68] For example, the CRISPR-Cas13a system can detect analytes that can directly cleave captured targets using the Cas13-crRNA complex,[69] and then the lateral cracks of the complex are activated to cleave nearby non-targeted RNA (quenched fluorescent RNA).[70] As a reporter, the quenched fluorescent RNA is cleaved and displays a fluorescence signal to respond to the results.

In addition, toehold switch and CRISPR-Cas can cooperate to detect analytes. For example, Pardee et al.[43] identified two strains by NASBA-CRISPR/Cas9 (NASBA, nuclear acid sequence-based amplification) cleavage method (Figure 2B). The nucleic acid sequence of a strain can be used as the target sequence. With the participation of reverse transcriptase and

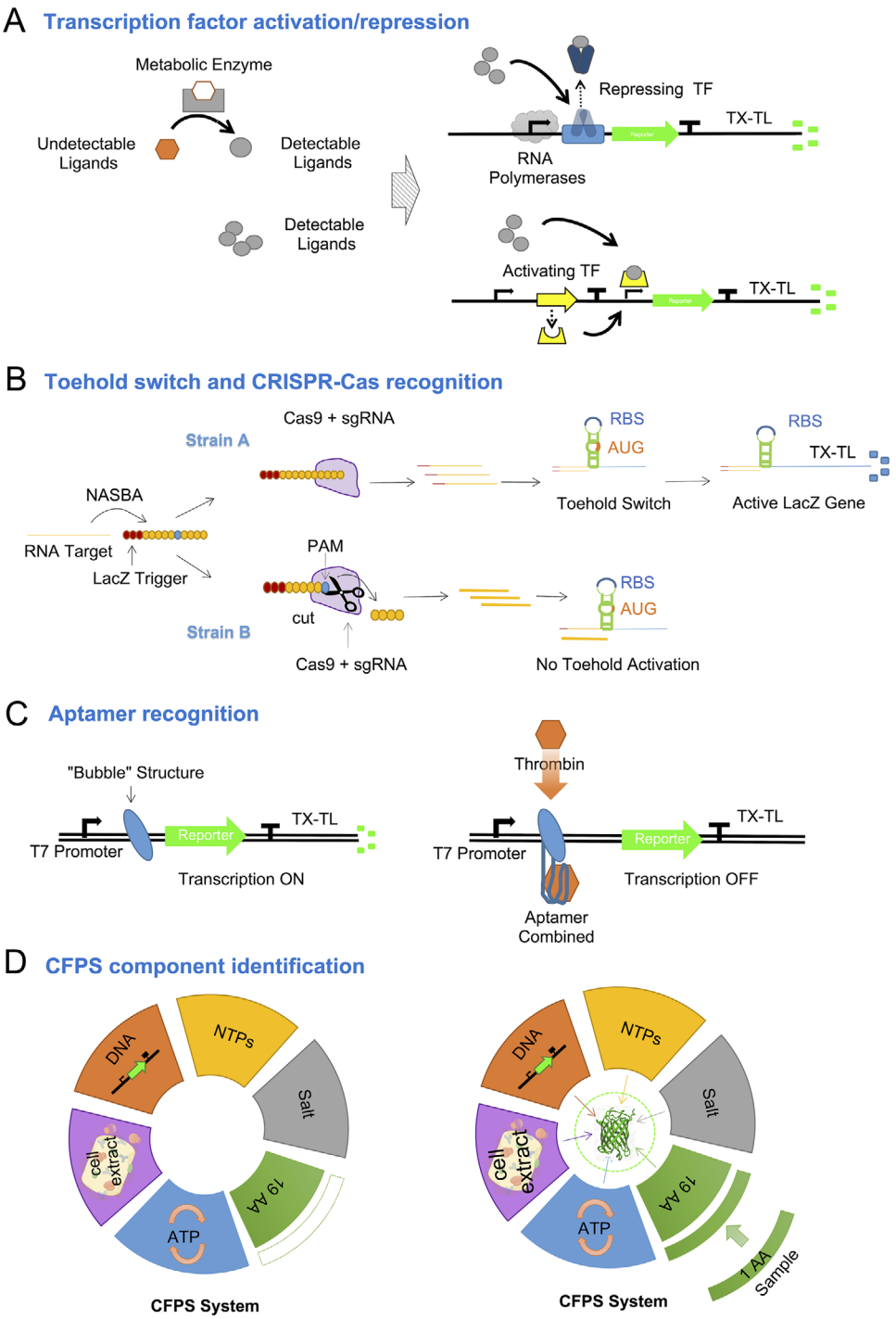

Figure 2. Recognition-response mechanisms of cell-free biosensor systems. A) After the ligand is identified, reporter genes can be activated by binding activated transcription factors or releasing inhibitory transcription factors. Unknown ligands can be converted into detectable ligands by using metabolic enzymes before the identification. B) Cell-free biosensors use NASBA-CRISPR/Cas9 cleavage system to identify different strains of virus. C) Nucleic acid ligands are used to identify thrombin. When thrombin is absent, the reporter protein is successfully expressed, but the translation is blocked in the presence of thrombin. D) When samples containing amino acids are added, incomplete CFPS is activated, leading to successful expression of the reporter protein.

T7 RNA polymerase, the proto-spacer adjacent motifs (PAM) and trigger sequence (lacZ trigger) can be introduced in target sequence by isothermal amplification.[71] Cas endonuclease is mediated by sgRNA to recognize the PAM of the target sequence for fixed-point cutting. The uncut sequence contains the trigger RNA, which can be detected by the paper-based toehold switch sensor and finally identified as the strain type by the color change.[72]

Nevertheless, there are some challenges to this method. The isothermal amplification process is susceptible to contamination, which could lead to off-target products and false positives. However, this phenomenon can be minimized by using sequentially specific fulcrum switch sensors, and CRISPR-Cas mediated amplification of downstream selection.[73] Multiplexing for CRISPR-Cas systems is also a considerable challenge.[74,75]

2.1.3. Aptamer Recognition

Due to its small size, high stability, high affinity, and high specificity, aptamers can not only separate and combine with any selected molecule (including metal ions, small molecules, peptides, proteins, and even materials surfaces) but also can be easily modified in any position.[76,77] Therefore, they are suitable for use as recognition elements of sensors. Aptamers are single-stranded DNA or RNA molecules, most of which are obtained by a combinatorial biology technique called SELEX (systematic evolution of ligands by exponential enrichment).[78] In the cell-free environment, the single-stranded region of the aptamer is easily regulated by phage polymerase, which regulates the transcription and translation of the aptamer sequence through the T7 promoter. For example, Iyer et al. used nucleic acid aptamers to identify thrombin. They inserted thrombin-bound ssDNA aptamers in the downstream of the T7 promoter, creating a "bubble" structure downstream that binds to the aptamers in the presence of thrombin, blocking transcription of the T7 RNA polymerase (Figure 2C). Finally, the presence of thrombin can be detected by the expression of the reporter gene.[55,79]

Although this method has high stability and specificity, its sensitivity needs to be further improved. In addition, the identification mechanism lacks high-quality aptamers for clinically important targets in medical applications. The aptamer technology must be extensively tested in clinical sample substrates to establish the reliability and accuracy.

2.1.4. CFPS Component Identification

In addition to the recognition mechanism mentioned above, there is another recognition mechanism that utilizes the cell-free translation system. So far, this mechanism has only been applied to amino acid detection. The design of a cell-free system selectively removes a specific amino acid, and it then adds the sample, which contains missing components from the system, such as amino acids in the patient's plasma sample.[80] (Figure 2D) Aminoacyl-tRNA synthase can be used as a recognition element to identify the amino acid in the sample and then produce aminoacyl tRNA. After the CFPS system is complete, the translation machinery is activated to produce protein output. Jang et al.[53] used this principle to design cell-free biosensors to detect amino acids. This cell-free biosensor not only can accurately detect the amino acid content in the sample but also has a high sensitivity.

In addition to using the recognition mechanism of the cell-free translation system, most of the recognition mechanisms are encoded in the genetic circuit and then added to the cell-free hybrid system for characterization. Cell-free synthetic biology can use genetic circuits to achieve complex regulation. For example, a series of *E. coli* promoters and transcriptional activators can cascade and combine to produce arbitrarily complex sensors for regulatory functions. Therefore, cell-free biosensors with multiple recognition mechanisms can be designed to detect analytes. This extends the types, levels, and ranges of target analytes to be detected.

2.2. Signal Output

The signal of cell-free biosensors is mainly output in the form of optical or electrochemical signals. Here, optical reporters can be summarized into three categories, including fluorescent (*gfp, deGFP, rfp, mScarlet*), colorimetric (*lacZ*), and bioluminescent (*lux, luc, NanoLuc*) reporters.[34,81] After the identification, the downstream reporter gene is activated and expressed in the cell-free system. Various output methods are available for monitoring the proteins expressed by the reporter genes, including fluorescence, luminescence, visible color changes, etc.

The most common method is to report the fluorescence of proteins based on GFP, which is a simple, convenient and intuitive signal output method in laboratory operation, and it does not require reaction substrates (**Figure 3**A). GFP expressed by the cell-free system could not glow by itself, and the fluorescence only can be generated by the light excitation. Therefore, the fluorescence output representing the gene expression level needs to be measured by means of absorbance detection.[31] However, the obvious disadvantage of GFP is that the background signal is high, which makes the sensitivity of GFP low.

In addition, the signal can be output by the luciferase gene (*lux/luc*) (Figure 3B).[33] The luciferase experiment is extremely sensitive. It has no signal background and has a wide linear range (up to 7–8 orders of magnitude). Luciferase needs to be mixed with luciferin and other required chemicals to produce fluorescence. Generally, luciferase assay reagent is added to the test system, and the luciferase level is measured in the plate reader after the reactant mixing. Moreover, the application of luciferase also has certain limitations, such as short half-life, poor repeatability, and certain requirements on oxygen.

Enzymes catalyze substrate molecules that can produce strong exponential signals than fluorescent proteins. β-galactosidase (β-gal) can lyse synthetic substrates, resulting in color changes, fluorescence, or chemiluminescence, which allows the output of LacZ-based sensors to be adjusted for different conditions. However, β-gal has some dependence on the substrate. The colorimetric method based on the enzyme activity of β-gal can directly observe the color changes with the naked eye, and the substrates detected by colorimetric method include O-nitrophenyl-d-pyrine galactoside, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside, and so

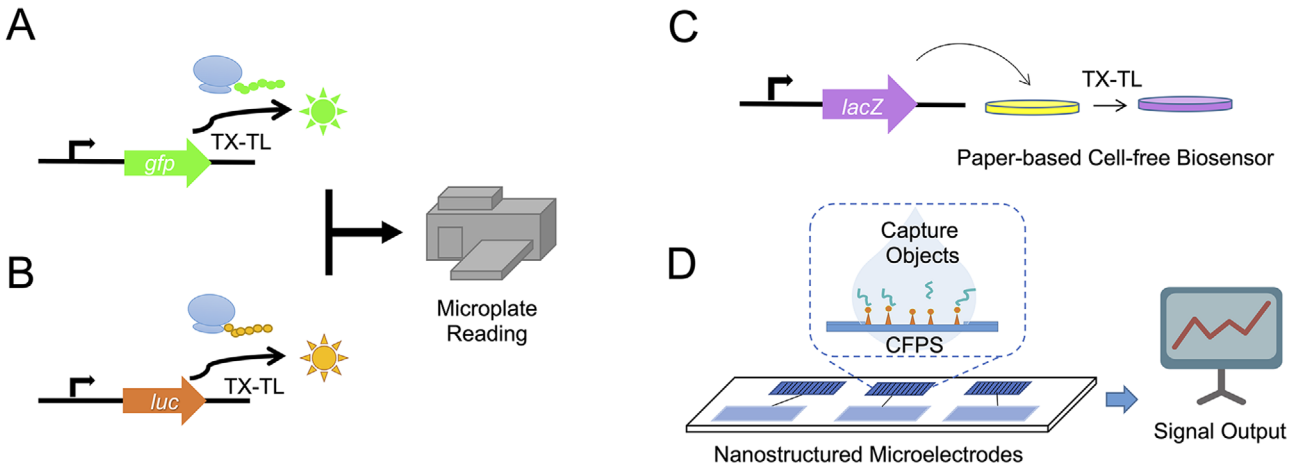

Figure 3. Signal output of cell-free biosensor systems. A) The reporter gene *gfp* is expressed in the CFPS system and output in the form of optical signal. B) The reporter gene *luc* is expressed in the CFPS system and output in the form of optical signal. C) The reporter gene *lacZ* is expressed in a paper-based cell-free system, and the color change on the measured paper can be easily detected by the naked eye. D) The biosensor based on the cell-free genetic circuit captures the analyte through the nanostructured microelectrode to generate the electrochemical signal output.

on.[34,44,45] This signal output method is suitable for paper-based cell-free biosensors (Figure 3C). Lopreside et al.[34] analyzed multiple gene reporters and concluded that *deGFP* may be a more reliable reporter gene for regular cell-free biosensors, because it worked well for both mercury and quorum-sensing molecular sensors. Therefore, the sensor design can select the appropriate cell-free system and output reporter protein to improve the sensitivity and the detection speed of the sensor.

Except for the optical signal output, cell-free biosensors can also be output by electrochemical measurement. A variety of material electrodes combined with cell-free system and electrochemical signals can be generated, while the electrodes can capture the target. Capacitance-voltage and constant-capacitance measurements are employed to monitor the capacitance changes induced by surface modification steps for sensor preparation as well as interactions between receptor and its ligands.[82] Mousavi et al.[47] designed an extensible reporter enzyme system, in which a sensor based on a cell-free gene circuit cleaves specific DNA sequences in cell-free system solution, generating electrochemical signals when the newly released strands are trapped on the surface of nanostructured microelectrodes (Figure 3D). This method is in sharp contrast to the traditional optical reporter, which not only has limited multiplexing ability but also has a dependence on ventilation and reduced sensitivity in turbid solutions.[47,83] These limitations can be overcome by electrochemical measurements. The electrochemical system will enable the multiplexing of gene-based circuits for portable detection and will facilitate the development of electronic devices related to synthetic biology.

Some researchers have developed specific cell-free biosensors with more direct signal outputs. For example, Jang et al.[54] designed a strategy to interface the molecular process of protein biosynthesis with the sensing mechanism of a personal glucose meter (PGM) to assay amino acids. The synthetic invertase convert sucrose into glucose, and PGM is used to detect electrons generated during the oxidation of glucose into gluconolactone by glucose oxidase.

3. Improvement of Cell-Free Biosensors in Practical Application

To keep up with the development of practical applications, the cell-free biosensor is required to be more portable, stable, and cost-efficient. These requirements have led to continuous improvement of cell-free biosensors. Generally, cell-free biosensor reagents are often refrigerated or frozen during storage or transportation. This method of storage is not convenient, which may hinder the field deployment. Therefore, researchers developed the lyophilization technology to improve the storage stability of cell-free biosensors.[43,84] The cell-free system ensures that it remains active after lyophilization and does not adversely affect the efficiency. By freeze-drying, the cold-chain storage problems can be overcome to stabilize the long-term and high-temperature storage of the cell extract, which can last up to 3 months to a year and reduce the necessary storage space.[27,85] Moreover, the stability of cell-free biosensors can be improved in other ways. For example, components can be encapsulated in the liposomes.[86] or polymer substrates to maintain the system stability.[24]

However, there are still some disadvantages of the cell-free freeze-drying platform. For example, the system needs to be stored in inert gas and silica gel desiccation packages,[45] and the activity of cell-free system decreases after freeze-drying. To solve these problems, some researchers have developed methods. For example, Karig et al.[87] investigated using the non-reducing disaccharide trehalose to protect cell-free system components during oven drying. This method can not only stabilize the system activity but also greatly improve the storage temperature of the system.

To facilitate the field monitoring, biosensors need to improve the portability. Cell-free systems can be freeze-dried with appropriate supports (e.g., microtubules and microporous plates). Beyond that, the cell-free system also can be introduced onto a cheap matrix simple paper,[45] where the freeze-drying and rehydration can reduce the chance of contamination and false positives. Because of the small size of the freeze-dried reagent required for

the paper-based cell-free biosensor, this greatly reduces the cost and facilitates the field testing. Some researchers have developed a two-filter system suitable for use with a smartphone that can output results digitally through the phone, which is more convenient.[31] Jang et al.[54] connected the sensor directly to the personal glucose meter, using mobile technology to facilitate the output reading for end users. These methods provide a more convenient way for site investigation.

Furthermore, due to the influence of many factors (e.g., site, operator, preparation of cell extracts, and preparation of auxiliary reagents),[88] cell-free systems prepared in different batches may be slightly different. In order to improve the stability and standards of cell-free biosensors, several approaches can be taken to reduce the batch-to-batch variation of the crude extract-based cell-free system. There are a few ways to reduce variability, including optimized extract preparation, fully solubilizing the master mix components, further purification of template DNA, large batch preparation and automatic preparation, and so on.[21,89–91] In addition, PURE systems can be developed to improve sensor standards. The PURE system for cell-free protein synthesis reduces the level of contaminated proteases, nucleases and phosphatases, and provides a more precise method of preparation, a higher reproducibility and better flexibility of the modular system. Because there is no metabolic side effect of amino acid library like in crude extract-based cell-free system, the PURE system is more stable.[23]

The PURE system has been commercialized to make them available for a variety of applications, with strict quality control. However, the commercial PURE system is expensive, and the production cost of the PURE system is very high, so the general sensor design still uses the crude extract system. Therefore, in order to standardize the cell-free biosensors, more time and energy should be spent on the development of the PURE system to improve the efficiency and reduce the cost. At present, some researchers have developed a cheaper way to produce the OnePot PURE system.[92] In the future, this method can be used to study and extend new applications to save costs.

Some researchers have also made much progress in improving the sensing response of cell-free biosensors. First, different reporter genes have different detection efficiency. Therefore, a suitable output reporter protein needs to be selected to improve the sensitivity and speed of the sensor.[34] Second, optimizing transcription factor concentration can reduce the LOD of the sensor.[9] Third, selecting the appropriate promoter, RBS, and degradation tags can reduce the expression and improve the dynamic range of the cell-free biosensors to improve the response of them.[50] Finally, Pandi et al.[19] optimized cell-free biosensors to monitor enzymatic production by applying a TF-doped extract, using a preincubation strategy with the TF plasmid, or reactivating two-step cell-free reaction.

In addition, based on the broad promise of cell-free biosensors, they could use synthetic biology tools that have been demonstrated on cell-based biosensors to improve their sensitivity and selectivity, and extend their function.[93] Modular and tunable genetic amplifiers can be introduced into the sensor. These powerful amplifiers can be incorporated into a variety of gene regulatory networks and can be used to improve the sensitivity and output dynamic range of transcription-based biosensors.[94,95] Sensors can also use genetic circuits such as AND and NOT logic gates[96] to modulate and integrate multiple input signals to detect multiple environmental signals simultaneously.[14] In the future, with the introduction of these tools, the application range of the cell-free biosensor will be more and more widely.

Cell-free synthetic biology methods have been applied to the production of biomaterials, including biopolymers, cellulose materials, and nanoparticles.[97] For example, P-gel has been used to produce a set of model proteins, including green fluorescent protein (GFP).[98] These cell-free smart materials offer greater flexibility beyond their use in the laboratory and in the field. By applying more intelligent and functionalized biomaterials to cell-free biosensors, more categories of cell-free biosensors will be developed in the future.

4. Applications

At present, we are all faced with the situation of frequent environmental pollution and human diseases. Some kinds of environmental pollutants and diseases (e.g., heavy metals,[99] cancer,[100] and Coronavirus disease 2019[101]) seriously threaten the living environment and health of human beings. To detect these hazards more quickly and accurately, biosensors with low cost and high efficiency are urgently needed. In this context, cell-free biosensors have made remarkable achievements in the rapid detection of environmental pollutants and disease pathogens (**Figure 4**).

4.1. Environmental Detection

Many chemical pollutants exist in the environment, which pose some serious threats because they are invisible or hard to spot. The most common harmful substances are heavy metals, such as arsenic and mercury,[31] as well as toxic small molecules and antibiotics.[29,50] The environmental protection agency (EPA) stipulates that some harmful pollutants cannot exceed a certain level in the environment, so it is necessary to test the present level of these substances in the environment. A number of cell-free biosensors have been developed and designed to detect the presence or content of hazardous substances in environmental samples.

When inorganic heavy metals enter the body from the environment, they accumulate in certain organs of the body, cause chronic poisoning, and harm health. Researchers have designed a number of cell-free biosensors for detecting heavy metals. For example, a paper-based mercury biosensor was designed by the iGEM team Peking 2010.[35] By adjusting the plasmid concentrations and adding new translation enhancement sequences to optimize the cell-free mercury sensor, it increased the yield of the CFPS reaction and enabled accurate quantification of the analytes. It can detect 6 μg L^{-1} of mercury concentration. The paper-based cell-free biosensor has a low cost and is convenient for detection. In addition, the team built a 3D-printed box that can be connected to a phone for real-time readings, extending the detection platform to real-time applications.

There are also some cell-free biosensors designed to detect toxic organic molecules. For instance, Alam et al.[35] developed a cell-free biosensor platform, a ligand-induced activated RNA output sensor (ROSALIND, RNA output sensors activated by ligand

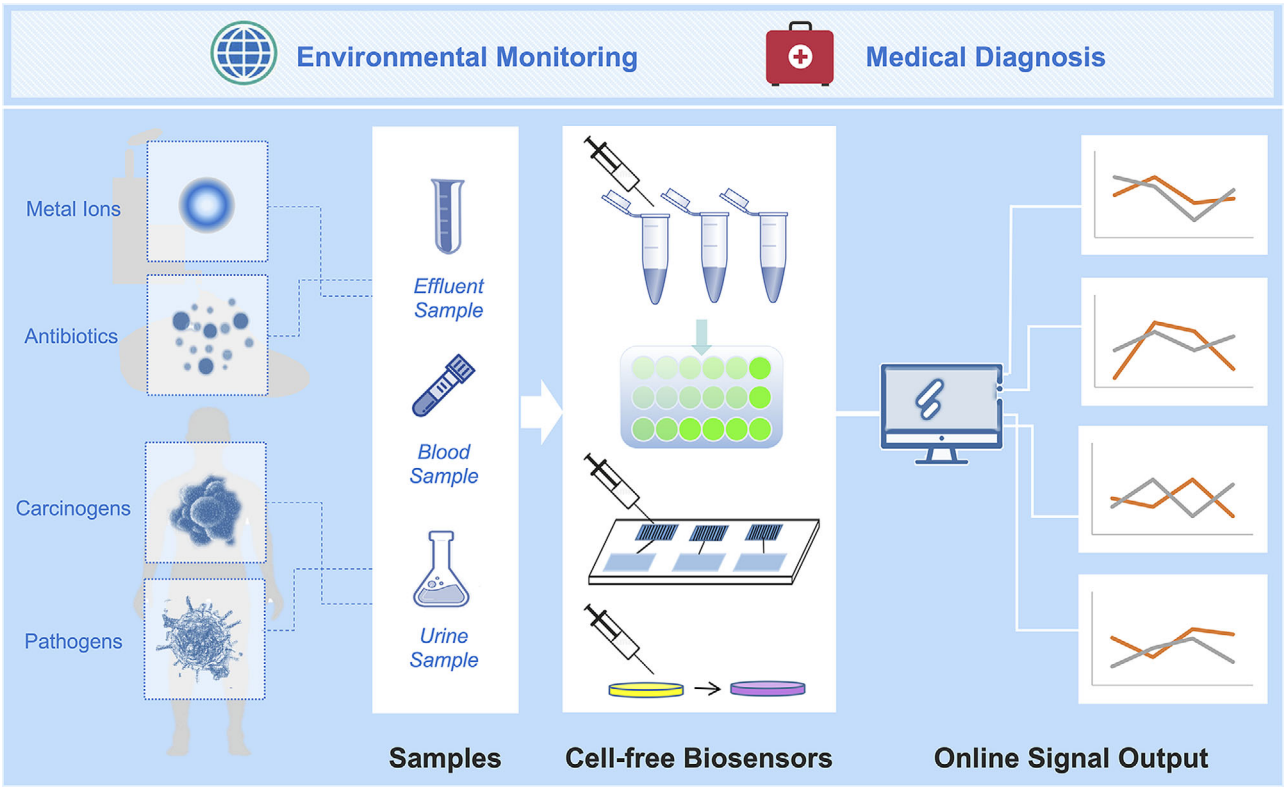

Figure 4. The applications of cell-free biosensors. Cell-free biosensors can quickly and efficiently detect environmental pollutants and human diseases, including heavy metals, antibiotics, pathogens, and even clinical samples such as blood and urine. The output signals are detected by enzyme markers and other instruments, and the data are obtained online for analysis.

induction), which uses a variety of TFs to detect harmful pollutants in water samples, such as toxic PPCP (pharmaceutical and personal care product) compounds (salicylate, benzalkonium chloride, and uric acid). However, when testing non-laboratory samples (e.g., drinking water and lake water), the sensitivity of the sensor may be reduced due to the interference of the molecular components in the samples. Therefore, the matrix effect of these test samples (e.g., environmental water samples) needs to be further explored in future work.

Pesticides are also a potential hazard to the environment. If they enter the human body from the environment, they will cause harm to human health.[102] Silverma et al.[48] developed a cell-free biosensor to detect the herbicide atrazine. They combined that CYA (cyanuric acid) cell-free sensor with a reconstituted cell-free metabolic pathway that converts atrazine to CYA through three steps in a one-pot reaction. Using a modular approach, the sensor can be capable of identifying high concentrations of Atrazine (10-100 μM). The combined metabolism and biosensing strategy will be applicable for the rapid cell-free detection of pesticides and other water contaminants, as well as environmental biomarkers and human performance analytes.

The presence of antibiotics in the environment is harmful to the ecological balance and human health. Therefore, its presence in the environment needs to be monitored in a timely manner. Duyen et al.[41] have developed a simple biological sensor based on colorimetric paper. The detection limits of paramycin, tetracycline, erythromycin, and chloramphenicol were further estimated to be 0.5, 2.1, 0.8, and 6.1 μg mL^{-1}. However, this sensor is not specific. It directly inhibits bacterial protein synthesis through antibiotics, so that any factors affecting TX-TL will affect its accuracy. So this kind of sensor needs further improvement. Pellinen et al.[33] designed another cell-free biosensor for the detection of tetracycline hydrochloride (Tc-HCl). Tc-HCl has been detected at concentrations below 10 ng mL^{-1}. The maximum residue limit for Tc-HCl in EU milk samples is 100 ng mL^{-1}, and the limit for Tc-HCl in commercial microbial inhibition tests is 30 to 500 ng mL^{-1}. It can be seen that the detection limit of cell-free biosensors can reach the ppb level, which has reached the environmental standard level for the detection of some pollutants. The test results can be used to judge whether the samples under test meet the standards or not. Although the sensitivity of current cell-free biosensors can reach a certain level, some substances with lower detection limits still exist or will appear in the future, which requires us to continuously improve and optimize the cell-free biosensors.

These examples demonstrate that cell-free biosensors have been successfully applied to the detection of toxic substances in the environment. Due to its low detection limit and fast and convenient characteristics, cell-free biosensors can quickly detect pollutants, determine the safety limit of analysis samples, and timely monitor the environment for timely prevention or repair. Through the continuous optimization of cell-free biosensors, it has a broad prospect in environmental monitoring.

4.2. Clinical Biomedical Applications

Because of the high sensitivity and fast response time of cell-free biosensors, they can quickly judge the diagnosis result, which occupies an important position in biomedicine. The existing cell-free biosensors mainly focus on the detection of clinically relevant carcinogens, bacteria, and pathogens (e.g., endocrine-disrupting chemicals (EDCs), amino acids (AAs) and N-acyl hyperserine lactone (AHL)).[37,38,52,103] These sensors can be used to screen pathogens that can be detected in blood, urine, and sputum samples from clinical patients.

The well-known chemicals called endocrine-disrupting chemicals (EDCs),[51,104] widely exist in the environment, food, and personal care products. Exposure to EDCs can lead to acute and chronic diseases, including cancer and diabetes. Salehi et al.[105] designed a hERβ-CFPS (human estrogen receptor β, hERβ) biosensor to detect endocrine xenoestrogens (XEs, one kind of the EDCs) in human blood and urine. Because these test samples (blood, urine, and sputum samples from clinical patients) are complex, they can interfere with the cell-free system to a certain degree and may alter the delicate calibration of protein synthesis reactions, making the test process less smooth. In order to solve this problem, some measurements can be taken to pretreat the sample. They added RNA enzyme inhibitors to the samples, which can reduce the impact of urine or blood samples on the CFPS system. This can simplify the detection process, which can be used for more complex analytes and expand the application range of cell-free biosensors.

The virus is rapidly pathogenic, and some are highly infectious. The severity of the virus infection and its unknown complications make timely diagnosis of the virus critical to the human health, which also can limit the rapid spread of the virus. In this context, some cell-free biosensors are designed to detect viruses. Pardee et al.[43] developed a rapid diagnostic cell-free biosensor based on a toehold switch to detect Zika virus from the plasma of a viremic macaque. They further improved the sensitivity of the sensor by isothermal amplification. By the NASBA-CRISPR Cleavage Assay, the sensor can distinguish between different strains (Zika virus and dengue virus). Cell-free biosensors can also be used to detect other viruses, such as Ebola virus,[45] Norovirus,[44] and novel coronavirus SARS-CoV-2.[106] In a word, cell-free biosensors can detect not only the presence or absence of a single virus but also different types of viruses.[43]

The content of amino acids also has a great impact on human health. The lack of amino acids can lead to abnormal physiological functions, affect the normal progress of antibody metabolism, and lead to diseases. It can be used as an early detection method for several types of cancer.[107,108] Jang et al.[53,54] designed a CFPS biosensor to detect amino acids in fetal bovine serum (FBS) samples. The amino acid content was successfully detected, and the detection limit was less than 100 nM. However, the types of amino acids detected by cell-free sensors based on crude extracts are limited, so they suggested using the PURE system for cell-free biosensors. It can detect more kinds of amino acids (Asp, Asn, Glu, and Gln) than the crude extract system, and the detection threshold is lower.[109]

Quorum sensing (QS),[110] is one system employed by many bacterial species, including *Pseudomonas aeruginosa*, to regulate cooperative and pathogenic behaviors. *P. aeruginosa*, a Gram-negative bacterium, is the dominant pathogen affecting persons with Cystic fibrosis by their late teens.[111] QS molecules can be used as an indicator of patient detection. Wen et al.[38] designed a modular DNA-encoded biosensor in cell-free protein expression systems can be used to measure a bacterial sputum samples. By optimizing the cell-free system and sample extraction, they demonstrate that the quorum-sensing molecule 3-oxo-C12-HSL (AHL) in sputum samples from cystic fibrosis lungs can be quantitatively measured at nanomolar levels using cell-free biosensor system.[37] Their experiments demonstrated that modular cell-free biosensors could rapidly and cheaply detect clinically relevant patient samples associated with infection status.

These examples show that cell-free biosensors have significant advantages in medical diagnosis and clinical sample testing. They can be used for early detection of disease and also can respond quickly to virus outbreaks or the occurrence of genetic variations and mutations to help monitor the spread of disease. The achievements of cell-free biosensors in environmental detection and medical diagnosis are remarkable, however, there is some lag in commercial applications of cell-free biosensors, which will be the focus of future development.

5. Conclusion

The cell-free system with unique advantages provides a novel design platform for the development of biosensors. Considering the openness and rapid prototyping capability of CFPS, the CFPS system can open up more design space for the construction and optimization of biosensors and genetic circuits. Cell-free biosensors based on TF, RNA, and toehold switch can reduce the number of cycles required to design and help build complex genetic networks. CFPS system can also be integrated with mathematical modeling and high technologies (e.g., automation and microfluidics) to unlock the potential of cell-free biosensors.[112]

In the practical applications of cell-free biosensors, the lyophilization technology based on the portable paper platform can maintain the stability of the system, which promotes its portable use and reduces the cost. However, there are still many challenges to be addressed. The decline of the activity after the storage is a problem that needs to be further improved. To improve in situ detection, multiplexing functions, the sensitivity and the specificity of cell-free biosensors need to be further optimized.[8] In the future, the cell-free biosensors could use synthetic biology tools that have been demonstrated on cell-based biosensors (e.g., engineering modular genetics, logic gates, and amplifying circuits) to improve their sensitivity, selectivity, and extend their function.[14,94,95,113]

In addition, cell-free biosensors can use CFPS systems other than *E. coli*, such as yeast and mammalian cells, to develop more sophisticated biosensors.[114,115] By improving the sensor recognition mechanisms and combining cell-free systems with other materials (e.g., silicon), more types of functional cell-free biosensors can be developed. It might expand the detection range of cell-free biosensors to sense the odor, air, temperature, light, and osmotic pressure. With further evolution of cell-free biosensors, besides environmental monitoring and human health diagnosis, they will increasingly expand their applications to food testing, classroom education, and others, making them more practical and commercial.[52,97,116,117]

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Conflict of Interest

The authors declare no conflict of interest.

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