



Regular article

Reengineering cell-free protein synthesis as a biosensor: Biosensing with transcription, translation, and protein-folding



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ABSTRACT

Biosensors hijack and reengineer biological systems for highly specific detection of diverse molecules. However, many traditional biosensor devices have slow response times and high operating costs and require specialized training and immobile lab equipment. In an effort to address these limitations, researchers have begun using cell-free protein synthesis (CFPS) systems as biosensors. CFPS-based systems provide the advantages of speed, portability, and robustness at a relatively low cost. Here, we review CFPS-based biosensors according to the three fundamental stages of CFPS: transcription, translation, and protein folding.

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1. Introduction

Biosensors have been employed in a wide variety of fields including clinical diagnosis, environmental monitoring, food safety, and biowarfare agent detection [1]. Biosensors commonly provide a highly sensitive, very specific assay; however, many of these diagnostic devices have slow response times and high operating costs and require specialized training and immobile lab equipment [2]. Thus, there is a need to develop more rapid, portable, and inexpensive detection methods.

A promising approach to addressing these limitations is utilizing cell-free protein synthesis (CFPS). Early on, CFPS played an important role in determining how amino acids are incorporated into proteins and in deciphering the genetic code [3]. Since this time, researchers have used CFPS for many other applications. To date, CFPS systems have been applied as (1) a protein production platform for the expression of diverse therapeutics, vaccines, and industrial biocatalysts [4–10]; (2) a synthetic biology platform for *in vitro* chemical biosynthesis [11], synthetic enzyme pathway expression [12,13], and nucleic acid circuitry [14,15]; and (3) a research platform for high-throughput advanced protein evolution [16–19], protein-polymer and protein-surface conjugation optimization [20–22], unnatural amino acid incorporation [21,23–26],

protein microarray fabrication [27,28], and genome engineering [29,30].

Recent developments in CFPS systems have further increased the possibility of applying CFPS technology to biosensors. Some of these recent advances include demonstrating that CFPS is viable in a wide range of environmental samples including raw sewage, human urine, and human blood [31–34]; demonstrating that CFPS systems can be lyophilized and maintain activity after being stored at temperatures as high as 37 °C for months [35–37]; and reducing the cost of cell-free components significantly with lysate-based systems [35,38]. Cell-free systems also eliminate cell membrane transport barriers and allow for high-throughput assays.

While many previous reviews have focused on biosensing [2,39–41] or CFPS [42,43] individually, and others have generally discussed *in vitro* biosensors [43,44], here we specifically review cell-free biosensors that utilize the process of CFPS (Table 1). This review is organized according to the three fundamental stages of cell-free protein synthesis where biosensing has been engineered to occur: transcription, translation, and protein folding (see Figs. 1–3).

2. CFPS biosensing at transcription

Since 2004, researchers have developed CFPS-based biosensors at the transcriptional level [45,46] for a number of applications, including disease diagnostics, analyzing crosstalk between bacterial species, and the detection of toxic metals [46–48]. These transcription-based biosensors are engineered to repress transcrip-

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Table 1
Summary of CFPS biosensors discussed.

Level of CFPS Biosensing	Summary of process	Target molecules	Detection level	Response time	Reference
Transcription	A repressor protein prevents transcription of DNA encoding an indicator protein if the analyte is not present. If the analyte is present, it binds to and releases the repressor protein, and transcription and translation of the indicator protein occurs.	Tc, HgCl ₂	Tc: 10–10,000 ng/mL HgCl ₂ : 1–10,000 nM	Tc: <90 min HgCl ₂ : 30 min	[45] ¹
		3O-C ₁₂ -HSL, a <i>Pseudomonas aeruginosa</i> quorum-sensing (QS) molecule	1–10,000 nM	30 min	[47] ²
		SCB1, a <i>Streptomyces coelicolor</i> QS molecule.	5 nM	30 min	[46] ³
		QS molecule.	0.125–2.5 nmol	30 min	[48] ⁴
		C ₈ -AHL, an <i>Escherichia coli</i> QS molecule	1–5 μM	1 h	[49] ⁵
		3O-C ₁₂ -HSL, a <i>Pseudomonas aeruginosa</i> QS molecule	10 nM–1 μM	Not reported	[50] ⁶
Translation (component sensor)	An essential molecule for CFPS is not supplied, so that translation of an indicator protein occurs only if the missing molecule is present in the sample tested.	12 amino acids: Ala, Cys, Gly, His, Pro, Ser, Thr, Trp, Asp, Asn, Glu, Gln	0.1–1 μM	1 h	[33] ⁷
Translation (toehold switch sensor)	A toehold switch sequesters an mRNA's ribosome binding site. When a target RNA sequence hybridizes with the switch, the mRNA structure changes to facilitate ribosome binding and translation of the indicator protein.	RNA from the Ebola virus genome	3 nM	1 h	[54] ⁸
		RNA from the Zika virus genome	1 fM	30 min	[34] ⁹
Protein folding	A reporter protein produced by CFPS folds incorrectly unless the analyte is present.	endocrine disrupting molecules (EDC)	9 nM	30 min	[32] ¹⁰

¹ Pellinen, T.; Huovinen, T.; Karp, M., A cell-free biosensor for the detection of transcriptional inducers using firefly luciferase as a reporter. *Anal Biochem* 2004, 330 (1), 52–7.

² Chappell, J.; Jensen, K.; Freemont, P. S., Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Res* 2013, 41 (5), 3471–81.

³ Wen, K. Y.; Cameron, L.; Chappell, J.; Jensen, K.; Bell, D. J.; Kelwick, R.; Kopniczy, M.; Davies, J. C.; Filloux, A.; Freemont, P. S., A Cell-Free Biosensor for Detecting Quorum Sensing Molecules in *P. aeruginosa*-Infected Respiratory Samples. *ACS synthetic biology* 2017, 6 (12), 2293–2301.

⁴ Yang, Y. H.; Kim, T. W.; Park, S. H.; Lee, K.; Park, H. Y.; Song, E.; Joo, H. S.; Kim, Y. G.; Hahn, J. S.; Kim, B. G., Cell-free *Escherichia coli*-based system to screen for quorum-sensing molecules interacting with quorum receptor proteins of *Streptomyces coelicolor*. *Appl Environ Microbiol* 2009, 75 (19), 6367–72.

⁵ Kawaguchi, T.; Chen, Y. P.; Norman, R. S.; Decho, A. W., Rapid screening of quorum-sensing signal N-acyl homoserine lactones by an in vitro cell-free assay. *Applied and environmental microbiology* 2008, 74 (12), 3667–3671.

⁶ Seto, J.; Buske, P.; Laurie, M., On a Robust, Sensitive Cell-Free Method for *Pseudomonas* Sensing and Quantification. *bioRxiv* 2018, 256438.

⁷ Jang, Y.-J.; Lee, K.-H.; Yoo, T. H.; Kim, D.-M., Complementary cell-free translational assay for quantification of amino acids. *Analytical chemistry* 2017, 89 (18), 9638–9642.

⁸ Pardee, K.; Green, A. A.; Ferrante, T.; Cameron, D. E.; DaleyKeyser, A.; Yin, P.; Collins, J. J., Paper-based synthetic gene networks. *Cell* 2014, 159 (4), 940–54.

⁹ Pardee, K.; Green, A. A.; Takahashi, M. K.; Braff, D.; Lambert, G.; Lee, J. W.; Ferrante, T.; Ma, D.; Donghia, N.; Fan, M., Rapid, low-cost detection of Zika virus using programmable biomolecular components. *Cell* 2016, 165 (5), 1255–1266.

¹⁰ Salehi, A. S.; Shakalli Tang, M. J.; Smith, M. T.; Hunt, J. M.; Law, R. A.; Wood, D. W.; Bundy, B. C., Cell-Free Protein Synthesis Approach to Biosensing hTRβ-Specific Endocrine Disruptors. *Analytical chemistry* 2017, 89 (6), 3395–3401.

tion of an indicator protein's DNA unless a target analyte is present (see Fig. 1). A repressor protein restricts expression of an indicator protein by binding the repressor-protein binding site on the DNA upstream of the gene encoding indicator protein. When the target analyte is present in the solution being tested, it dimerizes with the repressor protein, which causes the repressor protein to dissociate from the RBS. The indicator protein gene can then be transcribed into mRNA and translated into indicator protein.

GFP is most commonly used as an indicator protein, but using enzymes has the advantage of a greater control over the signal intensity and a stronger overall signal. For example, researchers using the β-galactosidase enzyme as their indicator protein reported a 10-fold enhancement in signal intensity when they replaced the chromogenic substrate X-Gal with the luminescent substrate Beta-Glo [49]. In addition, enzymes catalytically process substrate molecules, resulting in exponentially stronger signals than fluorescent proteins. Their catalytic rate can also be tuned not only by using different substrates, but also by changing the temperature, substrate concentration, and chemical composition of the reaction environment, which provides more control over optimizing the sensor for the desired sensitivity and accuracy.

One application of transcription-based CFPS biosensors is improving infectious disease diagnostics. In 2013, Chappell et al. developed a transcription-based CFPS biosensor using GFP as the

indicator protein and LasR as the repressor to detect 3O-C₁₂-HSL [47], a quorum-sensing (QS) molecule used by *Pseudomonas aeruginosa*, a bacterial species often contracted by patients with cystic fibrosis [46]. Quorum sensing molecules (QS) are used to coordinate activities within a bacterial community, including biofilm formation and production of virulence factors. In 2017, Wen et al. showed that in clinical samples Chappell et al.'s CFPS-biosensor had greater specificity for the correct QS molecule, less cross-activation by non-target QS molecules, and a lower detection limit (down to 5 nM of 3O-C₁₂-HSL, compared to 22 nM) than the corresponding cell-based biosensor [46]. In 2018, Seto et al. improved upon Chappell et al.'s biosensor by introducing hydrogel microscale agarose beads that encapsulated the cell-free lysate and the transcription/translation processes to provide additional portability and robustness [50].

Another use of transcription-based biosensors is the identification of crosstalk between bacterial strains of different species. In 2009, Yang et al. verified that SCB1, a QS molecule used by *Streptomyces coelicolor*, can deactivate the Scb repressor *in vitro* [48]. They used the Scb repressor, which is specific to *S. coelicolor*, to develop a transcription-based CFPS biosensor similar to that described above and illustrated in Fig. 1. Beyond SCB1, they tested QS molecules used by foreign strains of bacteria to see if they would also deactivate the Scb repressor and cause the biosensor to produce a signal. Two QS

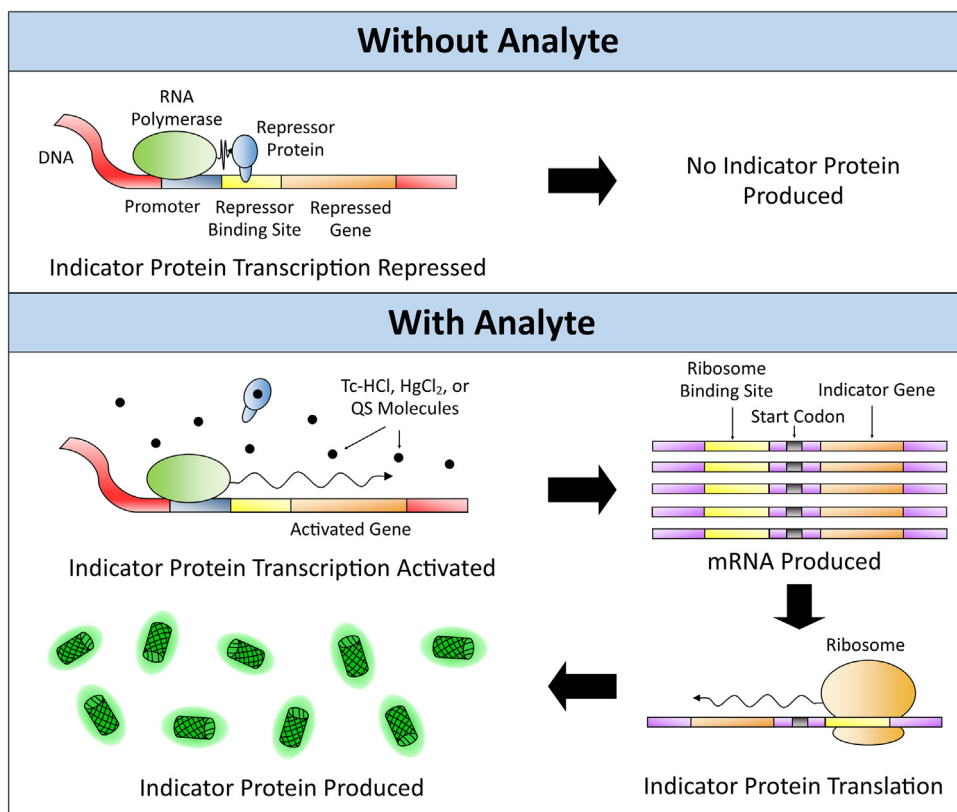


Fig. 1. CFPS Biosensing at Transcription.

molecules from foreign strains were found to also deactivate ScbR: VB-C, a QS molecule used by other *Streptomyces* species, and C₆-HSL, a QS molecule used by Gram-negative bacteria. This finding indicates that transcription-based biosensors have the capability to identify crosstalk between bacterial species previously thought to have no direct communication. It also demonstrates the need to extensively screen for crosstalk prior to applying a transcription-based biosensor to clinically test for infections.

Potential also exists for transcription-based CFPS biosensing beyond the sphere of QS molecule detection. Pellinen et al. compared *in vivo* and *in vitro* approaches to using transcriptional regulation to detect tetracycline hydrochloride (Tc-HCl) and mercuric chloride (HgCl₂) [45]. For each analyte, the *in vivo* and the *in vitro* biosensing approaches used the same DNA sequence, repressor protein, and firefly luciferase indicator protein. The *in vitro* approach enabled a wider range of Tc and HgCl₂ detection (10–10,000 ng/mL compared to 100–1,000 ng/mL for Tc and 1–10,000 nM compared to 2–200 nM for HgCl₂) and had a shorter induction time due to the absence of cell membranes. These results imply that CFPS biosensors can assess the bioavailability of toxic metals like Tc and HgCl₂ in a wider detection range and with a faster response time than cell-based biosensors.

3. CFPS biosensing at translation

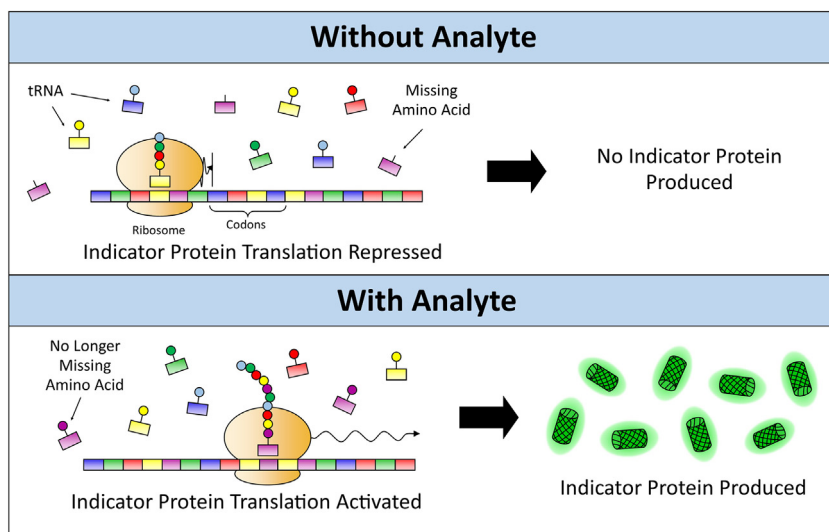
In addition to using CFPS systems as biosensors at the transcription-level, sensing at translation is a promising approach. Although translation-level biosensing lacks the earliest stage of signal amplification during transcription (which transcription-level biosensing does have, as illustrated in Fig. 1 by the many mRNA molecules produced from a single DNA template), it maintains the translation-level signal amplification (as illustrated in Fig. 2 by the many proteins produced from a single mRNA) and further catalytic

amplification if an enzyme is used as the reporter protein. The discussion of translation-level CFPS-based biosensors below is broken up into two parts: sensors that have been developed to detect chemical components necessary for protein translation and sensors that utilize translation activation control such as RNA toehold switch biosensors.

3.1. CFPS component sensors

The most straightforward approach to using CFPS for biosensing is detecting molecules that are necessary for transcription and translation, such as amino acids, ribonucleotides, and even small ions such as Mg²⁺ that have to be present in optimal conditions for protein synthesis to occur. To date, this approach has solely been explored for amino acid detection at the translation level. Jang et al. observed that by withholding one amino acid from the CFPS reagents, expression of reporter protein GFP could be correlated to the concentration of the withheld amino acid added, after a 1 h CFPS reaction at 30 °C (Fig. 2) [33]. This worked for 8 amino acids (Ala, Cys, Gly, His, Pro, Ser, Thr, and Trp), where the concentration of GFP production corresponded linearly to the amino acid concentration down to 0.1–1 μM. However, for 4 amino acids tested (Asp, Asn, Glu, Gln), GFP was expressed at the same level regardless of whether these amino acids were withheld. This is attributed to enzymatic production of the corresponding enzymes by endogenous machinery in the lysate. To overcome this obstacle, the authors demonstrated that the PURE CFPS system, which includes a minimal set of individually purified proteins and cofactors needed for transcription and translation, was effective in sensing the last 4 amino acids (Asp, Asn, Glu, Gln) down to ~20 μM. However, the PURE system is ~1,000-fold more expensive in reagent costs than the lysate-based CFPS system [13,16,26,51]. This might justify exploring genetically engineering the bacterial strain

A. Missing CFPS Component



B. Toehold Switch

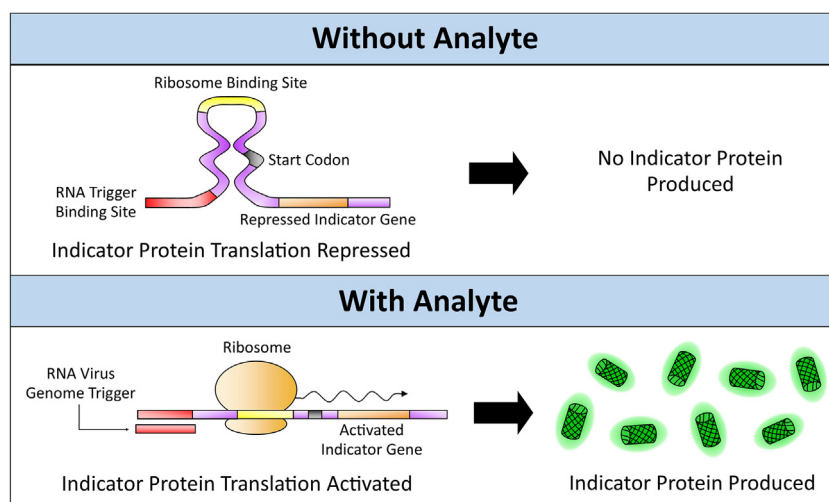


Fig. 2. CFPS Biosensing at Translation. (A) Missing CFPS components biosensor mechanism applied to amino acids. (B) Toehold switch biosensor mechanism.

used to produce the CFPS lysate to eliminate side reactions that produce these corresponding enzymes.

Amino acids are directly related to human health and abnormalities [52] and also could be used as an early detection method for several types of cancer [53]. Conventional methods for the detection of amino acids, including electrochemical, chromatographic, and spectroscopic approaches, require lengthy amounts of time for pretreatment and detection, experienced operators, and expensive modern apparatuses that are usually not portable [52]. Jang et al.'s CFPS amino acid biosensor not only used a fairly simple method, but the results showed that it was faster than cell-based methods, required less sample pretreatment, and had greater potential for field use. Biological samples of fetal bovine serum (FBS) were tested to verify the accuracy of this method, but reportedly due to the presence of inhibitory components such as nucleases, the signal expression was originally low. Heat-treatment of FBS samples followed by ultrafiltration and removal of aggregated protein significantly improved the quality of the CFPS reaction and the accuracy of amino acid detection in the FBS, yielding results almost identical to those of the traditional HPLC (high-performance liquid chromatography) assay [33].

3.2. RNA toehold switch sensors

Another approach to translation-based CFPS biosensing was recently demonstrated by Pardee et al., who reengineered the cell-based toehold switch biosensor developed by Collin's research group and combined it with paper-based sensing methods to develop a CFPS biosensor capable of detecting Zika virus. This translation-based CFPS biosensor uses an RNA toehold switch that represses expression of an indicator protein unless a specific RNA trigger is present (Fig. 2). The toehold switch sequesters the mRNA's ribosome binding sequence (RBS) and start codon in a hairpin loop; upon hybridizing with an RNA trigger sequence, the toehold switch changes conformation and the mRNA is translated, producing the indicator protein [54].

Diagnosis of Zika virus in remote places is critical for controlling the spreading of the virus, especially in South America. Antibody-based detection of Zika virus has a poor performance due to cross-reactivity in patients with previous exposure to other flaviviruses [55]. On the other hand, biosensors that detect viral RNA using loop-mediated isothermal amplification (LAMP) or reverse transcription loop-mediated isothermal amplification (RT-LAMP) [56] need trained personnel with sophisticated and expensive

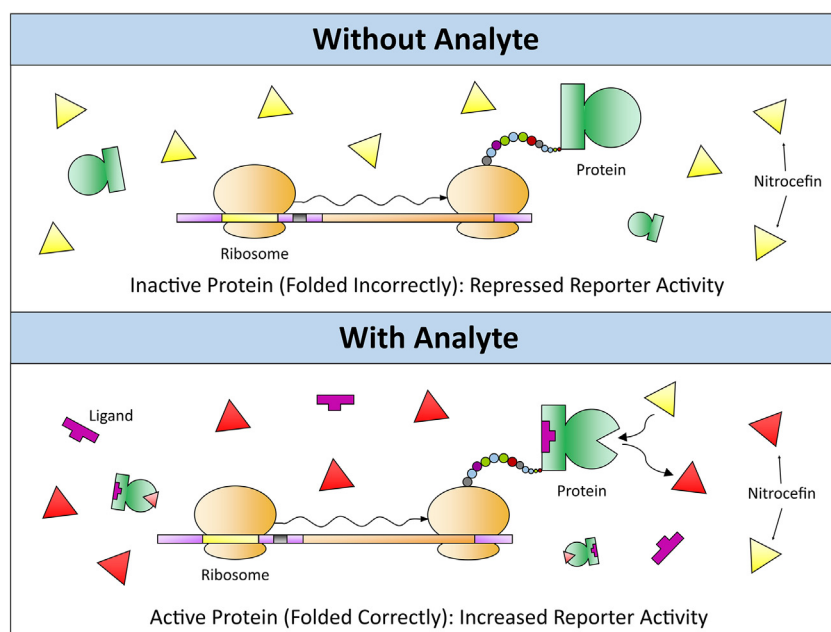


Fig. 3. CFPS Biosensing at Protein Folding.

apparatuses, which makes them unsuitable for field usage in remote locations with limited resources like Africa, South America, and Asia. Yet it is in these remote locations that infections are predominant, which creates an urgent need for simple, inexpensive, and accurate detection techniques [34,56].

The CFPS biosensor developed by Pardee et al. distinguishes between the Zika virus and other virus strains that have genomes with comparable RNA sequences, including Dengue and Chikungunya, and when combined with CRISPR/Cas9 technology, it can even distinguish between American and African strains of the Zika virus [34]. By combining this CFPS biosensor with nucleic acid sequence-based amplification, Pardee et al. detected the target Zika RNA molecule at concentrations as low as 1 fM. This is comparable to the clinically relevant concentration of 1.2 fM for serum and is much lower than the clinically relevant concentration of 365 fM for urine [34].

Pardee et al. also used toehold switch technology combined with paper-based sensing methods to develop cheap, portable CFPS biosensors for Ebola virus genome sensing [54]. Paper-based biosensors use lyophilized CFPS systems that are stable for lengthy periods of time at room temperature and suitable for use in remote places [54]. The freeze-drying of amplification-step reagents and toehold switches on paper reduced the chance of contamination and false positives. It also reduced costs to \$0.10–1.00 for each test, which is substantially lower than even the amplification step alone due to the low volume of freeze-dried reagents required for the paper sensor [34]. Toehold switch technology has potential to be expanded to diagnose many additional diseases, as toehold switches for a specific RNA sequence can be rapidly designed *in silico* [34,54].

4. CFPS biosensing at protein folding

Beyond the promising transcription- and translation-level CFPS biosensors discussed above, CFPS systems have also been engineered to use the protein folding process immediately following translation as a mechanism for biosensing. Salehi et al. introduced the Rapid Adaptable Portable *In vitro* Detection biosensor (RAPID) as a biosensor platform that detects endocrine disrupting chemicals (EDCs) when they bind to an allosterically-activated chimeric

protein while the chimeric protein is folding (Fig. 3) [31,57–59]. EDCs are chemicals that inhibit or mimic the natural hormones of the endocrine system disrupting metabolism, homeostasis, differentiation, development, and reproduction [60–62]. As such, EDCs are linked to the development of cancer, developmental disorders, and other epigenetic dysfunctions [63,64]. The chimeric protein of RAPID includes a nuclear hormone receptor binding domain that targets EDC ligands during protein folding. If EDCs are present in a sample being tested, the protein folds differently than normal, and its reporter enzyme domain is activated and begins to convert yellow nitrocefin into a red product (Fig. 3), a color change that can be recognized by the naked eye. Sensors for the human estrogen receptor beta and the human thyroid receptor beta have been successfully developed, detecting EDCs down to the 9 nM level [31,65].

The RAPID CFPS biosensor is a robust, scalable, and portable system capable of sensing EDCs in samples of raw sewage, human blood and human urine. It also has potential for detection in tap water, pond water, snow, storm/run-off water, and soil water samples and has been shown to be functional with diverse EDC. A lyophilized version for portable in-field detection that gives a read-out in less than 30 min has also been developed. Since the sensing step is triggered by protein folding regulation, RAPID harnesses the speed and robustness of CFPS to provide fast and accurate colorimetric assay readouts even in extreme conditions, without the necessity of purification or complex stabilization steps required by other EDC biosensors [31,65].

5. Future perspectives and conclusion

Significant potential exists for biosensors at all stages of CFPS. Transcription-based biosensors and translation-based toehold switch biosensors alike have potential for use in detecting additional pathogens for disease diagnostics. For example, transcription-based biosensors can be used to detect additional QS molecules, while translation-based toehold switches could be developed for a broader range of target RNAs and RNA virus genomes [54].

Translation-based CFPS component biosensors have potential for use in medical diagnostics, specifically as an early detection

method for several types of cancer [53]. Jang et al.'s work on amino acid biosensors [33] could be furthered by engineered to detect additional amino acids by removing enzymes capable of interconverting amino acids, so that additional amino acids can be detected. In addition, CFPS component biosensors could be engineered to sense for ribonucleotides, small ions such as Mg^{2+} , and other molecules necessary for transcription and translation.

To potentially increase portability and robustness, the reactants for translation- and protein-folding-based biosensors could be implemented in hydrogel microscale agarose beads, as has been done with transcription-based biosensors [50]. Moreover, while CFPS biosensors primarily use inexpensive *E. coli*-based systems [32–34,46], an *Agrobacterium tumefaciens*-based CFPS biosensor has been reported [49]. In addition, other non-*E. coli*-based systems, including yeast, wheat gram, and mammalian systems could become necessary as more complex biosensors are developed [66–69].

Overall, CFPS systems show great promise for use in biosensing. When compared to cell-based sensors, CFPS biosensors have the distinct advantages of (1) functioning in cytotoxic environments, (2) requiring less time for a response, (3) enabling rapid transport due to the absence of a cell wall, (4) allowing for high-throughput sensing, and (5) facilitating portable in-field use through lyophilization technology. Effective CFPS biosensors have been developed with mechanisms at the transcription level, translation level, and protein folding level as detailed above. While challenges remain and additional engineering is needed to achieve the sensitivity desired for many applications, researchers have demonstrated the extreme robustness and versatility of these systems with recent successful assays in raw sewage, blood, and urine [65].

Declaration of interest

None.

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