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Cell-free biosensors for biomedical applications

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ABSTRACT

Recent years have witnessed a rapid increase of the use of cell-free systems for biosensor engineering. Building on the past decade of synthetic biology research mostly performed in living cells, researchers have developed a variety of cell-free applications capable of detecting antibiotics, clinical biomarkers, or nucleic acid sequences from viral and bacterial pathogens. Cell-free systems allow for simple, fine-tuning of circuit components stoichiometry and provide a non-living alternative to whole-cell biosensors that could expedite their path through regulatory approval. Additionally, many of these systems have been shown to exhibit increased stability upon lyophilization, potentially enabling point-of-care applications and use in low-resource settings where continuous cold-chain maintenance may be difficult. Here we discuss the latest advances in cell-free biosensor research and highlight some key challenges to address in order for the field to bring its full potential.

Keywords: Cell-free, Biosensors, TXTL, Point-of-Care, Diagnostics

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Introduction

Over the last twenty years, the number of publications on 'cell-free' protein synthesis systems has steadily increased (**Figure 1**). Although the history of cell-free systems dates back to the deciphering of the genetic code[1], recent years have seen cell-free protein synthesis systems adopted by the field of synthetic biology, as they can be used to rapidly prototype biological circuit design[2], study the origin of life via a 'bottom-up' approach[3], and develop biosensors. Part of this rapid expansion has been fueled by a variety of methods for producing cell-free extract, the cytoplasmic component of the reactions containing the polymerases, ribosomes, and other machinery required for protein synthesis. These methods vary in scale from milliliters to liters of culture and lysis methods ranging from French press and sonication to even simply freeze-thawing[4–7]. Additionally, the commercial PURE system contains all of the purified components for protein synthesis without the RNases and proteases from extract-based systems, and it can even be rapidly and cheaply produced in-house[8,9]. Combined with new toolboxes for high protein output and the development of biological circuits [10], there are now a broad range of targets, detection mechanisms, outputs, and stability and robustness techniques implemented in cell-free biosensors (**Figure 2**).

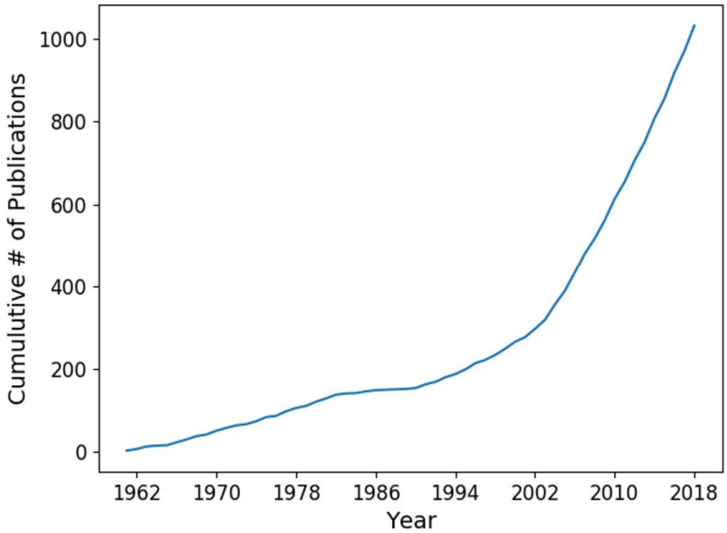

Figure 1. Publications on "cell-free protein synthesis" systems have steadily increased in recent years. Data from Web of Science (WoS, Thomson Reuters) search using the topic "Cell-free Protein Synthesis".

While many other *in vivo* biosensors have been developed under the domain of synthetic biology, they have already been extensively reviewed[11–14] and will not be included here. Rather, we will focus principally on three aspects of cell-free biosensors. First, their use in detecting small molecules including ions, biomarkers, and antibiotics; second, the development of cell-free technologies for detecting nucleic acid sequences from viruses and other pathogens; and third, the pre-processing procedures involved in using complex samples and the variety of techniques used to improve sensor stability including lyophilization.

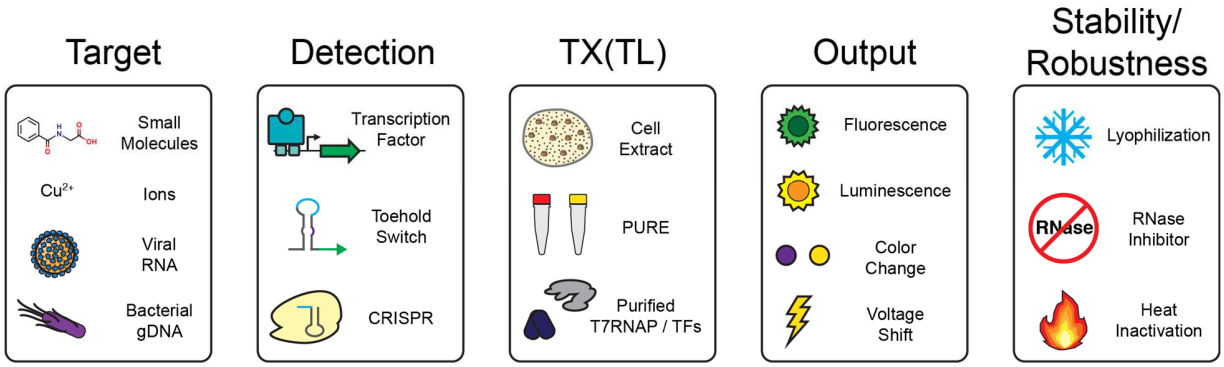

Figure 2. Cell-free biosensors use a variety of methods and systems for detection. Sensors can be developed for small molecules, ions, or viral RNA or bacterial genomic DNA. Detection strategies include transcription factor activation/repression, RNA-based ribosome binding site sequestration in toehold loops, or CRISPR-based RNA recognition. Transcription and/or translation [TX(TL)] can be performed using cell extract, the complete transcription and translation PURE system, or using selectively purified polymerases and transcription factors. Signal output can then be measured as fluorescence, luminescence, visible color change, or even voltage shifts. Finally, cell-free sensors can exhibit increased robustness via lyophilization, addition of RNase inhibitors, or heat inactivation of clinical samples before detection.

Detecting small molecules with cell-free biosensors

Detecting ions, antibiotics, and other small molecules

Transcription factor-based biosensors use allosteric transcription factors discovered in nature to control expression of a reporter gene upon binding to particular small molecules. While used extensively with *in vivo* biosensors, they have also been utilized to engineer cell-free biosensors capable of responding to a range of inducer molecules. Gräwe et al. used the transcription factors MerR and BlcR to build cell-free biosensors to detect mercury ions and gamma-hydroxybutyrate[15]. Furthermore, they constructed a 3D-printed box setup with filters compatible with cell phone diagnostics, expanding the diagnostic platform toward point-of-care applications. Another approach has been to use cell-free systems to detect ribosome-interfering antibiotics through analyzing how they reduce expression of a constitutively expressed reporter. Duyen et al. added paromomycin, tetracycline, chloramphenicol, and erythromycin to cell-free reactions in the PURE system and were able to detect each at concentrations between 0.5-1 µg/mL at a reaction pH between 6 and 10; additionally, they detected paromomycin spiked into environmental water samples[16].

While most transcription factor-based biosensors require previously existing transcription factors that respond to a given compound, the field has recently expanded with the incorporation of metabolic engineering strategies. Recent work used the transcription factor BenR to detect benzoic acid, then expanded the range of detectable compounds by incorporation of the metabolic enzymes HipO and CocE, which convert hippuric acid and cocaine, respectively, into benzoic acid[17] (**Figure 3a**). This methodology is scalable using the SensiPath and RetroPath algorithms, which search multiple databases to find metabolic enzymes and transcription factors to convert and detect previously undetectable molecules[18,19]. This framework has been

further expanded to detect benzamide and biphenyl-2,3-diol and combine the relative strength of four different inputs to build a cell-free metabolic perceptron[20].

While most transcription factor-based biosensors monitor detection via transcription and translation of a reporter protein, such as green fluorescent protein, other systems bypass the translation step by using fluorescent aptamers. Alam et al. recently developed the RNA Output Sensor Activated by Ligand Induction (ROSALIND) system that combines freeze-dried polymerases, transcription factors, and DNA templates to produce fluorescent aptamers for ligand detection[21] (**Figure 3b**). They engineered sensors for tetracyclines (tetracycline, doxycycline, oxytetracycline, chlortetracycline), macrolides (erythromycin, azithromycin, clarithromycin, roxithromycin), small molecules (3-hydroxy benzoic acid, salicylate, benzalkonium chloride, uric acid), and metals (zinc[II], copper[I/II], cadmium[II], lead[II]); additionally, the authors were able to increase or decrease the sensitivity of the sensors and demonstrate functionality in environmental water samples. Finally, some research has used cell-free protein synthesis to produce proteins that can be coupled to electronics for increased sensitivity. Chen et al. produced the nematode olfactory receptor ODR-10 via an *E. coli* extract cell-free system for detection of diacetyl and functionalized it to the surface of electrolyte-insulator-semiconductor sensors via a His6-tag and anti-His6-tag aptamers[22]. They showed concentration-dependent linear response to diacetyl from 0.01 nM to 1 nM and specificity vs. 2,3-pentanedione, butanone, and isopentyl acetate. These examples show the diversity of approaches being brought to the development of transcription factor-based cell-free biosensors.

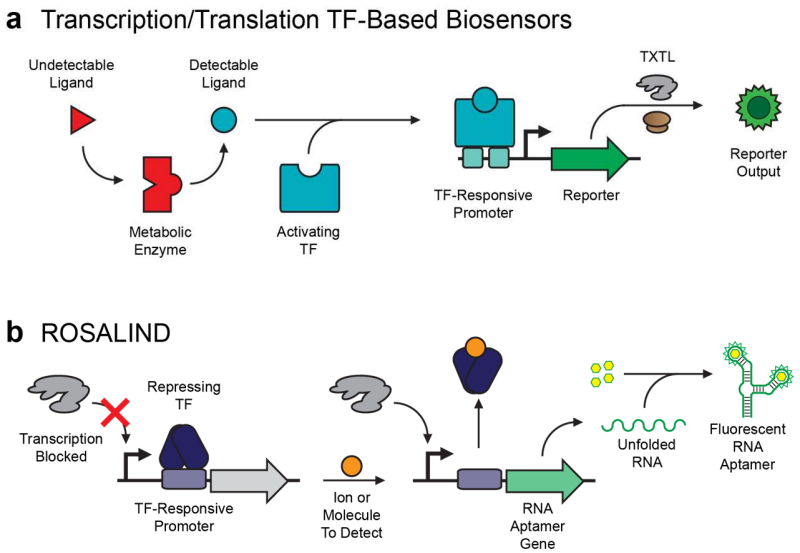

Figure 3. Transcription factor (TF)-based cell-free biosensors detect small molecules or ions via transcription/translation (TXTL) or by transcription alone. a) Binding of a ligand to its activating transcription factor results in expression of a reporter protein. Alternatively, metabolic engineering-based sensors can detect additional ligands for which there is no known transcription factor by initially transforming the ligand into a detectable ligand using a metabolic enzyme. b) ROSALIND uses ligand binding to release a repressing transcription factor, thereby permitting transcription of a fluorescent RNA aptamer. Figure 3a and Figure 3b are adapted from Voyvodic et al.[17] and Alam et al.[21], respectively.

Detecting small molecules from complex samples

While most sensor development is initially conducted by adding purified inducers to reactions, field-deployable biosensors will need to remain functional in complex sample conditions, such as those found in clinical samples like blood and urine. Removing the protein production machinery from within the cell membrane has many advantages in sensor development, but permitting the added sample to directly interact with this machinery while maintaining sensor function is a non-trivial task. Some work has circumvented this interference by including pre-processing steps for added clinical samples. Wen et al. developed a cell-free sensor for the quorum sensing acyl-homoserine lactone (AHL) 3OC12-HSL capable of detection at levels under 10 nM[23]. To detect 3OC12-HSL in the sputum of patients, they extracted the AHLs using an organic solvent that was then dried and rehydrated before testing. The resulting cell-free sensor was able to detect the molecules in patients infected with *P. aeruginosa* and showed reasonable agreement with LC-MS quantification.

A few cell-free sensors have been tested in the presence of unprocessed clinical samples. Salehi et. al developed a hybrid cell-free fusion protein for a nuclear hormone receptor disruptor, human thyroid receptor beta, capable of functioning in up to 40% raw sewage[24]. Additionally, they tested the inhibition of different environmental samples (tap, pond, snow, storm, and soil water), wastewater treatment samples (raw sewage, post clarifier, post biological, post filter, and effluent) and blood and urine on constitutive GFP expression and found that in particular blood and urine exhibited strong inhibitory effects (25% blood or 4% urine resulted in near total inhibition). However, in a later work they found that adding an RNase inhibitor partially recovered expression in urine and significantly recovered expression in blood[25]. Using this upgraded system they detected an estrogen receptor disruptor, E2, in reactions containing up to 20% blood or 10% urine. Finally, our recent work developing sensors for benzoic acid, hippuric acid, and cocaine produced proof-of-concept detectors for each inducer: benzoic acid was detected in commercial beverages and quantified against LC-MS results, endogenous hippuric acid was detected in human urine samples and similarly quantified, and cocaine could be detected in human urine at previously clinically-detected levels[17]. These examples illustrate that while conducting cell-free protein synthesis reactions in the presence of complex samples has its challenges, they are not unsurmountable and sensors can be developed to detect endogenous compounds even in complex environments.

Detecting pathogens via nucleic acids with cell-free biosensors

The ability to detect pathogenic organisms in a cheap, fast, and portable fashion is a key engineering problem to bringing diagnostic technologies to much of the developing world. Many new diagnostic platforms being engineered focus on detecting nucleic acid sequences of these pathogens, as they are highly specific and can be pre-processed via amplification steps to facilitate lower detection limits. One such cell-free diagnostic platform uses toehold switches to control the expression of an output gene; these RNA-based switches create a loop upstream of the gene that sequesters the ribosome binding site until it binds to a trigger RNA, which unfolds the loop and permits translation[26] (**Figure 4a**). Pardee et al. designed toehold switches and trigger RNAs based on Ebola mRNA; additionally, the assay could distinguish between Sudan and Zaire strains. Two years later, the same group developed a rapid detection platform for Zika virus, wherein the workflow permitted the design, validation, and production of cell-free sensors

in as little as five days that could detect clinically relevant concentrations of Zika virus in three hours[27]. This detection limit was made possible through the use of an isothermal nucleic acid sequence-based amplification (NASBA) step that reverse transcribes the target RNA and then through transcription produces many more copies before detection, allowing detection of trigger RNA at concentrations as low as 3 fM. The fast development and low detection limits of these technologies, along with other toehold-based sensors for detecting genomic DNA of the gut microbiota[28] demonstrate great promise for developing point-of-care sensors.

Another nucleic acid-based cell-free sensor of note is the Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) system[29] (**Figure 4b**). Gootenberg et al. take advantage of the ability of Cas13a to not only bind to and cleave a target RNA strand, but once bound to non-specifically degrade surrounding RNA. Using a reporter/quencher pair separated by RNA, they were able to detect Zika and Dengue virus lentiviral particles, Zika virus in human serum and urine, and bacterial culture genomic DNA. Additionally, the system can differentiate between base pair mutations from viral RNA or human genotyping. The following year, the SHERLOCKv2 improved on this technology with multiplexing using orthogonal enzymes (Cas13, Cas12a, and Csm6), quantitatively measuring down to two attomolar, increasing sensitivity, and incorporating a lateral flow readout[30]. The extremely low limit of detection provides great promise for these nucleic acid based cell-free biosensors going forward.

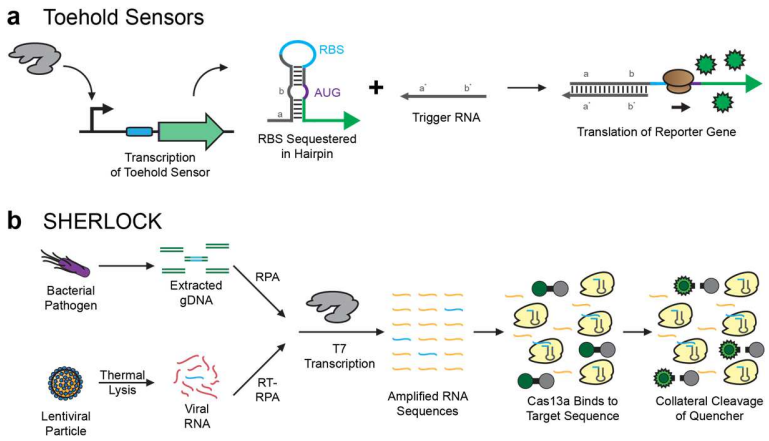

Figure 4. Nucleic acid cell-free biosensors detect target sequences by releasing ribosome binding site (RBS) sequestration or CRISPR-based cleavage of quenched reporters. a) Transcription of a reporter protein is inhibited by sequestration of the RBS in a toehold loop. Binding to the target trigger RNA permits translation and sensor output. b) SHERLOCK detects genomic DNA extracted from bacterial pathogens or viral RNA freed from lentiviral particles via thermal lysis. Amplification occurs via a combination of recombinase polymerase amplification (RPA), or reverse-transcription RPA (RT-RPA), and T7 polymerase transcription. Purified Cas13a proteins then bind to the target sequence via a guide RNA and collaterally cleave a quenched fluorescent reporter for detection. Figure 4a and Figure 4b are adapted from Pardee et al.[26] and Gootenberg et al.[29], respectively.

Sample processing and sensor stability

Preserving cell-free reactions via lyophilization

One of the most difficult aspects of providing diagnostics to rural areas in low-resource nations is the temperature control required. Many diagnostics require refrigeration or freezing and this

cold-chain must be maintained from production to user applications. Additionally, even in areas with much greater access to refrigeration, the development of point-of-care diagnostics would be greatly aided by stability at room temperature storage. Most cell extract and buffer components are traditionally stored at -80°C, potentially producing an obstacle to field deployment. However, Pardee et al. first showed in their paper developing toehold sensors that they could lyophilize the transcriptional and translational machinery along with the DNA as pellets or on paper and then later rehydrate to start the reaction[26]. These freeze-dried components could then be stored in tubes at room temperature for up to one year, although they did show an ~80% decrease in signal after that period of time. Similarly, they and others have shown that freeze-dried components can be used to produce therapeutics, thereby potentially enabling on-demand, on-site manufacturing of both diagnostics and treatments[24,31–33].

Several groups have looked at a variety of other storage conditions on sensor stability. Salehi et al. tested the stability of standard or lyophilized extract stored at -80°C, -20°C, 4°C, or 25°C for up to one year[33]. While the non-lyophilized extract showed a rapid decrease in efficacy at higher temperatures, the lyophilized extract was much more stable; however, it had lost all activity after one year. Karig et al. also investigated using the non-reducing disaccharide trehalose to protect cell-free components during oven drying[34]. By separately drying the reaction buffer and extract at 37°C, they could later recombine them to produce pyocin capable of killing *P. aeruginosa* after 136 days, although at lower dilution levels than fresh reactions. Lyophilization has also been shown as an effective means of killing any remaining bacteria that survive the lysis process[35], a vital component for future devices to pass through regulation. Additionally, autolysing cell lines can be lyophilized and rehydrated to create functional extract *in situ*, and Didovyk et al. demonstrated that such a method can produce a mercury sensor with a detection limit of 25 nM, similar to previous *in vivo* versions of the same biosensor[7]. Lastly, while the progress made in lyophilizing cell-free biosensors is promising and considerable, it should be noted that many successful sensors rely solely on strong, constitutive protein production through a robust T7 polymerase-based system and that other sensors that rely on activation or repression with native *E. coli* polymerase may require additional troubleshooting to remain functional after lyophilization.

Pre-processing steps for complex samples

Another challenge for cell-free biosensors is the very fact that they have no physical barrier to shield them from the samples introduced. While this can have many benefits, the delicate calibration of protein synthesis reactions can be greatly altered by the introduction of complex mixtures. A few solutions have been proposed to address these issues. For cell-free protein synthesis systems, work from the Bundy group has shown that the incorporation of RNase inhibitors can greatly reduce sample interference, particularly for blood samples[25]. This can facilitate extraction-free reactions, as we have shown by detecting endogenous hippuric acid in urine that requires only a dilution step in water[17]. Additionally, the pre-processing procedure HUDSON (heating unextracted diagnostic samples to obliterate nucleases) was developed for the SHERLOCK platform to allow for direct detection in clinical samples[36]. After a 10-25 minute nuclease and viral inactivation step, Myhrvold et al. were able to detect Zika viral RNA in

whole blood, serum, saliva, and urine at clinical concentrations (<100aM). While there still remain several hurdles to improve the robustness of many cell-free biosensors, the steps made thus far are promising.

Conclusion

While in this review article we have focused primarily on cell-free biosensors that are based on transcription and/or translation, there are certainly many other categories that we were not able to include, such as protease sensors that can achieve ultrasensitivity[37]. Additionally, many types of cell-free biosensors have been encapsulated into liposomes, recreating a controllable barrier between the reactions and the surrounding environment. These have been used to create synthetic microreactors, osmotic shock and temperature sensors, and even enable communication between cell-free liposomes and bacterial biosensors[38–41]. Furthermore, cell-free reactions have also been used to functionalize biomaterials, which could lead to additional categories of cell-free biosensors in the future[42]. Finally, recent work has even brought cell-free biosensors into high schools to teach and inspire the next generation of synthetic biologists. Using freeze-dried cell-free reaction components, teachers can instruct students on how to produce a range of fluorescent proteins[43], create biosensors to differentiate genomic DNA between different fruit species[44], learn about the mechanisms of antibiotics[45], and even conduct experiments with CRISPR/Cas9[46]. With an increase in the amount of research being performed and inspiring future generations by putting cell-free technology in their hands from a young age, the future looks bright for cell-free biosensors.

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Competing Interests

The authors declare no competing interests.

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** of outstanding interest

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