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Cell-free synthetic biology for environmental sensing and remediation

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The fields of biosensing and bioremediation leverage the phenomenal array of sensing and metabolic capabilities offered by natural microbes. Synthetic biology provides tools for transforming these fields through complex integration of natural and novel biological components to achieve sophisticated sensing, regulation, and metabolic function. However, the majority of synthetic biology efforts are conducted in living cells, and concerns over releasing genetically modified organisms constitute a key barrier to environmental applications. Cell-free protein expression systems offer a path towards leveraging synthetic biology, while preventing the spread of engineered organisms in nature. Recent efforts in the areas of cell-free approaches for sensing, regulation, and metabolic pathway implementation, as well as for preserving and deploying cell-free expression components, embody key steps towards realizing the potential of cell-free systems for environmental sensing and remediation.

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Introduction

Microbes are found in nearly every realm on earth, ranging from thermal vents to Antarctic ice. The spectrum of sensing and metabolic activities that microbes exhibit to thrive in these environments has long inspired efforts to harness microbial biology for sensing and metabolic engineering applications. Sensing, for example, has been achieved with a wide range of different biological components, including enzymes, antibodies, receptor proteins, and nucleic acids [1]. Meanwhile, remediation has been accomplished even using natural microbes, although genetic engineering has also been used to improve metabolic efficiency of contaminant degradation [2].

To date, most biosensors utilize either a small set of purified biological components interfaced with a transducer, or whole cells that are simply modified to express reporter genes inserted downstream of ligand-activated promoters [1]. Most bioremediation efforts are similarly straightforward, focusing on either the use of natural cells or on the optimization of existing metabolic pathways. Synthetic biology offers transformative tools for improving both biosensing and bioremediation performance by expanding the range of sensor and remediation targets, and increasing the sophistication of sensor and regulator implementation. However, practical application of the resulting synthetic systems is hindered by safety concerns associated with the release of genetically modified organisms (GMOs) into the environment.

The emergence of cell-free synthetic biology offers a promising mechanism for circumventing GMO release [3,4], allowing deployment of gene networks and metabolic pathways without the risk of unbridled replication and spread of new microbial strains in the wild. Beyond safety, cell-free systems offer a host of other benefits as well. For instance, cell-free systems can operate in the presence of toxins that would inhibit or kill live cells. This means that key sensing and metabolic components, such as transcription factors and enzymes, can be produced in higher concentrations than in living cells, leading to improved sensitivity and efficiency. It also means that environmental chemicals are better tolerated, including those that are the target for sensing or remediation [5]. In addition, in cell-free platforms, all energy resources can be devoted to the engineered application, as opposed to supporting self-replication. Finally, the potential for evolution, which can undermine or even abolish engineered function, is largely removed in cell-free contexts.

Cell-free protein expression systems typically consist of a cell extract, which contains machinery essential for transcription and translation, as well as a number of components to fuel expression, including buffers, nucleotides, amino acids, and energy sources. Although cell-free protein expression systems have been used for decades to investigate biological phenomena and produce proteins

that are difficult to express in living cells, cost, yield and scale have historically prevented their adoption in sensing and bioremediation applications. Fortunately, these barriers have been recently removed thanks to new advances in cell-free preparations [6,7]. This has made possible a range of novel biosensing and bioremediation applications such as spill tracking, source pinpointing, and remediation *in situ*. The potential application space made possible by new advances in cell-free technology is the focus of the current review. First, we discuss sensing, including sensing modalities and integration of sensors into regulatory networks. We then touch on recent advances that facilitate the implementation of remediation pathways in cell-free systems. Finally, we discuss practical needs for applying cell-free systems, namely the unique challenges of cell-free systems as compared to living cells, as well as extension of shelf-life and the encapsulation of components for robustness in application contexts.

Sensing

Sensing modalities

Several different approaches for generating responses to ligands have been demonstrated in cell-free systems. These approaches include the use of receptors and other ligand responsive transcription factors [8], as well as an array of strategies based on leveraging DNA or RNA structures for regulation (*e.g.*, aptamers) [9]. The use of receptors is exemplified by the detection of bacterial quorum sensing signals using engineered genetic constructs in cell-free systems [10,11,12•,13]. These gene circuits express a bacterial quorum sensing receptor, which can form a complex with cognate quorum sensing molecules, subsequently enabling activation of a promoter expressing a reporter protein. This ability to detect chemical signatures of bacteria illustrates the potential for leveraging cell-free systems for pathogen detection. Besides quorum sensing receptors, other transcription factors that regulate downstream promoters upon ligand binding include the mercury binding transcription factor MerR [5], and the tetracycline binding transcription factor TetR [14].

While transcriptional regulator proteins offer robust performance, many sensing targets have no known regulator. By contrast, powerful selection procedures are available for identifying aptamers [15,16]. Therefore, a number of different cell-free sensing strategies have employed aptamers. In general, when a ligand binds an aptamer region, the aptamer changes conformation, resulting in a corresponding alteration in enzymatic activity, transcriptional efficiency, or translational efficiency, depending upon the precise implementation. Iyer and Doktycz, for example, demonstrated a DNA aptamer-based approach for engineering ligand responsive promoters in cell-free systems [17]. Specifically, they placed a DNA aptamer sequence near a T7 promoter such that ligand binding to the aptamer regulated transcription. The majority of approaches, however, rely on RNA aptamers (*e.g.*, riboswitches). For instance, Ogawa presented an approach for designing riboswitches that function in eukaryotic cell-free systems and demonstrated responses to theophylline, FMN, tetracycline, and sulforhodamine B [18]. In addition to DNA and RNA aptamer approaches, more recently, a novel RNA regulation approach was designed for sensing specific RNA sequences [19]. Pardee *et al.* utilized this method to detect Ebola [20] and Zika [21] RNA in *Escherichia coli* extracts.

Few direct comparisons have been made to date between cell-free sensors and their counterparts in more traditional sensors (*e.g.*, nano-bio sensors or whole cell sensors) in terms of sensitivity and specificity. A cell-free theophylline riboswitch in a cell-free translation system [18] and an aptamer-based electrochemical biosensor for theophylline [22] exhibited different, yet overlapping dynamic ranges of detection (3–100 μM vs. 0.2–10 μM). Similarly, cell-free and whole cell receptor-based sensors have been compared and exhibited fairly similar response characteristics [13]. As more cell-free biosensors are constructed and characterized in the future, the key determinants of sensitivity and specificity may be elucidated for each sensing modality. Meanwhile, by comparison to whole cell biosensors, the cell-free context may offer several sensitivity advantages. First, it may be possible to produce key receptors in higher concentrations than can be achieved in living cells. Second, it has been shown that cell-free systems can avoid problematic false negatives that arise in whole cell biosensors when ligands reach levels that are toxic to cells [5].

Collectively, the diversity of sensing options that have been demonstrated in cell-free systems suggests that sensors can be developed for a wide variety of targets. Future approaches may additionally leverage the amenability of cell-free protein expression systems for producing other components such as membrane receptors [23] and antibodies [24].

Complex regulation

The above sensing modalities offer basic sensing and response function; however, the deeper potential of synthetic biology lies in leveraging gene circuits to implement complex regulation. This regulation may be used to process multiple inputs and correspondingly regulate one or more outputs (reporters or remediation products). In addition, specificity may be generated through digital logic. For instance, a logical AND gate of multiple sensors with imperfect specificity may generate a response with an overall high specificity. This approach to improving specificity is analogous to the recent use of dual aptamers, whereby two aptamers were used to target different sites of a ligand in order to achieve highly specific detections, in a nanoparticle hybrid sensor [25]. While few dual

aptamers have been identified, synthetic gene networks facilitate the combination of sensors without the need for dual aptamer discovery. Indeed, using synthetic biology, multiple sensing modalities (*e.g.*, transcription factors, receptors, aptamers) can even be combined into a single sensor read-out.

A number of developments in cell-free synthetic biology lay the groundwork for implementing sophisticated sensors that rely on logic gates and complex genetic circuit designs. Many of the necessary components, for example, are contained within two cell-free "toolboxes" that have recently been developed. The first toolbox contains sets of *E. coli* promoters and transcriptional activators that can be cascaded and combined [26] to generate arbitrarily complex regulatory functions for sensor implementation. The second toolbox presents detailed characterization of additional components, including ones for regulating mRNA and protein stability [27••]. These components can provide orthogonal mechanisms for orchestrating complex system responses to different signals. In addition to the two toolboxes, various multi-input phage-derived and *E. coli*-derived promoters have been tested in cell-free systems [26,28,29]. Multi-input promoters are particularly helpful for integrating multiple sensor responses and thus for implementing regulatory functions such as digital logic. Leveraging these and other similar components, several regulatory systems have been demonstrated in cell-free systems. These include transcriptional cascades [26,30], inducible feedback circuits [14,26], digital logic [29], a pulse generator [27••], oscillators [31], and a system that emulates *Drosophila* pattern formation [28].

Remediation

Remediation pathways

Natural microbes have been found to degrade an enormous variety of environmental contaminants, including fuel, chlorinated solvents, pesticides, creosote, benzene, toluene, polychlorinated biphenyls, dioxins, nitro-aromatics, and chlorinated ethenes [32,33]. The potential of genetic engineering for realizing vast improvements in remediation efficiency has long been known [2]. Moreover, through metabolic engineering and synthetic biology, the range of remediation targets can also be extended [34]. As suggested above, however, concerns over GMO release have prevented large scale application of engineered organisms for bioremediation. Fortunately, the extension of synthetic biology efforts to cell-free systems may overcome the barrier of safety concerns. In addition, the growing field of cell-free metabolic engineering capitalizes on a number of other benefits of cell-free protein expression [35]. Cell-free protein expression, for example, both broadens the range of enzymes that can be incorporated into a synthesized metabolic network and extends the range of active enzyme concentrations beyond what can be realized *in vivo*. For instance, Li *et al.* demonstrated cell-free production of active multi-copper oxidases. These are enzymes used for applications such as wastewater decolorization that have proved difficult to produce efficiently *in vivo* [36]. In addition to toxic or difficult to make end products, cell-free systems can also avoid issues of toxic intermediates, energy constraints and loss of function through evolution that are often problematic with living cells.

One of the most important advantages of a cell-free approach, however, is ease of engineering. Just as the cell-free context simplifies prototyping and optimization of genetic regulatory circuits, it also facilitates construction and optimization of metabolic pathways. When engineering metabolic pathways in living cells, time consuming genetic modification and subsequent transformations are required to test different enzyme choices or to tune relative enzyme concentrations to balance flux. By contrast, in cell-free systems, this testing and optimization may be performed by either adding different relative concentrations of the DNA components encoding different pathways [37,38] or, alternatively, by mixing cell extract variants that contain different components [39••,40••]. While cell-free metabolic engineering efforts to date have primarily focused on industry, energy, and medical applications, the same underlying techniques could easily benefit efforts to implement environmental remediation pathways.

Complex regulation

As with sensing, bioremediation may also benefit from some of the more complex regulatory mechanisms that have been developed in traditional synthetic biology platforms. This would enable more refined responses to environmental contaminants, potentially providing safer, less invasive and less ecologically damaging intervention. For example, depending on the organisms selected, one problem with bioremediation using natural microbes is their tendency to consume oxygen. In marine environments, for instance during an oil spill, this can result in anoxic conditions that are deadly to marine life. By regulating bioremediation in response to oxygen levels, low oxygen thresholds could be avoided. While this might slow the overall remediation process, the net effect on marine life may be improved. Notably, integrating complex regulation into bioremediation technology requires additional biosensors engineered to work in concert with remediation steps.

Practical considerations

Scale and standardization

Many field applications in environmental sensing and bioremediation require large scales of operation. In 2011, Zawada *et al.* demonstrated 100L scale cell-free production of a human therapeutic [7]. Impressively, scale-up was linear, which bodes well for scaling up variations of cell-free systems optimized for

environmental applications. The emergence of companies such as Sutro and GreenLight that leverage cell-free protein synthesis illustrates the growing practicality of cell-free systems for large scale, commercial applications. To our knowledge, commercial sensing and remediation applications of cell-free protein expression have not yet been developed. However, Thermocyclomics has investigated the use of cell-free extracts of thermophiles to remediate organic pollutants [41]. Together, these examples point towards the growing practicality of cell-free systems for large scale, commercial sensing and remediation applications.

Although there has been significant progress in scaling up cell-free reactions, additional efforts in the realm of standardizing cell-free systems would be helpful, particularly for synthetic biology applications. To date, protein yield has primarily been used to assess the efficiencies of different cell-free systems. However, yield assessments can vary significantly as a result of a number of factors, including the particular protein chosen for yield measurement, the expression construct used, and the method for assessing yield. In addition, yield does not capture dynamics of expression, which may be important for applications that require either fast expression or long duration. A helpful complementary approach to relying on absolute yield information would be to compare genetic component and cell-free reaction mixture performances independently using standardized systems for the other constituent. Component performance, for example, could be evaluated with widely available commercial kits, which undergo more stringent quality control. The PURE system, a minimal cell-free system derived from individually purified proteins as opposed to cell extracts, offers one appealing choice [42]. For reaction mixture performance, a library of expression constructs of GFP-tagged proteins of different sizes was recently constructed and made available through Addgene [43]. These constructs were designed to express proteins in essentially any in vitro translation system, thus their use may help to facilitate comparisons of cell-free preparations between labs.

Challenges of the cell-free context

Many of the genetic components that function in living cells also function in cell-free systems [13,44,45•]. However, there are several key differences that can complicate design of biosensing and bioremediation applications. First, unlike living cells, cell-free systems have no mechanism for producing or harvesting energy. Although the goal of sustaining cell-free reactions through inexpensive feed sources remains an active area of research [46], currently, cell-free systems have a limited operation period. While this may be viewed as a safety advantage, it may also cause operational challenges. That said, all energy resources in a cell-free preparation can be devoted to the engineered application, as opposed to supporting self-replication, helping to extend the lifetime of these systems.

Another, more subtle challenge of cell-free systems is that fundamental kinetics of a given gene circuit can differ relative to its kinetics in living cells [47]. As a result, performance of the gene circuit in living cells and cell-free systems is often qualitatively similar, yet quantitatively different [13,14]. For instance, Chappell *et al.* tested a library of promoters and a library of ribosome binding sites in *E. coli* and *E. coli* extracts. Although they showed that relative rates were strongly correlated in the two systems, absolute transcription and translation rates often differed between live cell and cell-free contexts [13]. In addition, for many cell-free preparations, natural *E. coli* promoters and their derivatives do not yield strong transcription. These differences can complicate biosensor and bioremediation design, where absolute yield, rather than relative read, may be a key system parameter. One strategy for circumventing transcription issues is to use viral promoters, such as T7, T3, and SP6, along with corresponding modifications to these promoters to enable regulation [14,28]. Alternately, it is also possible to optimize cell extract preparation to improve transcription from natural host promoters and their derivatives, facilitating efforts to tap into the wide and growing diversity of genetic constructs developed in living cells [48].

Besides potential differences in transcription and translation rates, degradation rates of mRNA and proteins are often much slower in cell-free contexts. Indeed, many cell-free preparation methods have been specifically optimized to reduce these degradation rates. In addition, the lack of volume expansion caused by cell growth leads to slower reduction of concentrations. These slower degradation rates are ideal when simple production of proteins in high yield is sought. By contrast, the design of dynamic cell-free systems, which would be required for implementing sophisticated logic gates and genetic circuits, would require faster component turnover. Previous cell-free systems with dynamics include a pulse generator and an oscillator. These have relied on degradation machinery such as MazF for mRNA degradation and ClpXP for targeted protein degradation [27••,31], and a similar design may be necessary for sensing applications, particularly if accurate reporting of fluctuating signals is desired.

Shelf-life obstacles

Applications such as environmental sensing and remediation would greatly benefit from technologies that facilitate the storage and delivery of cell-free protein expression reagents [49]. Cell-free reagents are typically stored at −80°C, but practical use in the field would be much more feasible for cell-free systems that demonstrated significant shelf-life at ambient environmental temperatures. The number and diversity of proteins and reagents

needed for cell-free protein expression make this a challenging problem. Nonetheless, a few efforts have focused on drying and preserving cell-free protein expression components.

Pardee *et al.* lyophilized cell-free reagents and used them to both implement biosensor gene networks capable of detecting Ebola [20] and Zika virus [21] and also to produce a wide array of therapeutics [38]. Importantly, lyophilized pellets of cell-free reagents exhibited expression after a year of room temperature storage, while dry reagents in paper could be reconstituted for biosensor operation. Although testing of the pellet-based approach relied on an inert gas atmosphere (N_2) and a silicon desiccant package [20], this format of preservation would be amenable to storage and distribution of small biosensors that could be distributed in Mylar pouches and activated on demand. Other efforts have focused on the inclusion of stabilizing reagents to improve preservation without the need for specialized environments [50,51]. Smith et al. separately examined the preservation of *E. coli* extracts and the preservation of sets of additional components needed to fuel protein expression [52]. Recently, they demonstrated room temperature preservation of cell extract for a year, and also demonstrated the production of a human therapeutic using dried and reconstituted reagents [53]. Extending these efforts to realize long-term resilience, even above room temperature, in a scalable fashion will greatly facilitate applications in environmental remediation [54].

The system/environment interface

Recently, several platforms have been developed to facilitate protein expression and even purification using cell-free protein expression components. To date most of these efforts have targeted therapeutic applications, such as the production of medicine on demand [55,56]. For environmental sensing and remediation, however, a key requirement is to realize sufficient protection and consolidation of reagents to enable transcription and translation, while still allowing biosensor components to access target ligands and remediation products to access the environment. One option is the use of liposomes. Cell-free protein expression in liposomes has been widely explored, and is of particular interest in the realm of membrane protein purification [57]. Recently, cell-free systems encapsulated in liposomes were used to demonstrate both the synthesis of and the response to a bacterial quorum sensing signal, showing how encapsulated cell-free reagents may be engineered to interact with the environment [12•]. However, a key issue to overcome for environmental applications is stability. Another possible encapsulation strategy that might offer better stability is the use of polymer substrates. For instance, cell-free protein expression has been demonstrated in alginate beads coated with silica to enhance environmental resilience [58]. In addition, DNA microgel formats have been developed for cell-free expression [59]. Recent studies have shown that encapsulation techniques do not significantly reduce bioremediation efficacy when using live cells [49]. It remains to be seen whether cell-free systems adapt equally well to different encapsulation strategies.

Conclusion

Cell-free systems offer a practical and flexible context for leveraging the power of synthetic biology for environmental sensing and remediation. Recent developments in cell-free sensing, regulatory networks, and metabolic pathway implementation pave the way towards sophisticated artificial cells that sense multiple conditions, regulate responses, and efficiently break down contaminants in a highly controllable fashion. Additional progress in shelf-life improvements and robust encapsulation set the stage for practical deployment. Future efforts may leverage features unique to the cell-free environment, for instance facilitating the coupling of DNA computing approaches to more conventional gene regulatory networks. In addition, methods for producing and optimizing efficient cell-free systems from a wider diversity of organisms will capitalize on the diverse array of sensing and metabolic capabilities observed in nature [60]. Collectively, through these advances, promise to bring about an expanded set of sensing and remediation targets, an extended range of operation contexts, and a degree of control over the duration and scale of remediation activity *in situ* that cannot be currently realized with living cells.

Declaration of interest

JHUAPL has filed an application on behalf of D.K.K. with the US Patent and Trademark Office on work related to the preservation of cell-free protein expression reagents.

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