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REVIEW PAPER

The Use of Cell-free Protein Synthesis to Push the Boundaries of Synthetic Biology

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Abstract Cell-free protein synthesis is emerging as a powerful tool to accelerate the progress of synthetic biology. Notably, cell-free systems that harness extracted synthetic machinery of cells can address many of the issues associated with the complexity and variability of living systems. In particular, cell-free systems can be programmed with various configurations of genetic information, providing great flexibility and accessibility to the field of synthetic biology. Empowered by recent progress, cell-free systems are now evolving into artificial biological systems that can be tailored for various applications, including on-demand biomanufacturing, diagnostics, and new materials design. Here, we review the key developments related to cell-free protein synthesis systems, and discuss the future directions of these promising technologies.

Keywords: cell-free protein synthesis, synthetic biology, recombinant proteins, metabolic engineering, artificial cells

1. Introduction

Advances in biotechnology have brought revolutionary changes across diverse sectors of industry, including the pharmaceutical, fine chemicals, food, energy, and environment. Bioprocesses based on the cultivation of genetically engineered cells have enabled commercial-scale production of therapeutic recombinant proteins that are now invaluable for the treatment of many major diseases. In addition, along with recent advances in synthetic biology and metabolic engineering, microbial cell factories harboring manipulated metabolic pathways have been developed to produce numerous chemical products, providing a vital contribution to global efforts to tackle climate change by reducing dependence on petroleum. However, the methodologies developed to date have several intrinsic limitations associated with the use of living cells. For instance, cell-based methods barely provide the opportunity to adjust the molecular environment of gene expression, and exogenously introduced genes can only be expressed within a narrow range of physicochemical cytosolic conditions. In addition, the productivity and titer of heterologous products are limited by the regulatory mechanisms that maintain the viable physiology of cells [1-4]. Finally, living cells are not ideal chassis for testing genetic circuits in synthetic biology; while synthetic biology seeks to optimize biological systems via design-build-test-learn (DBTL) cycle, the requirements to transform and grow cells limit the seamless operation of this cycle substantially.

Many of the constraints of cell-based systems can be addressed by employing the approach of cell-free synthesis. Instead of relying on the growth of transformed cells, cell-free synthesis systems harness the synthetic machinery of cells after extraction into an artificial environment. By decoupling biosynthesis reactions from the cellular physiology, the cell-free approach expands the boundaries of genetically programmable biological functions. In particular, cell-free synthesis systems are gaining rapid interest in the field of synthetic biology, which requires the design and testing of large sets of DNAs in a high-throughput manner. Unlike cell-based gene expression, nucleic acids in various configurations can be used to direct cell-free synthesis, including chemically synthesized or polymerase chain reaction (PCR)-amplified linear DNA. Consequently, cell-free synthesis systems can serve as programmable breadboards

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to accelerate the DBTL cycle for developing novel bioprocesses. There seems to be a clear consensus in the synthetic biology community that cell-free systems will find increasing applications in numerous disciplines, including the design of novel biological systems, pathway development, synthesis of artificial proteins, and biosensors. These expectations have been reflected in the surge in the volume of related publications in recent years. This review describes the recent developments in cell-free synthesis systems and discuss how these platforms can contribute to the growth of synthetic biology. We also survey recent efforts to connect the biological molecular machinery with artificial structure, devices and instruments, with the goal of engineering and augmenting the capabilities of biological systems.

2. Optimization of the Biochemical Conditions for Cell-free Protein Synthesis

Following Nirenberg and Matthaei's demonstration that the translational machinery in an extract of *Escherichia coli* cells retained its ability to translate mRNA, cell-free synthesis systems were originally conceived as an analytical tool to study the molecular process of translation [5-7]. Subsequently, the pioneering work of Spirin [8] demonstrated that the cellular translational machinery can operate *in vitro* with unexpectedly robust stability and high efficiency, which inspired the successive studies that resulted in the development of cell-free protein synthesis systems with markedly improved productivity [8-11]. Via optimization of reaction conditions [12,13], template preparation methods [14-17] and ATP regeneration schemes [18-20], cell-free systems are now capable of producing recombinant proteins in mg/mL concentrations [21,22]. While this remarkable improvement promises expanding applications that have not been achievable previously, there is sufficient scope for further enhancement of the productivity of cell-free protein synthesis. The concentration of proteins in bacterial cells is approximately 200 mg/mL. Assuming a doubling time of 30 min, bacterial cellular proteins are produced at an approximate rate of 400 mg/mL/h. Although it is not realistic to expect cells to produce recombinant proteins at such a rate, it would seem that the present bioprocesses are not harnessing the full synthetic power of cells. The intrinsic ability of cells to produce recombinant proteins is limited by the fact that a substantial portion of cellular resources is directed towards the reproduction and maintenance of the cells. In addition, the semi-permeable cellular membranes limit both the supply of chemical resources to the translational machinery and the removal of inhibitory by-products. These are intrinsic constraints of producing recombinant proteins inside the cells. By contrast, cell-free protein synthesis methods are not hampered by the constraints of the cellular physiology, making it possible to funnel the flux of resources to the production of target

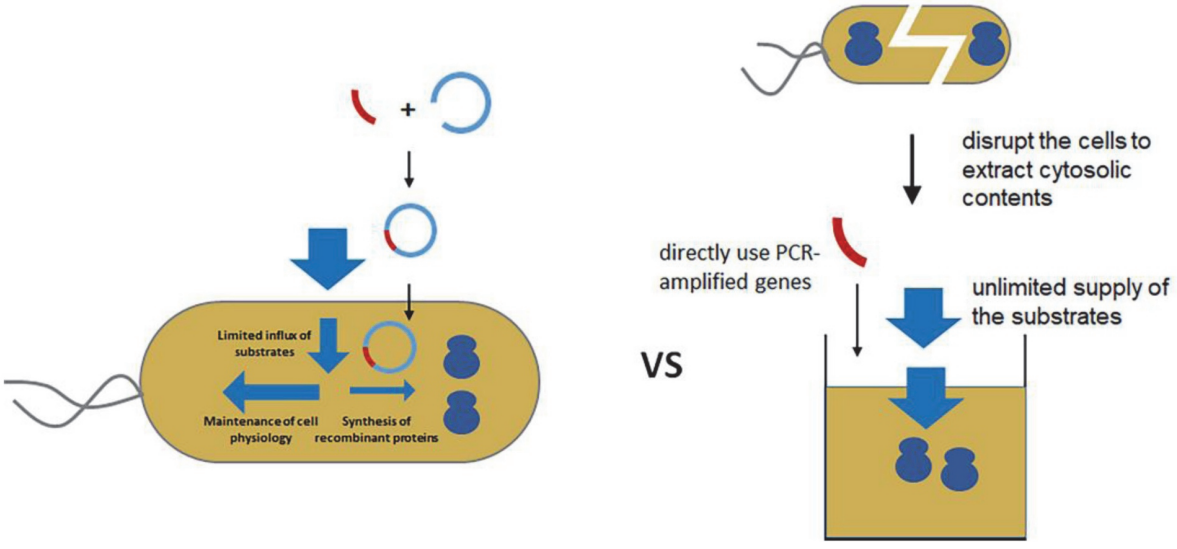

Fig. 1. Cell-free synthesis can potentially reach the theoretical yields of recombinant protein production. Cell-free protein synthesis harnesses the synthetic machinery of deconstructed cells. In principle, due to the lack of requirement to support the maintenance and growth of cells, the substrates in the reaction mixture can be fluxed entirely to the synthesis of recombinant proteins encoded in the template DNAs. While residual cellular enzymes still can cause unproductive consumption of the substrates, this can be addressed by biochemical or genetic inactivation of those inhibitory enzymes. In addition, unlike cell-based gene expression methods (left), cell-free systems accept polymerase chain reaction (PCR)-amplified DNA as the direct template for protein synthesis (right), which greatly enhances the throughput of protein generation.

proteins (Fig. 1). Furthermore, the open nature of cell-free protein synthesis enables the use of biochemical and genetic tools to remove unnecessary or inhibitory components from the cell extracts. For example, by preparing cell extracts from the *E. coli* cells pre-conditioned with high concentration of glucose, Kim and Swartz [23] developed an efficient cell-free protein synthesis system that uses glycolytic intermediates for ATP regeneration. Genome engineering methods also have been successfully applied to optimize the strains used to prepare the cell extract. Genomic modifications to the extract source strain can improve the stability of DNA [24-26], amino acid supply [27,28], and protein degradation [29,30]. In their recent report, Martin *et al.* [31] used multiplex automated genome engineering to create an *E. coli* strain harboring inactivating mutations in the genes encoding seven inhibitory proteins [31-33]. The extract prepared from this engineered strain was able to support cell-free protein synthesis with yields as high as 1.8 mg/mL. These findings support the expectation for the development of strains optimized to provide cell extracts that can utilize exogenously added substrates to produce target proteins close to theoretical yields.

3. Engineering the Template DNA for Maximal Synthesis of Recombinant Proteins

As aforementioned, advances in the productivity of cell-free protein synthesis have been achieved mainly through extensive optimization of the cell extract and chemical composition. However, although an optimized biochemical environment is necessary, it is not sufficient for the efficient production of proteins. Oftentimes, the yield of protein synthesis is limited by the nature of the nucleotide sequence of the template DNA. Despite recent advances in computational design of DNA, in general, predicted results require experimental verification. Cell-free protein synthesis provides a valuable platform for large-scale testing of protein expression from the libraries of template nucleic acids. The translational efficiency of an mRNA is affected heavily by the nucleotide sequences adjacent to the start codon (the downstream box). Ahn *et al.* [34], demonstrated that changes in the nucleotide sequence of this region affect the efficiency of gene expression dramatically and, to improve the synthesis of recombinant proteins with extremely low productivity, they randomized the first few codons of the template DNAs. In cell-free protein synthesis reactions, the variant template DNAs displayed a continuous distribution of recombinant protein expression levels, including those several-fold higher than the expression levels achieved using the native sequences. Notably, the relative expression levels of the variant genes were reproduced well when the same genes were expressed in *E. coli* cells, indicating that cell-free expression screening can be used to produce recombinant proteins at the desired levels during cell-based bioprocesses (Fig. 2).

Overall, these findings promise the evolution of cell-free protein synthesis systems into universal protein generators that can exceed the limits of the present cell-based processes. However, it should be noted that the present cell-free protein synthesis systems lack of mechanisms to replicate the translational machinery. This limits the scalability of cell-free protein synthesis. Up to date, cell-free protein synthesis systems have been scaled-up to 100 L [35].

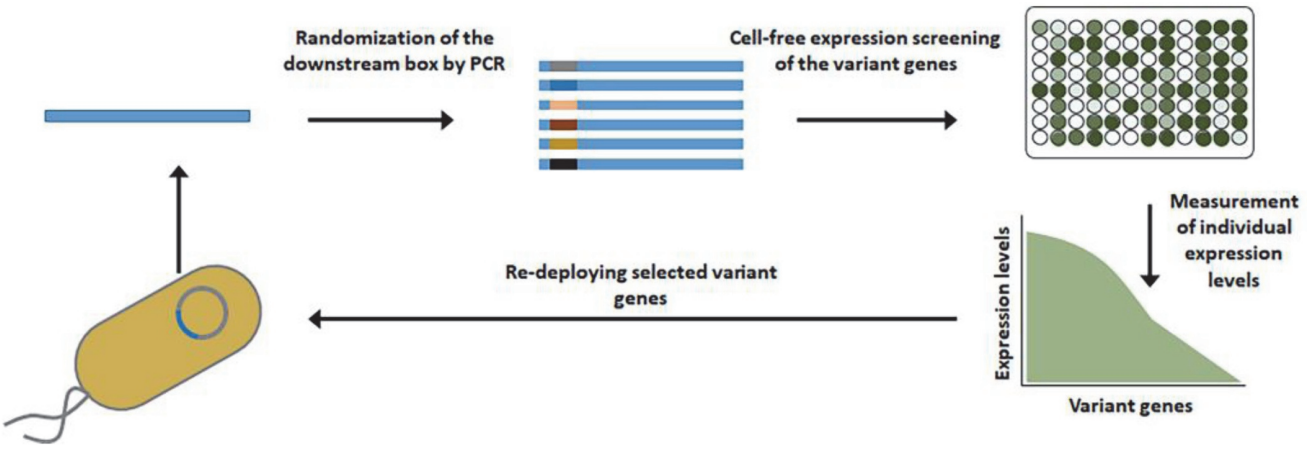

Fig. 2. Cell-free expression screening. The ability to produce recombinant proteins directly from polymerase chain reaction (PCR)-amplified DNA enables rapid and parallel evaluation of individual genes for their expression levels. This was successfully used to improve the synthesis of recombinant proteins with extremely low productivity by randomizing the downstream box sequences. Variant genes with desired expression levels are selected by high-throughput cell-free protein synthesis reactions, and re-deployed into the host cells for enhanced production of the recombinant proteins.

Therefore, it would be reasonable to find the use of cell-free protein synthesis systems for high-throughput generation of multiple proteins or rapid production of recombinant proteins in a pilot scale.

4. Co-expression of Multiple Genes in Cell-free Systems

In addition to their use for the production of particular proteins, cell-free systems are also being explored as a platform for the simultaneous production and assembly of multiple proteins. Unlike cell-based expression systems, in which co-expression of multiple genes is restricted by the compatibility of the expression vectors, in principle, cell-free protein synthesis systems can be programmed flexibly with multiple template DNAs. The ability of cell-free systems to accept multiple genes enables DNA-programmable synthesis of complex protein structures. Colant *et al.* [36] demonstrated that multi-protein virus-like particles (VLPs) are promising targets for cell-free synthesis. Unlike *in vivo* production of VLPs, cell-free synthesis enables the uncomplicated control of environments to enhance the soluble expression and assembly of the subunits. The cell-free approach is particularly useful for the production of VLPs with toxic intermediates or those that require the incorporation of non-canonical amino acids [37,38]. Furthermore, Shin *et al*. [39] reported that the large DNAs of bacteriophages can be expressed in a cell-free protein synthesis system to generate intact viral particles.

The facile programmability of cell-free synthesis systems also provides an excellent platform for building and testing synthetic metabolic networks prior to their deployment in living cells [40,41]. By using cell-free synthesis reactions as a breadboard of testing electronic circuits, this approach can reduce the time and labor required for pathway development dramatically. Indeed, recent results demonstrate the *de novo* generation of pathways by programming the reaction mixture for cell-free synthesis with required genes. Products of these approaches include N-acetylglucosamine [42], violacein [43], and n-butanol [44].

5. Controlling the Expression Levels of Multiple Proteins

Cell-free co-expression of multiple genes does not always result in a balanced production of the desired proteins. While the cytosolic contents, including ribosomes and translational factors, are regulated dynamically in living cells, protein synthesis in a cell-free system depends on the pre-loaded amount of translational machinery. As a consequence, different template DNAs in the reaction mixture can compete for the limited resources required for protein synthesis, which can lead to biased expression of proteins [45]. Although this limitation can be addressed in part by modulating the relative amounts of each template DNA, it can be difficult to determine optimal ratios of the multiple templates. As discussed above, the translational efficiency of an mRNA is determined largely at the initiation stage, which in turn is affected heavily by the nature of the nucleotide sequences adjacent to the start codon. Therefore, the translatabilities of co-expressed genes might be equalized if they have similar nucleotide sequences in the downstream box region. Indeed, Kasi *et al.* [46] were able to address biased expression by incorporating the identical nucleotide

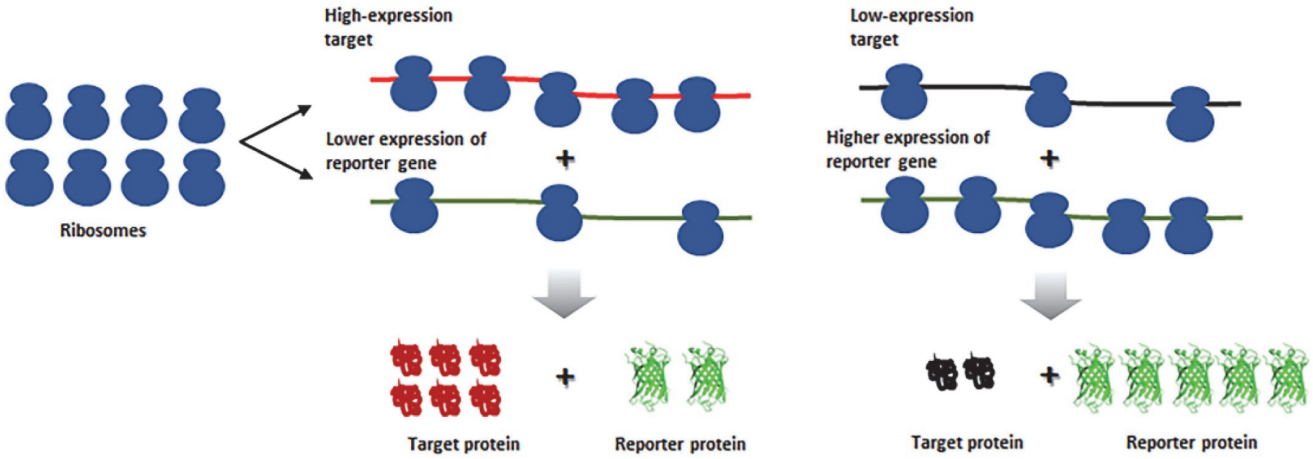

Fig. 3. Assessment of translational efficiency based on co-expression of a reporter gene. Different mRNA species should compete for a finite pool of ribosomes during cell-free protein synthesis. When a reporter gene is co-expressed with a target gene, the expression of the reporter gene is negatively affected by the translational efficiency of the target gene. Therefore, the expression level of the co-expressed target gene can be estimated by the measurement of the signal from the reporter protein.

sequences in front of the co-expressed genes. Combined with engineering of the downstream box sequences, these findings suggest that the relative expression levels of multiple DNAs can be modulated precisely in a cell-free synthesis system.

On the other hand, competition for limited amounts of ribosomes between co-expressed genes can be used to determine the translational efficiency indirectly. Using the gene of green fluorescence protein (GFP) as a common reporter co-expressed with target genes, Park *et al.* [47] demonstrated that GFP fluorescence in the reaction mixture inversely correlated with the expression level of the target genes. This method enables prognostic assessment of translational efficiency simply by measuring GFP fluorescence (Fig. 3).

6. Toward Artificial Cells

The construction of minimal cells with specialized functions is one of the ultimate goals of synthetic biology. Artificial cells can be built by a top-down approach, in which the genomes are edited to carry minimally required genes, or by a bottom-up approach which involves assembly of the nonliving materials [48]. While total synthesis of 'life' is beyond the reach of present technology, harnessing the existing biosynthetic machinery, re-compartmentalization of engineered cell-free synthesis system offers a more plausible route to the bottom-up approach. Ideally, artificial cells should be able to produce desired products by genetic programming, capable of reproducing themselves, and controllable by external stimuli. Different approaches explored in these aspects include the containment of cell-free systems (using emulsions, hydrogels, liposomes, polymersomes, and proteinosomes), and the use of dividing vehicles, *in vitro* replication of DNA and ribosomes, and inter-compartment communications [49-55]. Although there are many challenging technological hurdles, presently primitive forms of artificial cells will continue to evolve to acquire more cell-like functions (Fig. 4).

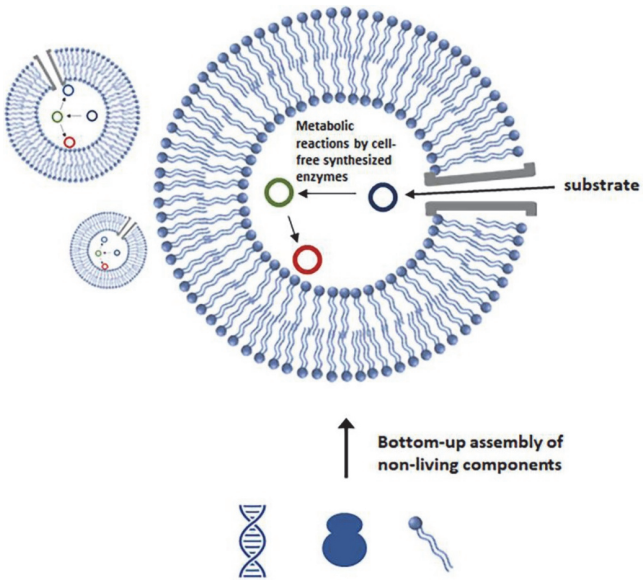

Fig. 4. Bottom-up construction of artificial cells using cell-free synthesis systems. While building biological systems starting from molecular level is unrealistic, by the use of synthetic machinery extracted from cells, cell-free protein synthesis provides a compromised route to the bottom-up construction of artificial cells. When integrated with the techniques that enable the reproduction of compartments, cell-free synthesis systems would serve as synthetic cytosols to decode the programmed DNA inside the artificial compartments.

7. Conclusion

Since the invention of PCR, the seemingly simple principles of nucleic acid amplification have brought fundamental changes in biotechnology. The enormous success of the technology to amplify nucleic acids *in vitro* has lured the scientists to seek protein equivalents of PCR. Obviously, it is not achievable to exponentially amplify proteins because they cannot template the self-replication reactions. However, protein synthesis is also an enzyme-catalyzed process, and thus combined use of the components for transcription and translation reactions should allow *in vitro* production of proteins from the template nucleic acids. The highly demanding complexity to build a functional set of components can be addressed by using the crude extract of cells, which comprises the idea of cell-free protein synthesis based on crude cell extracts. Since the first observation that crude cell extracts maintain translational activity under carefully optimized conditions, extensive studies have been made to improve the productivity and convenience of cell-free protein synthesis. As a result, cell-free protein synthesis is now accepted as a competitive option for the production of recombinant proteins. Furthermore, the unique feature as a programmable homogeneous solution makes cell-free synthesis systems a plug-and-play platform to build complex networks of biological reactions required for synthetic biology. Indeed, cell-free synthesis technology is now pursed as a platform to mimic and augment cellular functions, including metabolic production of chemical compounds, recognition and response to specific targets, and operation of artificially designed genetic circuits. It will soon be witnessed that a wide range of biological products presently obtained from living cells are developed and manufactured by various cell-free synthesis systems of various configurations.

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Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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