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Cell-free synthetic biology: Orchestrating the machinery for biomolecular engineering

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ABSTRACT

Due to inherent complexity, incompatibility, and variability in living cell systems, biomolecular engineering faces significant obstacles. To find novel solutions to these issues, researchers have turned to cell-free synthetic biology (CFSB), a relatively young field of study. Biochemical processes can be triggered *in vitro* through cell-free synthesis, providing a wider range of options for biomolecular engineering. Here, we provide a survey of recent advances in cell-free synthesis. These have sparked innovative studies in areas including the synthesis of complex proteins, incorporation of unnatural amino acids, precise post-translational modifications, high-throughput workflow, and synthetic biomolecular network regulation. CFSB has transformed the studies of biological machinery in a profound and practical way for versatile biomolecular engineering applications.

1. Introduction

Biomolecular engineering refers to the practice of manipulating biomolecules at a molecular level. The primary goal is to identify and manufacture useful biomolecules, and then to engineer and manufacture artificially active biomolecules with completely unique physicochemical features. There has been the extensive application of this technology in the fields of life sciences, including medicine, energy, agriculture, and the environment.[1,2] There has been a lot of development in this area over the past few decades, particularly in the use of biological approaches for molecular modification. It allows for increased freedom and efficiency in the design and production of valuable biological macromolecules, particularly proteins with specific functions.[3,4] However, at the present time, the majority of biomolecular engineering efforts are concentrated on living cells despite the fact that these are severely constrained by the physical membrane barrier and the complex internal environment, which includes the redox environment, the crowding environment, the synergy between organelles, uncontrollable protein degradation, uncontrollable protein modification, and others.

Within this framework, the advancement of cell-free synthetic biology (CFSB) method expands the capacity of natural biological systems for biomolecular engineering and opens up new avenues for biological manipulation.

Cell extracts from either prokaryotes or eukaryotes are used in cell-free synthesis, together with nucleotides, DNA or RNA templates, amino acids, energy molecules, cofactors, and salts, to perform transcription and create proteins.[5] This strategy allows for the creation of target molecules to be prioritized due to the absence of consumption induced by cell expansion. This open method facilitates rapid substrate addition and product purging without requiring passage through the cell wall.[3] In addition, a novel method of high-level expression and high-specificity mutation screening is presented through the direct use of linear DNA templates.[6] Because of these benefits, cell-free systems have been increasingly applied to the study, development, and manufacturing of biomolecules. In this overview, we highlight the advances in biomolecular engineering made possible by CFSB and emphasize its benefits and challenges.

Abbreviations: **CECF**, continuous-exchange cell-free; **CFCF**, continuous flow cell-free; **CFSB**, cell-free synthetic biology; **CHO**, Chinese hamster ovary; **PTM**, post-translational modification; **PURE**, Protein synthesis Using Recombinant Elements system; **SIMPLEX**, single-molecule PCR-linked *in vitro* expression; **UNAA**, unnatural amino acid.

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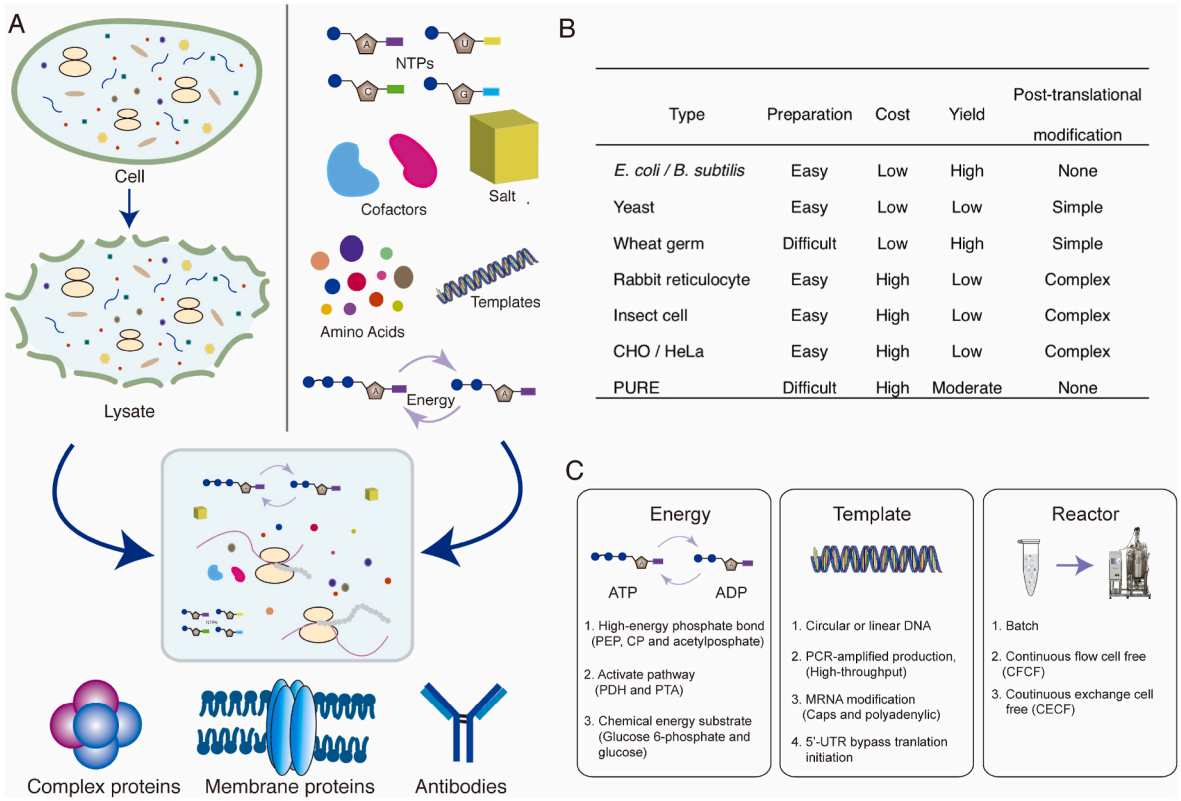

Type	Preparation	Cost	Yield	Post-translational modification
E. coli / B. subtilis	Easy	Low	High	None
Yeast	Easy	Low	Low	Simple
Wheat germ	Difficult	Low	High	Simple
Rabbit reticulocyte	Easy	High	Low	Complex
Insect cell	Easy	High	Low	Complex
CHO / HeLa	Easy	High	Low	Complex
PURE	Difficult	High	Moderate	None

Fig. 1. Cell-free systems. **(A)** Schematic overview of cell-free biosynthesis. **(B)** Comparison of several cell-free systems. **(C)** Processes made in energy regeneration, templates, and scale-up.

2. Development

The earliest example of cell-free applications can be traced back to 1907, when the German Nobel Laureate Eduard Buchner carried out alcoholic fermentation with broken yeast cells for the first time.[7] Then, in 1922 another Nobel Laureate Otto Meyerhof explained the glycolytic pathway with cell-free systems, having important influence on the development of modern biological chemistry.[8] Until 1961, Marshall Nirenberg constructed a cell-free system from *E. coli* and received the Nobel Prize in Physiology for his outstanding contributions to the discovery of genetic codes.[9]

These days, eukaryotic cells are being used in cell-free synthesis methods, which originally arose from prokaryotic cells. Molecular biology, genetic circuit design, and protein engineering are just a few examples of the many applications of cell-free systems that highlight the many benefits of this approach to biomolecular engineering. However, the previous cell-free synthesis approach is unable to meet the objectives of industrial manufacture even in the fundamental research due to difficulties with cell extract preparation, poor stability, and low yields. Cell-free synthesis has emerged as one of the most cutting-edge technologies and pioneering research subjects due to the ways of extending the reaction time and enhancing the protein synthesis. Biomolecular engineering has come a long way in the previous decade, with much attention paid to optimizing reaction conditions, energy sources, and reactor designs for use in fundamental biological research and commercial production.

3. Cell-free systems

There has been a lot of interest in cell-free synthesis as a viable alternative tool for biomolecular engineering due to its high throughput, efficiency, and ease of use. Exogenous or endogenous RNA polymerase can be used to transcribe either circular plasmid DNA or linear PCR-amplified templates into messenger RNA. T7 RNA polymerase, which is derived from bacteriophages, and T7 promoters are the most popular combination in cell-free synthesis systems due to their high activity and promoter specificity. Then, with the help of exogenously provided amino acids, tRNA, and energy substrates, the mRNA templates are translated into proteins by the cell's endogenous ribosome and translation factors (Fig. 1A). Since there is no physiological constraint, and protein expression may be performed rapidly, cell extracts provide the basis of most cell-free synthesis.

Recent years have seen the development of cell-free systems utilizing a wide range of prokaryotic and eukaryotic cells (Fig. 1B). Successful expression of many proteins that were previously impossible to express *in vivo* has been achieved using cell-free systems. Due to its high commercial value and the ease with which extracts can be prepared, the *Escherichia coli*-based system is one of the most extensively used cell-free systems. A number of prokaryote-based extracts, including those from the model industry bacteria *Bacillus subtilis*, *Corynebacterium glutamate*, and *Vibrio natriegens*,[10] and from notable temperature-tolerant active thermophiles,[11] have also evolved into cell-free systems. The inability to undergo post-translational modifications (PTMs) prevents the production of many eukaryotic proteins in prokaryotic systems. Cell-free lysates produced from several yeast strains have shown to be a useful tool for investigating *in vitro* eukaryotic protein expression.[12] However, a major drawback of cell-free yeast systems is the inefficiency of translation initiation. Wheat germ cell-free synthesis method, which originates in eukaryotes, is highly prized because it produces the largest yields of post-translationally modified proteins.[13] In comparison to *E. coli* cell-free systems, wheat germ cell-free synthesis systems are superior for the expression of a wide variety of soluble and functional proteins. Another cell-free protein synthesis system from a eukaryote that can do PTMs is the rabbit reticulocyte system.[14] Manufacturing on a large scale, however, is challenging. Cell-free expression methods based on mammalian cell lines were developed for biomolecular engineering

to address the needs of producing complicated PTMs of macromolecules. In particular, CHO cells have established themselves as the industry standard for manufacturing complex therapeutic proteins.[15] In addition, drug and virus manufacturing were both accomplished by utilizing HeLa cell-free extracts.[16] Cell-free synthesis systems serve specialized purposes and exhibit unique qualities.

Compared to cell-based expression, cell-free synthesis offers several advantages, including greater control over system additives. This control is further enhanced with a system called the PURE system,[17] which consists of over 100 purified transcriptional and translational elements. The transcriptional elements include DNA template, T7 RNA polymerase, nucleotides, and salts. The translational elements include tRNAs, tRNA synthetases, translation factors, ribosomes, enzymes for energy regeneration, and salts. With the PURE system, all elements are known, nuclease and protease activities are reduced, and the concentration of each element can be optimized for maximum protein expression. However, the PURE system is usually used for fundamental research, not as popular as cell extract-based systems for its difficult preparation and high cost, as well as lower yields.

4. Use of cell-free systems

There have been advancements in lowering costs, stabilizing systems, and increasing synthesis scale, making cell-free synthesis systems better suited for protein foundation assay and adaptable commercial production. In addition to its benefits, cell-free synthesis offers a fresh approach to biomolecular engineering obstacles associated with low-expression proteins.

4.1. Unnatural amino acids

Having promising prospects in the research and engineering of proteins and their interactions with other substances, the introduction of unnatural amino acids (UNAAs) in protein sequences paves the way for intriguing new possibilities to change proteins for chemical modification. In recent years, protein site-specific modification using UNAA encoding technology has been employed to successfully provide proteins with novel biological features.[4] Co-translational incorporation of more than a hundred UNAAs into proteins has been reported.[18] Some intriguing possibilities for UNAA integration are offered by cell-free systems. Due to the possible toxicity of UNAA incorporation-related components, researchers are able to precisely control their concentrations benefiting from the reaction environment's transparency. Large or electrically charged UNAAs can now be added to reaction systems without encountering a physical barrier. Cell-free systems have excellent UNAA integration capabilities, making them a promising tool for the use of new proteins.

4.2. Toxic protein

Cell-free systems have been used to synthesize native proteins and proteins that are hazardous to cells due to their versatility and ability to synthesize any desired proteins. Joachim described pierisin-1b′s potent toxicity in a cell expression environment,[19] noting that it exhibited particular ADP-ribosyltransferase activity on deoxyguanosine residues of DNA, which led to the induction of apoptosis and cell death. For pierisin-1b expression, cell-free system was utilized. Onconase is a cytotoxic and difficult-to-produce protein, but Salehi et al. were able to rapidly express it in a soluble and active form.[20] With the demand of new biomedical development, the synthesis of toxic proteins will show a new development trend.

4.3. High-throughput biomolecular screening

In addition to their groundbreaking impact on the production of single proteins, modern cell-free systems have made their mark in fast-evolving fields like high-throughput technology. Clarifying the structure, function, and interaction of every protein encoded by genes remains a formidable problem in the post-genomic era. Establishing high-throughput protein expression technology that is both fast and effective is so crucial. Cell-free synthesis allows for the functional interpretation of protein-coding sequences from any genome with unmatched throughput by using PCR products directly as templates. The solubility-improving fusion partners were screened from a pool of highly abundant *E. coli* by expressing hundreds of fusion constructs directed against a range of cytokine proteins in a cell-free expression system.[21] Nakano et al. combined the benefits of PCR amplification and plate screening into a new high-throughput approach called single-molecule PCR-linked *in vitro* expression (SIMPLEX), for the generation and screening of protein mutant libraries.[22]

5. Optimization/improvement of cell-free systems

Because of issues with yield, cost, and scaling up, cell-free systems have been used mostly in niche areas of biology and industry. The constant effort has been made to optimize energy regeneration, expression templates, and reaction modes to meet these requirements (Fig. 1C).

5.1. Energy transformation

Regeneration ability in cell-free systems is far lower compared to that of cell-based complex living systems. Because there are so many steps in translating DNA into protein that require ATP or an equivalent, ATP regeneration is crucial to the effectiveness of *in vitro* synthesis.[23] The high cost of energy substrates used in cell-free synthesis is another key factor. Thus, researchers are working hard to perfect techniques of ATP regeneration that are both inexpensive and effective, thereby increasing the viability of the cell-free synthesis system beyond its current application in the lab.[24] Regenerating ATP from ADP is possible through the incorporation of secondary energy molecules containing high-energy phosphate linkages, such as phophoenol pyruvate, creatine phosphate, and acetylphosphate. In addition, glucose 6-phosphate, an intermediary in glycolysis, was found to be an energy substrate. Thanks to the regeneration mechanism, cell-free protein synthesis may be provided at a much lower cost.

5.2. Template stabilization

Another issue limiting synthesis efficiency is the stability of the genetic template. Templates in cell-free systems might be plasmids (which are circular) or PCR products (which are linear). The rational sequence design efficiently generates large numbers of stable linear DNA templates.[6,25] Separate transcription of template mRNA is possible in a transcription system. Natural mRNA in eukaryotes is typically modified with caps and polyadenylic acid prior to translation start, however, this presented a challenge. Several publicly available sequences, including TMV-derived wheat germ-based sequences, have been shown to be useful for initiating eukaryotic translation after minor modifications.

5.3. Correct folding

Though the vast majority of cytoplasmic proteins can be produced, complicated protein synthesis remains a formidable challenge and an active area of study.[26,27] To begin, disulfide bond formation is typically critical. Correct disulfide bonds can be generated by pretreatment of the cell extract with iodoacetamide, use of oxidized and reduced glutathione, and coupling with disulfide bond isomerase. Second, the use of native chaperones or synthetic chaperones such nanogel helps keep the protein from clumping together and promotes proper folding.

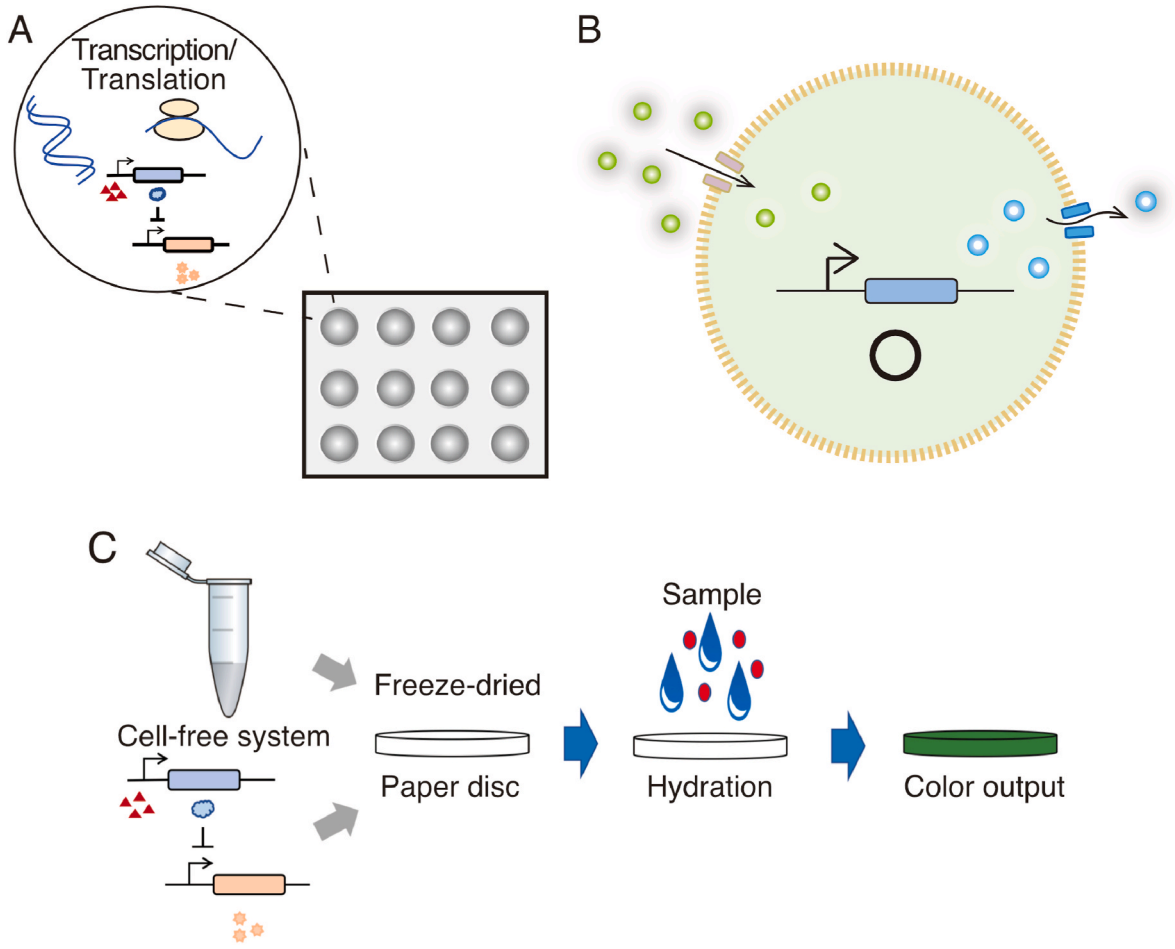

Fig. 2. Applications of cell-free systems. (**A**) Gene circuits. (**B**) Minimal cell. (**C**) Paper-based cell-free synthesis.

5.4. *Post-translational modification*

One of the most important ways that proteins' functions are modulated is by post-translational modification, which adds complexity and variety to protein structure. Cell-free systems can perform modifications such as glycosylation[28] and phosphorylation.[29] Glycosylation is diverse and complicated because of the polysaccharide structure's intricacy. As long as microsomes are added to the system, glycosylation can be finished. It will be a major breakthrough if the glycosylation modification can be precisely achieved with defined structural coponents *in vitro* in the future.

5.5. *Reactor modification*

The current cell-free reactors are largely split into batch, continuous flow cell-free (CFCF), and continuous-exchange cell-free (CECF), and are designed for processing small amounts ranging from tens of microliters to several milliliters. Traditional batch systems could only guarantee a reaction time of a few hours. The breakdown of mRNA, the accumulation of amino acids and by-products, the depletion of ATP and GTP, and the inhibition of the translation mechanism all contributed to the halt in production. The CFCF reactor was developed to increase the reaction duration to 40 hours.[30] Zawada also created a cell-free system based on *E. coli* that can fold at any scale with simple downstream purification steps, ultimately achieving production at titers of 700 mg/L in 10 hours using a volume of 100 L.[31]

6. Potential applications in biomolecular network analysis

6.1. *Gene circuits*

The "gene" in synthetic biology forms a network to enable cells to carry out the desired functions. However, there is still the issue of synthetic components not being compatible with the host cells for this new idea to work. When it comes to platforms, cell-free synthesis is highly adaptable. However, the transcription repertoire of current cell-free systems is extremely limited, consisting of only a few bacteriophage regulatory elements, such as T7 RNA polymerase, which severely restricts the ability to design synthetic gene circuits *in vitro*. As a result (Fig. 2A), a set of sigma factors that can be used in synthetic circuits were developed and organized into a toolbox.[32]

6.2. *Minimal cell*

In addition, synthetic biologists have been working on artificial cells in order to encode transcription and translation machinery and regulate the entire cellular dynamics. Top-down or bottom-up methods are now being used in the development of minimal cells. Using cell-free systems, one can build a minimal cell as part of the bottom-up strategy (Fig. 2B).[33–35] Understanding subcellular activities and emergent behaviors in a more cell-like environment and developing novel signaling pathways to achieve specific cellular behaviors are both aided by the creation of a robust platform that permits the assembly of artificial cells.

6.3. Gene network on paper

Cell-free synthetic gene networks with the properties of sterility and abiogenesis could be embedded onto the paper (Fig. 2C).[36] Using the bare essentials of transcription and translation, this was primarily accomplished by freeze-drying the cell-free system onto paper, which was then used by adding water.[37,38] Applications in biological detection and unstable drug production in the field are enhanced by the portability and stability of this technology when stored at ambient temperature.

7. Concluding remarks

Biological topics that are challenging to study *in vivo* can now be addressed with cell-free synthesis. Recent advances in the field have revealed promising industrial uses for cell-free systems, which have been put to use in fields ranging from basic biology to biomolecular engineering. Large-scale manufacturing of recombinant protein medicines, high-throughput functional protein screening, and site-specific mutation are all examples of biomolecular engineering in which cell-free systems have been implemented; yet, they still have some limitations that need to be addressed. The potential for cell-free synthesis to play an increasingly important role in biomolecular engineering has prompted researchers to focus on improving reaction stability and post-translational modification. By orchestrating the machinery for biomolecular engineering in advantageous cell-free synthetic systems, natural and unnatural proteins with diverse structures and functions will be designed and synthesized, promoting the fast development of biocatalysis and biomedicine fields.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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