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Cell-free synthetic biology: Engineering in an open world

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ABSTRACT

Cell-free synthetic biology emerges as a powerful and flexible enabling technology that can engineer biological parts and systems for life science applications without using living cells. It provides simpler and faster engineering solutions with an unprecedented freedom of design in an open environment than cell system. This review focuses on recent developments of cell-free synthetic biology on biological engineering fields at molecular and cellular levels, including protein engineering, metabolic engineering, and artificial cell engineering. In cell-free protein engineering, the direct control of reaction conditions in cell-free system allows for easy synthesis of complex proteins, toxic proteins, membrane proteins, and novel proteins with unnatural amino acids. Cell-free systems offer the ability to design metabolic pathways towards the production of desired products. Buildup of artificial cells based on cell-free systems will improve our understanding of life and use them for environmental and biomedical applications.

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1. Introduction

Advances in DNA sequencing and gene editing technologies have endowed the synthetic biologist with unprecedented power to program cells at will. The ability of synthetic biology to engineer biological functions holds great promises for applications ranging from biomedical to biofuel research. For the most part, synthetic biology is still tied to the living cell. One major advantage of using the living cell is its self-reproduction. However, the daunting complexity of living cells and the barriers of cell membrane make engineering difficult, and therefore make synthetic biology face four insurmountable challenges [1]: hard to standardize, unwieldy complexity, incompatibility and variability. From the standpoint of synthetic biology, it is highly desirable for these problems to be overcome using a standardized set of better engineering solutions.

To address these challenges, an emerging interdisciplinary approach has been adopted: cell-free synthetic biology. Cell-free synthetic biology system activates biological machinery without the use of living cells. It allows direct control of transcription, translation and metabolism in an open environment. Three types of cell-free systems have been well developed. One is extract-based

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system. The system is composed of crude extract with basic transcription and translation functions, DNA templates, energy regeneration substrates, amino acids, nucleotides, cofactors, and salts. Most commonly used organisms providing the extracts are *Escherichia coli* [2], *Saccharomyces cerevisiae* [3], rabbit reticulocyte [4], wheat germ [5], and insect cell [6]. The other one is purified system, such as the PURE system which consists of a toolbox of purified *E. coli* translational components [7]. The third one is synthetic enzymatic pathway system, which consists of numerous enzymes for implementing complicated bioreactions [8]. The ability of cell-free systems to harness a cell's capabilities unimpeded by cells opens new opportunities for the academic research and industry applications. Briefly, reduced dependence on cells drives the increase in engineering flexibility. As a result, *in vitro* cell-free systems have many advantages over traditional *in vivo* cell systems (Table 1) [8–10], which include controllable transcription, translation and post-translational modification, convenient high-throughput screening format, accelerated design-build-test-learn cycle, high synthesis rate and product yield, easy production of soluble membrane proteins and complex proteins, easy incorporation of unnatural amino acids (uAAs), high tolerance for toxic substrates or products, and good ability to focus on particular metabolism.

These features make cell-free synthetic biology serve as a versatile platform for engineering biological parts at three different levels of protein, metabolism and cell (Fig. 1). Cell-free synthetic biology can be an enabling technology for innovating medical diagnostics and therapeutics, developing complex metabolic system, making functional biomolecules, and producing sustainable bioenergy and biochemicals.

2. Cell-free protein engineering

Protein engineering is an important method to produce valuable proteins for basic and applied research. Currently, engineering proteins still relies on cell-based approaches, but many problems are difficult to deal with. Synthesis of functional proteins in cells usually faces some challenges, including insoluble expression, low protein yield, variability in expression, incorrect folding and low stability. Protein synthesis in the open cell-free system is uncoupled from cell growth and therefore can be directly controlled using greater degrees of manipulation and less complexity [11]. The direct access to the reaction environment of protein synthesis makes engineering proteins much easier. Cell-free expression also allows protein engineering in high-throughput format for discovery of novel biomolecules [12], flexible strategies for post-translational modifications [13] and convenient chemical conjugation [14]. Due to the open nature of cell-free systems, various biological parts or man-made devices can be implemented into cell-free systems to improve the biomanufacturing and expand the applications of proteins [11,15,16].

2.1. Synthesis and folding

Proteins are increasingly a key part of modern medical care. A significant proportion of proteins used in biopharmaceutical research and industry are complex proteins, toxic proteins and membrane proteins, which are difficult to produce *in vivo*. The primary advantage of cell-free systems is the ease of controlling and optimizing the reactions for better protein production.

Expressing complex proteins consisting of hetero subunits in cell-free systems is significantly beneficial as it allows co-translation of multiple mRNA to form bioactive complex proteins [17]. Other efforts to synthesize complex proteins are to encourage correct formation of multiple disulfide bonds. It could be achieved in cell-free systems by pre-treating the cell extracts, using redox buffers, adding disulfide bond forming enzymes or providing the chaperones [9,18].

Toxic proteins which interfere with cellular metabolic pathways and inhibit cell division are hard to express in high yields *in vivo*. Restriction endonuclease [19], cytolethal distending toxin [20] and the human microtubule binding protein [21] are typical examples of proteins toxic to cells. Since there is no cell growth, cell-free systems could serve as an excellent platform for the synthesis of those toxic proteins [17].

Constituting a significant fraction (20%–30%) of human genome [22], membrane proteins represent 60% of approved drug targets [23]. Overexpression of them in cells might lead to issues of accumulation as inclusion bodies. The protein concentration in cell-free system is approximately 20-fold less than that *in vivo* [24]. The dilution appears to be beneficial for protein folding. To assist protein folding, cell-free systems provide an attractive alternative to synthesize membrane proteins in the presence of surfactants or preformed liposomes, which mimics the environment of cellular membranes. It can prevent aggregation and enhance the solubilization of membrane proteins. Many membrane proteins have been successfully expressed in cell-free systems, such as G-protein coupled receptors [25], vaccine antigens [26,27] and tetracycline pump [28].

Table 1
Comparison of *in vitro* cell-free systems and traditional *in vivo* cell systems.

Feature	*In vitro* cell-free system	*In vivo* cell system
Manipulation of transcription and translation	Easy to control in an open environment	Hard because of cell membrane as the barrier
Post-translational modification	Hard	Easy
Self-replication	Hard	Easy
DNA template	Plasmids or PCR products	Plasmids or genomes
Synthesis of membrane proteins and complex proteins	Easy synthesis by adding surfactants or adjusting the system environment	Hard synthesis due to limited intracellular environment
Incorporation of unnatural amino acids into proteins	Easy	Hard
Ability to only produce the desired products	Easy achievement by focusing on the target metabolic pathways	Hard achievement due to complicated cellular metabolism
Toxic tolerance	High	Low
Integration with materials	Easy	Hard
Design-build-test-learn cycle	Two days	Two weeks
Biomanufacturing	High production rate	Modest production rate
	High product yield	Modest product yield
	Easy purification process without cell lysis	Cell lysis prior to product purification
Cost	Modest to high	Low to modest