Physical stimuli-responsive cell-free protein synthesis

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ABSTRACT

Cell-free protein synthesis has been developed as a critical platform in synthetic biology. Unlike the cell-based synthesis system, cell-free system activates transcriptional and translational mechanisms in vitro, and can control protein synthesis by artificially adding components or chemicals. However, the control method puts forward higher requirements in terms of accurate and non-toxic control, which cannot be achieved by chemical substances. For cell-free system, physical signal is a kind of ideal spatiotemporal control approach to replace chemical substances, realizing high accuracy with little side effect. Here we review the methods of using physical signals to control gene expression in cell-free systems, including studies based on light, temperature, electric field, and magnetic force. The transfer of these switches into cell-free system further expands the flexibility and controllability of the system, thus further expanding the application capability of cell-free systems. Finally, existing problems such as signal source and signal transmission are discussed, and future applications in pharmaceutical production, delivery and industrial production are further looked into.

1. Introduction

Cell-free protein synthesis (CFPS) has developed rapidly in recent years [1]. CFPS activates biological machinery without the use of living cells, allowing direct control of transcription, translation and metabolism in an open environment [2]. It eliminates membrane closure [3, 4], and is not restricted by most intracellular metabolic pathways, providing simpler and faster engineering solutions to design with extreme freedom than cell system. In CFPS, by adding specific substances, the biosynthetic reaction is initiated and completes within a few hours [5], offering rapid iterative “design-build-test” cycles [6]. Given the above superiority, CFPS has been widely used in protein synthesis, including biodrug [7], membrane protein [8], unnatural protein [9, 10], biological diagnosis [11–13], genetic circuit design [14,15], and high-throughput analysis [16]. However, different applications have specific requirements in time and space, and the transcription and translation process of the system needs precise initiation and control to better realize these applications.

Therefore, one of the major objectives and challenges for cell-free systems is appropriate spatial and temporal control of the transcription or translation. Openness is a unique characteristic of cell-free systems. The addition of inorganic ions and organics at a specific time can flexibly control the transcriptional and translation process in a cell-free system. In recent years, to address these challenges, researchers have proposed a spatiotemporal control method based on physical elements as stimulus signals, including light [4,5,17], temperature [18], electric field [19] and magnetic force [20] in CFPS. Physical signals with low toxicity and side effects are better than chemical substances at switching genes on and off [21]. More importantly, the physical signal can be flexibly adjusted in CFPS, so as to achieve immediate and local signal transmission and activation, and the rate will not be limited by other metabolic paths in vitro. The introduction of the physical signal switch into CFPS increases the potential of the system in health care, industrial production, synthetic biology education, and eventually achieving intelligent synthetic life.

Here, we mainly review the progress on the control of gene transcription and translation in CFPS by using physical signals with two types of classification, including direct response and indirect response (Fig. 1). Direct response involves directly changing the structure of the regulatory elements, while indirect response involves altering the position of the elements. In addition to summarizing the control principles (Table 1) and technical characteristics (Table 2) of each control method,
the application fields of the system also prospect, the possible defects are discussed, and the future research direction is further proposed.

2. Direct response - control of elements

Direct method is to change the state of the corresponding regulatory element by physical stimuli, which may be allosteric, phosphorylated, or specifically altered in the structure of the DNA. Specific elements that respond to physical stimuli can bind to gene promoters to determine whether genes are expressed or not. At present, two physical stimuli, light and temperature, have been well developed to respond directly to genetic elements, on the basis of which the applications of these systems in cell-free systems have been achieved (Table 1).

2.1. Control by light

Light possesses many advantages as an ideal genetic control switch, including fast input speed, good spatiotemporal conversion, low toxicity, and low cost of light source device. Light-controlled gene synthesis belongs to the field of optogenetics technology, which uses light to remotely control modified genes. At present, light control systems are mainly based on two-component systems (TCSs) [22], one-component systems [23] and photoreceptors [24]. Their introduction into CFPS could enrich the optogenetics research means, provide better guidance for the design of light control elements, and provide guidance for studying the interaction between proteins.

The two-component system (TCSs) depends on the phosphorylation between bicomponent proteins under different light conditions, ranging from ultraviolet light to near-infrared light, and light signal controls gene expression under the matched promoter [25]. Zhang et al. constructed two plasmids named pDark and pLight using TCSs YF1/FixJ that responds to blue light, to realize cell-free light-controlling gene expression with Escherichia coli extract under darkness and blue light in the batch, respectively [5]. This work successfully transplanted the TCSs into CFPS and achieved light control, further demonstrating its potential in education, healthcare and artificial cell construction. However, the control signal studied so far is limited to blue light, whose phototoxicity might destroy the expressed proteins, and the subsequent in vivo application of artificial cells constructed by CFPS is limited. To address these challenges, an alternative solution is to use TCSs with longer wavelengths, such as CcaS/CcaR responding to green light [26], Cph8/OmpR responding to red light [27], and BphP1/PpsR2 responding to near-infrared light [28]. It also could achieve flexible cooperative regulation of CFPS using different wavelengths of light, to enrich the applications of cell-free light-controlled system. However, the TCSs are intrinsically complex, relatively slow in signal relay and reversal, and less portable. All of these are the directions for further efforts and attempts to construct cell-free optogenetic systems in the future.

To avoid the possible drawbacks brought by TCSs, the system that only requires a single element to achieve control was developed, the so-called one-component system. Jayaraman et al. used EL222 protein-based blue light-inducible promoter to construct the light-controlled system in CFPS with E. coli extract [17]. EL222 protein forms homologous dimers when irradiated by blue light, and the dimers would bind to the PBind promoter, recruiting relevant RNA polymerase to the promoter region to initiate transcription [29]. This work proved the possibility of dynamic control of target gene expression, but this light control system is not currently being developed for engineering applications. However, in the one-component system, the components that can be selected to respond to long wavelengths are limited compared with TCSs, which may limit its development for CFPS.

In addition to the above light-controlled methods by directly
interacting macromolecular photosensitive proteins with promoters, organic small molecular structures that respond to specific wavelengths of light are also used to control the cell-free expression. Kamiya et al. tethered 2,6-dimethyl-4-(methylthio) azobenzene-4′-carboxylic acid (S-DM-Azo) units to the T7 promoter region and achieved photoswitching of gene expression using visible light in CFPS with PURE system [30]. In this system, azobenzene can induce reversible formation and dissociation of the DNA duplex upon light irradiation [31]. The system avoids complex protein-promoter interactions, but the sample preparation procedure was laborious, and heating to above the Tm of the template was required to induce photoisomerization, limiting its practical applications. In addition to azobenzene, the photoswitches commonly used also include stilbenes [32], hemithioindigos [33], spiropyrans [34], diarylidenes [35], and fulgides [36]. By modularizing these conversion units into DNA strands, it is also possible to reversibly control cell-free gene transcription [37].

At present, cell-free optogenetic system is the main means of direct response, but there are still some challenges to be solved for its development. The establishment of the system requires that the optically responsive elements originally existing in vivo should be transplanted into the cell-free system solution. To overcome the differences between in vivo and in vitro and improve the efficiency, the prototype design should be carried out, and the key influencing factors of the system should be explored. One important problem of cell-free optogenetic system is that light can be refracted in the solution. The effective transmission of light in solution is also the direction of future research.

2.2. Control by temperature

Temperature is another physical signal with high flexibility and stability. Compared with the light control, there are more methods to control and change the temperature of the system, including external triggers such as focused ultrasound, infrared light, and magnetic particle hyperthermia [38]. In general, there are heat-shock protein promoters [39], thermo-sensitive repressor [40], and RNA thermometers (RNATs) that could induce the transcription process [41]. Changes in temperature alter the structure of these elements, thereby altering the process of gene transcription. It is of great significance for the intelligent creation of artificial life systems to give cell-free systems with the ability to respond to temperature.

Though there have been various ways to achieve temperature control in cells, only RNATs have been transplanted into cell-free systems in previous studies. RNAT loop unfolds when the temperature exceeds a defined threshold, to release the RBS for subsequent gene expression. Jia et al. designed three different threshold temperature RNAT switches that can be activated at 35°C, 37°C, or 40°C in cell-free PURE system [18]. This study further used this system to create a temperature-sensitive protocell model for potential drug delivery applications. However, the expression of a high threshold switch under thermal activation is relatively low, which may be related to the stability of the secondary structure in the system.

From a technical point of view, heat-shock promoters and heat repressor proteins are also feasible means of abundant cell-free temperature control in the future. Compared with RNA thermometers, the interactions between promoters and related proteins are more complex

| Table 1 | Summary of the gene expression control systems in CFPS. |
|--------|--------------------------------------------------------|
| Sorting | Physical signal | Key elements | Mechanism | Induction Multiple | Ref. |
| Direct response | Light | Two-component system: YF1/FixJ | YF1 | 6 | [5] |
| | | One-component system: EL222 | EL222 | >10 | [17] |
| | | Azobenzene-tethered photosensitive T7 promoter | FixJ | 7.1 | [30] |
| Temperature | RNA thermometer | RNA thermometer | RNA polymerase | 11 | [18] |
| Indirect response | Electric field | DNA compartment on a chip | DNA compartment | – | [19] |
| | | DMF board | DNA compartment | – | [42] |
| Magnetic force | Magnetic beads coated with DNA | Magnetic beads coated with DNA | DNA | – | [20] |
and may be associated with metabolic pathways in the body, making transplantation more difficult. Another idea is about miniaturization, which has a great prospect in the application fields such as therapy and medicine. Currently, magnetic nanoparticles are used to be coated with microcapsules to generate heat under the action of alternating magnetic field, so as to achieve the purpose of heat induction.

3. Indirect response - control of contact

Indirect methods mainly use external physical signals to control the location of elements involved in transcription and translation, thus controlling the process of gene expression. This control mode does not change the activity and state of biological components, but its applicability is stronger and more flexible. The biomolecules under a nonuniform E-field could be trapped and polarized using dielectrophoresis (DEP). The other way is to turn on gene expression by manipulating the droplet’s motion directly with the electric field. It is also worth noting that, for the cell-free system, the manipulation using electric field can be carried out without considering the survival and growth of cells.

One way that the electric field controls cell-free gene expression is to use DEP technology. Efrat et al. used DEP to demonstrate the electric field on/off switch response of gene expression in the PURE CFPS system on a chip [19]. Various forms of DEP can be applied to trap, manipulate and separate biomolecules to controlling the contact between the DNA brush attached to the surface in the trap and the elements, such as ribosomes, RNA polymerases, newborn RNAs and proteins, so that controlling protein synthesis. Based on the electronic control system, the biochip was constructed and demonstrated protein synthesis oscillations successfully, which further improved the potential of electric control. However, the electrotherm effect may occur when the electric field interacts with the solution system, which affects the performance of the components in the system. In the future, by combining the design principles of integrated circuits with the powerful information processing capability of biological systems, the capability of biochips to process biological information can be expanded. At the same time, further development of genetic elements that can accurately respond to electric fields would enrich the means of electric field control.

An alternative approach to controlling gene expression using electric fields takes a more macroscopic approach. There are also studies achieved controlling gene expression into proteins using digital microfluidic (DMF), which can directly manipulate single droplets. Liu et al. used real-time remote-controlled DMF technology to start cell-free synthetic reaction successfully [42]. By controlling the movement and mixing of the drops containing different components through real-time DMF, cell-free protein expression reaction started on the board. This work enables the automation of the preparation of cell-free system solutions, which can save time and manpower costs. However, limited by the number of plate electrode arrays, only the motion of small droplets can be controlled. Moreover, this control plate can only initiate the gene expression by electric fields, while it cannot be stopped without the addition of an exogenous transcriptional inhibitor, because the components in the droplet are sufficiently mixed.

Electric-field regulation is usually used to regulate complex neural signaling pathways in vivo, mainly to control the expression of some factors and enzymes [43], which is difficult to be transplanted to in vitro system. At present, the method of electric-field control in CFPS is relatively simple. In addition to controlling the location of key components, the combination of electric field and materials science improved the accuracy of spatiotemporal control, such as hydrogels that release substances in response to electric field stimuli [44].

3.2. Control by magnetic force

Magnetic force is a noteworthy control signal. Magnetic force has a stronger ability to penetrate tissues than light signals, and it does not cause global effects or local thermal damage like temperature signals, nor does it need to consider the potential harm of electric current to life activities. Although the regulation of magnetic field on life activities has been studied, the regulatory elements for gene expression have not been developed, so magnetic field control is still indirect.

The magnetic force controlling cell-free gene expression mainly relies on nanomagnetic beads. Finkler et al. developed a cell-free magnetic gene expression control system based on E. coli extract using the magnetic beads connected to target DNA [20]. Specifically, the DNA templates bound to magnetic beads can accumulate under magnetic control, thus stopping elements in the solution system from participating in DNA transcription and translation. This method can precisely regulate gene expression only through the spatiotemporal separation of nucleic acids. The magnetic control system is simple in principle and can be applied to microfluidics or non-regular amino acid embedded proteins. However,

Table 2

Summary of technical details in different CFPS systems. Citations indicate where more information about the systems in the context of cell-free can be found.

| Physical | Key elements | Cell-free system | Reaction format | Volume | Pros | Cons | Ref. |
|----------|--------------|------------------|----------------|--------|------|------|------|
| Light    | YF1/FixJ     | E. coli extract  | Batch; One compartment | 20 μL | Abundant components responding to different wavelengths | Slow signal relay and reversal rates; Poor portability | [5] |
|          | EL222       | E. coli extract  | Batch; One compartment | 25 μL | Good portability; Fast response | Limited response wavelength | [17] |
|          | Azo benzene- | PURE             | Batch; One compartment | –     | Avoiding complex protein interactions | Tedious preparation process | [30] |
|          | tethered promoter | RNAAT | Batch; One compartment | –     | Flexible and varied means of temperature control | Low expression | [18] |
| Temperature | DNA brush on a chip | PURE | Batch; Fixed in compartment on a chip | Compartment: R = 35 μm; H = 3 μm | Novel manipulation of cell-free expression | Reduced component performance by electrothermal effect | [19] |
| Electric field | DMF board | E. coli extract  | Batch; Multiple droplet compartments | 20 μL | Remoted control of cell-free expression | Limited control scale | [42] |
| Magnetic force | Magnetic beads coated with DNAs | E. coli extract  | Batch; One compartment | 24 μL | Simple control method and spatiotemporal principle | The attachment effectiveness of DNA on magnetic beads | [20] |
this control method requires attaching the target DNA to the nano-
particles, which cannot guarantee that each particle will adhere
effectively.

4. Conclusion and perspective
At present, the research on cell-free gene expression by the direct
response realized in CFPS is mainly based on the light and temperature
signals as switches. The signal response circuit originally existed in
the cell is transplanted to the cell-free system, and the protein expression
was successfully induced and controlled in vitro by using two kinds of
physical signals, light and temperature. The direct response method has
been preliminarily applied to simple dynamic demonstration, control
of pharmaceutical protein synthesis and artificial cells, showing certain
application potential. The indirect response is a simple control method
that can bypass the complex biological metabolic interaction network
to turn on and off the biological response of gene expression. The ability
of electric and magnetic forces to control the elements allows them to
respond indirectly, and the researchers have built systems for control-
ing gene expression that is sensitive and fast. Such systems have shown
potential for mass control of synthesis and non-natural amino acids
embedded in proteins, but have not been further explored for
application.

The physical signal-controlling system has been developed with
 certain control ability, but it still needs to be further improved. For a
system that responds directly, overcoming differences between in vivo
and in vitro can improve the control effect of components, so it is
necessary to screen out key influencing factors to reduce such differ-
ences. And rapid prototyping and high-throughput screening are also
advantageous cell-free systems. Besides, direct methods require several
time periods to respond, and the development of systems with shorter
response time will greatly improve its application ability. For systems that
respond indirectly, the flexibility of the control system can be broadened
by combining the control system with new materials. For example, the
electro-responsive hydrogels are currently being carried out in drug
delivery, and the combination of material and elements is worth
considering.

In addition, this system has great application potential in both Cell-free
engineering and manufacturing medical. CFPS controlled by phys-
ical signals has not been put into engineering manufacturing practice.
Engineering proteins with complex structures put forward higher
requirements on the synthesis ability, so it is necessary to further improve
the ability to modify the high-level structure of proteins in cell-free
systems. In recent years, the construction of artificial cells with cell-
free system has increased. Studies have used light and temperature
signals to control protein expression in artificial cells, preliminarily
proving that protein synthesis can be controlled at the cell scale. Since
artificial cells may be responsible for the production and delivery of
drugs in vivo, the transmission of physical signals in vivo and the activity
of protein production require further study.

In general, the development of controlled protein synthesis in cell-
free systems will greatly promote the development of targeted drug
synthesis and delivery technologies, and the flexible control of protein
production in vitro will further promote the progress in the field of in-
dustrial protein manufacturing.

Ethics approval
This article does not contain any studies with human participants or
experimental animals performed by any of the authors.

CRediT authorship contribution statement
Junzhu Yang: Conceptualization, Validation, Writing - original draft.
Yuan Lu: Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
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