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Development of a robust *Escherichia coli*-based cell-free protein synthesis application platform

Nan Jiang [1], Xuanwei Ding [1], Yuan Lu *

Key Laboratory of Industrial Biocatalysis, Ministry of Education, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

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ABSTRACT

Since the cell-free protein synthesis system is not limited by the cell growth, all the substrates are used to produce the protein of interest, and the reaction environment can be flexibly controlled. All the advantages allow it to synthesize toxic proteins, membrane proteins, and unnatural proteins that are difficult to make *in vivo*. However, one typical reason why the cell-free system has not been widely accepted as a practical alternative, is its expression efficiency problem. The *Escherichia coli*-based system was chosen in this study, and the model protein deGFP was expressed to explore a more efficient cell-free system. The results showed that Mg^{2+} with a concentration of 15 mM in the cell-free system with BL21 Star (DE3) as the extract could better synthesize protein. The smaller the vectors, the lighter the burden, the higher the protein synthesis. Simulating the crowding effect in the cell does not improve the protein expression efficiency of the optimized cell-free protein synthesis system. Based on the optimized system, the cell-free fundamental research platform, primary screening platform, and portable biomolecular synthesis platform were established. This study provides a robust cell-free protein synthesis toolbox with easy extract preparation and high protein yield. It also enables more researchers to reap the benefits from the cell-free biosynthesis platform.

1. Introduction

The aim of synthetic biology is to advance biological functionality through engineering, and rapid progress has been made [1–4]. Its ability to engineer biological functionalities holds excellent prospects for applications in bioenergy production [5], drug synthesis [6], and biosensor development [7]. However, the development of synthetic biology faces enormous challenges [8]: hard to standardize, unwieldy complexity, incompatibility, and variability. To overcome these problems, cell-free protein synthesis (CFPS) has received new attention as an emerging synthetic biology technology, because it is an open system, not limited by cell growth or cell membrane, and it allows the use of various reaction formats. CFPS systems have many advantages such as controllable transcription, translation, and post-translational modification, high synthesis rate and product yield, convenient high-throughput screening format, easy incorporation of unnatural amino acids, high tolerance for toxic substrates or products, and accelerated design-build-test-learn cycle [9]. Therefore, cell-free synthetic biotechnology has been used to make difficult-to-synthesize proteins, unnatural proteins, and complex proteins for various biological applications.

Despite its many advantages, the CFPS system has not been widely accepted as a practical alternative. The problem of low productivity is considered to be one serious obstacle to CFPS, so the optimization of the CFPS system is very important [10]. The extract is one of the most important components in the CFPS system. Its preparation and selection affect the CFPS efficiency [11–14]. Rational design of DNA template is one of the keys for efficient CFPS [15]. In most cases, a supercoiled plasmid DNA containing the gene of interest is used in cell-free systems because of its good stability [16]. The substrate concentration in CFPS is another key factor affecting the protein expression. The experimental conditions in CFPS must be precise to achieve optimal expression. Molecule density is another key plaguing CFPS systems [17]. In recent years, the crowding effects of macromolecules have been transferred to CFPS, which is an important function in living cells but ignored in cell-free systems. Molecular crowding is a natural state of cells, which could lead to a fundamental impact on cellular properties and then may affect the efficiency of CFPS [18]. In addition, metal cations are an indispensable part of biological machinery, involving many essential

* Corresponding author.
E-mail address: yuanlu@tsinghua.edu.cn (Y. Lu).
[1] These authors contributed equally to this work.

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tasks ranging from biomolecular structure stabilization to enzyme catalysis. The change of metal cation type and concentration could affect the protein synthesis process, thus affecting the CFPS efficiency [19–21].

CFPS has a broad application prospect in basic scientific research and industrial production. With the advantages of CFPS, it can be applied to the synthesis of difficult-to-express proteins, the embedding of unnatural amino acids, and the rapid screening of proteins. CFPS systems have successfully expressed high levels of biopharmaceutical protein and membrane proteins, such as virus-like particles and G protein-coupled receptors [22,23]. Also, an orthogonal translation system can be used to incorporate unnatural amino acids into natural proteins to synthesize unnatural proteins with new functions [24]. In addition, through residue-specific and site-specific incorporation, CFPS is becoming a platform for the production of enzymes with altered rapid screening functions [25]. It is believed that CFPS can promote the development of basic science, biomedicine, and high-throughput screening platforms.

In this paper, the issues that directly affect the CFPS synthesis were concerned. The selection of extracts, the size of the plasmid, the molecular crowding effect and the metal ion effect, which are important component parameters, were explored to improve the protein expression efficiency. The final optimization results were used in three various applications. Based on the optimized CFPS systems, the cell-free fundamental scientific research platform, primary screening platform, and portable biomolecular synthesis platform were established.

2. Materials and methods

2.1. Construction of plasmid

The target protein is the model protein deGFP. First, inserted the coding sequence of deGFP into the plasmid pET23a (Tiandz, Beijing, China) between NdeI and SalI. A PCR cassette was generated using the primers W006F and W006R with the pET23a plasmid template and W003F and W003R with homemade pET21b-deGFP plasmid template by a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Two linear fragments were assembled by Gibson. The plasmid pET23a-deGFP was constructed after adding the coding sequence for a 6xHis-tag to the deGFP gene by a PCR cassette (W005F/W009R) and Gibson assembly. Next, deleted the coding sequence of *rop* (W001F/W001R) and f1 *ori* (W002F/W002R) on the basis of pET23a (Supplementary Table S1). After PCR cassettes and Gibson assembly, the small plasmid pET23a△rf-deGFP was constructed (Supplementary Table S2).

2.2. T7 RNA polymerase preparation

T7 RNA polymerase was prepared from *Escherichia coli* BL21(DE3)/pAR1219 (Sigma-Aldrich Merck, Shanghai, China) as described previously [26]. Cells were cultured in 1-L shake flasks with LB growth media (1 % tryptone, 0.5 % yeast extract, 1 % NaCl). Cells were fermented at 37 °C with 200 rpm agitation in a rotary incubator shaker for an hour, and then IPTG induction was performed. Another two hours later, cells were harvested at an OD600 of 2.0 and centrifuged at 8000 rpm at 4 °C for 20 min by a M41041-F000 centrifuge (KUBOTA, Tokyo, Japan). Cells were washed by suspending in 5 mL ice-cold S30 (10 mM Tris-acetate, 14 mM magnesium acetate, and 60 mM potassium acetate) per gram of cell for twice and centrifuged at 8000 rpm at 4 °C for 20 min. The pellets were resuspended in 4 mL ice-cold lysis buffer (50 mM NaCl, 10 mM EDTA, 10 mM K_2HPO_4, 1 mM DTT, 10 mM β-mercaptoethanol, 1×Protease inhibitor, 5 % glycerin, pH 8.0) per gram of cell in preparation for cell lysis, ultrasonicated for 40 min (work 2 s and intermittent 6 s) and then centrifuged at 80,000 rpm at 4 °C for 20 min. Finally, the supernatant was dialyzed (6−8 KDa) twice in dialysis buffer (50 mM NaCl, 1 mM EDTA, 40 mM K_2HPO_4, 1 mM DTT, 20 % Sucrose, pH7.7) at 4 °C overnight. The suspension was centrifuged at 10,000× g for 30 min at 4 °C, and the pellets were discarded. The crude T7 RNA polymerase was flash-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Crude extract preparation

Cell growth for extract preparation was performed using BL21 Star (DE3) cells in 1-L shake flasks with inner concave baffles. Cells were cultured at 37 °C with 240 rpm agitation in a rotary incubator shaker. The fermentation was performed in 2xYT growth media (1 % yeast extract, 1.6 % tryptone, 0.5 % NaCl). Cells were harvested at early logarithmic growth phase at an OD600 of 3.2–4.0, about 3.5 h after induction by a M41041-F000 centrifuge (KUBOTA, Tokyo, Japan) at 8000 rpm at 4 °C for 20 min. Cells were then washed by suspending in 5-mL ice-cold Buffer A (1 mM dithiothreitol, 10 mM Tris base, 14 mM magnesium acetate, and 60 mM potassium glutamate) per gram of cell and centrifuged at 10,000 rpm at 4 °C for 10 min, and subsequently resuspended in 1-mL ice-cold buffer A per gram of cell in preparation for cell lysis. Cell suspensions were lysed with two passes through a JN-3000PLUS low temperature ultra-high-pressure continuous flow homogenizer (JNBIO, Guangzhou, Guangdong, China) with sample cooling for 1 min in an ice-water bath after the first pass. The lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C, and the pellet was discarded. The supernatant was carried forward for a run-off reaction by incubating at 37 °C with 120 rpm agitation for 80 min. The suspensions were centrifuged at 10,000 × *g* for 30 min at 4 °C, and the pellet was discarded. Finally, dialyzed (6−8 kDa) twice in Buffer B (10 mM Tris base, 14 mM magnesium acetate, and 60 mM potassium glutamate) at 4 °C overnight. The suspensions were centrifuged at 10,000 × *g* for 30 min at 4 °C, and the pellet was discarded. The extract was flash-frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Cell-free protein synthesis (CFPS) reactions

The CFPS systems included the necessary components as shown in Table 1. Combined transcription-translation reactions were carried out in 1.5 mL Eppendorf tubes at 30 °C for 12 h, unless otherwise noted. After the fermentation was performed in TB growth media (1.2 % tryptone, 2.4 % yeast extract, 0.4 % glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4), the plasmids were prepared using a Qiagen Plasmid Maxi Kit.

Table 1
The concentrations of components in CFPS reactions.

Components	Concentrations	Company
Potassium glutamate	175 mM	Vetec (Sigma-Aldrich), Shanghai, China
Ammonium glutamate	10 mM	Vetec (Sigma-Aldrich), Shanghai, China
Sodium oxalate	2.7 mM	Vetec (Sigma-Aldrich), Shanghai, China
Spermidine	1.5 mM	Vetec (Sigma-Aldrich), Shanghai, China
Putrescine	1.0 mM	Vetec (Sigma-Aldrich), Shanghai, China
Natural amino acids	2 mM	Vetec (Sigma-Aldrich), Shanghai, China
Magnesium glutamate	15 mM	Vetec (Sigma-Aldrich), Shanghai, China
Glutathione disulfide (GSSG)	4 mM	Solarbio, Beijing, China
Glutathione (GSH)	1 mM	Solarbio, Beijing, China
β-NAD	0.33 mM	Vetec (Sigma-Aldrich), Shanghai, China
CoA	0.27 mM	Sigma, Co.St.Louis, MO, USA
ATP	1.2 mM	Sangon, Shanghai, China
GMP, UMP, CMP	0.86 mM	Sangon, Shanghai, China
Phosphoenolpyruvate (PEP)	66 mM	Alfa Aesar, Shanghai, China
Folinic acid	34 μg/mL	Aladdin, Shanghai, China
E. coli tRNA mixture	170 μg/mL	Roche, Indianapolis, IN, USA
DNA template (plasmid)	15 μg/mL	Preparation methods in this study
T7 RNA polymerase	0.1 V	Preparation methods in this study
Extract	0.25 V	Preparation methods in this study

2.5. Protein synthesis determination

The protein yield was determined by diluting the reaction volume to 200 μL with MilliQ water (Millipore, Billerica, MA, USA) in flat-bottomed 96-well black microtiter plates. Measured the fluorescence with an Infinite 200 Pro microplate reader (Tecan Austria GmbH, 5082 Grödig, Austria). The excitation/emission wavelengths for deGFP was 485 and 510 nm, and mCherry were 587 and 615 nm, respectively.

^{14}C-Leucine-labeled protein synthesis was detected. Added 0.534 μL^{14}C -Leucine into 20 μL CFPS reaction system and then incubated reactions for 12 h at 30 °C. After the reaction, 3 μL reaction mixture was spotted onto both the R (total radiation), T (total protein) and S (soluble protein) filter paper strips corresponding to the correct experiment condition and replicate. The soluble fraction of the product protein was isolated by centrifuging the reaction mixture at 14,000 rpm for 15 min at 4 °C. The paper strips were dried under a heating lamp for 30−45 min. The amount of L-[U-^{14}C]-Leucine incorporated into the protein was measured using the trichloroacetic acid procedure described previously to precipitate the synthesized protein [27], which was quantified with a liquid scintillation counter. Once the liquid scintillation counting (LSC) has completed the counting, calculations were performed to determine protein yields.

The calculation formula is as follows (R = Total radiation LSC count, T = Total protein LSC count, S = Soluble protein LSC count):

$$\text{Total Protein } (\text{mg}/\text{L}) = \frac{(T/R) \times ([\text{Leu}] + [14C - \text{Leu}])}{(\text{Leu per protein}) \times (\text{protein MW})}$$

$$\text{Soluble Protein } (\text{mg}/\text{L}) = \frac{(S/R) \times ([\text{Leu}] + [14C - \text{Leu}])}{(\text{Leu per protein}) \times (\text{protein MW})}$$

2.6. Activity determination of TkADH

The activity of *Tk*ADH was spectrophotometrically determined at 40 °C by monitoring the substrate-dependent change in the absorbance of NAD(P)H at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}). Oxidation of alcohols was performed in a reaction mixture composing of 50 mM glycine-NaOH buffer (pH 9.0), 100 mM phenylethanol, and 1.0 mM NAD(P)$^+$. The reaction started by adding an appropriate amount of the enzyme into the reaction mixture in a total volume of 1 mL. One unit of ADH activity was defined as the amount of enzyme that oxidizes 1 μmol NAD(P)H per minute.

$$\text{phenylethanol} + \text{NAD(P)}^+ \xrightarrow{TkADH} \text{phenylethanol} + \text{acetophenon} + \text{NAD(P)H} + \text{H}^+$$

3. Results and discussion

3.1. Selection of crude extract from different E. coli strains

CFPS enables direct control of the protein synthesis by performing the reaction in a tube, wherein the transcription, translation, and protein folding machinery provided by cell extract are combined with energy sources to catalyze the target protein synthesis. Cell extracts are the key factors of CFPS, which can mimic the intracellular environment and provide the necessary translation elements, enzymes, and cofactors for protein synthesis. Hence, viable cell extract plays a crucial role in effective cell-free reactions, and cell choice is a key unit in CFPS. Here, host cells were screened, and cell culture conditions were explored to improve protein expression.

Based on *E. coli* which was the most widely used host background for protein expression, five commercially available types were tested in the initial screening experiment (Table 2). Origami B (DE3) which has trxB/gor mutant greatly facilitates cytoplasmic disulfide bond formation. Rosetta (DE3) expresses six rare tRNAs (AUA, AGG, AGA, CUA, CCC and GGA) and facilitates expression of genes that encode rare *E. coli* codons. Rosetta-gami B (DE3) combines the advantages of Origami B (DE3) and Rosetta (DE3). BL21 (DE3) is widely used host bacterium for protein expression on most proteins because of lon and ompT protease deficient. BL21 Star (DE3) has the rne131 mutant, which can improve the expression stability by enhancing the stability of mRNA in the strain. It was speculated that the genetic changes in BL21-based strains might affect their protein synthesis yield. By comparing extracts from each of the five strains prepared by the same method (see Materials and methods 2.3), the fluorescence value of the expressed protein with Rosetta (DE3) as the extract was the lowest (Table 2). When Origami B (DE3) was used as the extract, the fluorescence value was relatively high, but it was not the highest. The Rosetta-gami B (DE3), which combines the advantages of both, was also relatively low in fluorescence. Therefore, none of them were optimal extracts for the protein expression. The fluorescence value of the expressed protein with BL21 Star (DE3) as the extract was 1–2 times higher than that of other *E. coli* strains. BL21 (DE3) is suitable for the expression of non-toxic protein initiated by T7, while BL21 Star (DE3) carries out gene mutation based on it to make the mRNA more stable. Therefore, BL21 Star (DE3) might be suitable for the expression of deGFP, a non-toxic fluorescent protein, and maintained the stability of its template in the process of transcription and translation, so that it showed high expression. In order to get an efficient CFPS, BL21 Star (DE3) was chosen to continue the later research. It was worth noting that due to the different features of different extracts, the quality of the expressed protein was also different. The researchers can choose different extracts according to the purpose of the study.

Table 2
The expression of four plasmids in eight different *E. coli*-based systems. It showed the fluorescence value of the model protein pET23a-deGFP.

Escherichia coli	Derivation	Key feature(s)	pET23a-deGFP
BL21(DE3)	B834	lon and ompT protease deficient	45,496 ± 3618
BL21 Star (DE3)	BL21 (DE3)	*rne131* mutant, enhancing the stability of intracellular mRNA	64,638 ± 7462
Origami B (DE3)	B strain	*trx /gor* mutant, greatly facilitating cytoplasmic disulfide bond formation	59,115 ± 5735
Origami B (DE3) & Kan + Tet			42,126 ± 3513
Rosetta (DE3)	BL21	Expressing six rare tRNAs; facilitating expression of genes that encode rare *E. coli* codons	28,528 ± 4812
Rosetta (DE3) & Cam			1018 ± 65
Rosetta-gami B (DE3)	B strain	Combining Origami B (DE3) and Rosetta (DE3)	56,101 ± 15,027
Rosetta-gami B (DE3) & Kan + Tet + Cam			17,829 ± 5324

The microbiological contamination is encountered during the preparation of cell extracts, which are usually addressed by the addition of antibiotics. In order to explore whether the addition of antibiotics affected the protein expression of the extract, we also tested three different drug-resistant strains (Table 2). They were tested with or without antibiotics using different DNA templates, which expressed the model protein (deGFP). The result was obvious that the cell culture process without antibiotics were more suitable for cell extracts in CFPS (Table 2). The reason for this may be that the toxicity of the antibiotics themselves inhibits cell growth and affects DNA replication, which in turn affects the activity of the extract and thus hinders protein synthesis. Interestingly, the results showed that the addition of antibiotics to Rosetta (DE3) and Rosetta-gami B (DE3) had a greater effect. Probably it was because both Rosetta (DE3) and Rosetta-gami B (DE3) are resistant to chloramphenicol. Chloramphenicol has an inhibitory effect on the function of the inner ribosomes of growing cells and affects protein synthesis. However, although the addition of antibiotics could reduce

the expression level, in some special cases, such as some applications of cell-free high-throughput platforms, open and long-term operations are required. To avoid contamination, antibiotics need to be added to the culture medium during extract preparation. Although the expression level is low, chloramphenicol resistance can be avoided in plasmid design to obtain sufficient expression level.

Bacterial seed fluids were needed to be grown in fresh media for 3 h after overnight growth, and then the cell extract preparation was started (Supplementary Fig. S1). In this section, a total of five strains were screened, BL21 Star (DE3) which had the highest protein expression was selected as the extract, and the culture medium was further optimized. These steps increased the activity of crude cell extracts and protein expression, allowing for potential downstream scalability (see Materials and methods: CFPS reactions).

3.2. Selection of plasmid sizes

In most of the protocols for cell-free synthesis, protein synthesis is directed by plasmid templates, since the efficiency of the plasmid templates in cell-free synthesis is higher than those of the reactions utilizing linear templates. This is mainly due to the rapid decay of the linear templates by the exonucleases present in the cell-free extract [28]. According to the lowest cost principle, one of the points of concern is improving template utilization efficiency.

The choice of vector affects the efficiency of CFPS [29]. The pET system with the T7 promoter-driven system is the most widely used system yet developed for the cloning and expression of recombinant proteins in *E. coli*. It has been greatly expanded and now includes over 35 vector types designed for efficient detection and purification of target proteins. One of the vectors is pET23a (4289 bp), which is the smallest in Novagen. Also, BL21 Star (DE3) is mainly applicable to the high levels of the protein expression of T7 promoter expression vector. Therefore, the widely used and relatively simple pET23a was chosen as the original vector in the plasmid construction.

As DNA templates, two different sizes of plasmids were designed and compared. The larger one used pET23a as the vector. The smaller one used pET23a△rf (2872 bp) without the coding sequences of repressor of primer (*rop)* and f1 origin (f1 *ori*) (Fig. 1A). deGFP was still selected as the model protein. The wild-type *rop* protein is an RNA-binding protein involved in the regulation of the copy number of the ColE1 plasmid. At the same time, it can also promote the unstable RNA I-RNA II complex to become stable. The f1 *ori* is a bacteriophage replication initiation site, which facilitates the production of single-stranded DNA products. Theoretically, the deletion of *rop* and f1ori should not affect the replication of plasmids and the expression of proteins in CFPS. Although the plasmid vector pET23a△rf lacked *rop* and f1 *ori*, it still retained some of the necessary components of the vector such as T7 promoter and terminator, AmpR and MCS. Hence, it could perform the transcription and translation stably in CFPS.

To investigate the effect of plasmid size on protein expression, the fluorescence values of pET23a and pET23a△rf were analyzed under different extract conditions. The results showed that no matter which *E. coli* was selected as the extract, the relative fluorescence of pET23a△rf-deGFP (Fig. 1B) was always higher than that of pET23a-deGFP (Table 2). The reduction of plasmid size doubled the expression of Rosetta-gami B (DE3) and Rosetta (DE3). The expression of other extracts could increase by 10 %–50 %. Different strains have different genetic features, leading to different levels of increase. However, in any case, the protein expression of pET23a△rf-deGFP increased.

Moreover, when BL21 Star (DE3) was selected as the extract, with the increase of the plasmid concentration in the reaction system, the expression of the fluorescent protein first gradually increased and tended to be steady when the concentration reached about 0.06 mM. However, no matter how the plasmid concentration changed, the protein expression level of pET23a△rf-deGFP was always higher than that of pET23a-deGFP (Fig. 1C). Therefore, pET23a△rf-deGFP was selected as the DNA template. Deleting the coding sequences unrelated to target protein transcription and translation made the plasmid size smaller, which meant the burden of plasmid became smaller, and the proportion of the target protein encoded gene was larger than before. The results indicated that plasmid size could be an important parameter for protein expression in the CFPS system. This was an interesting discovery, but the

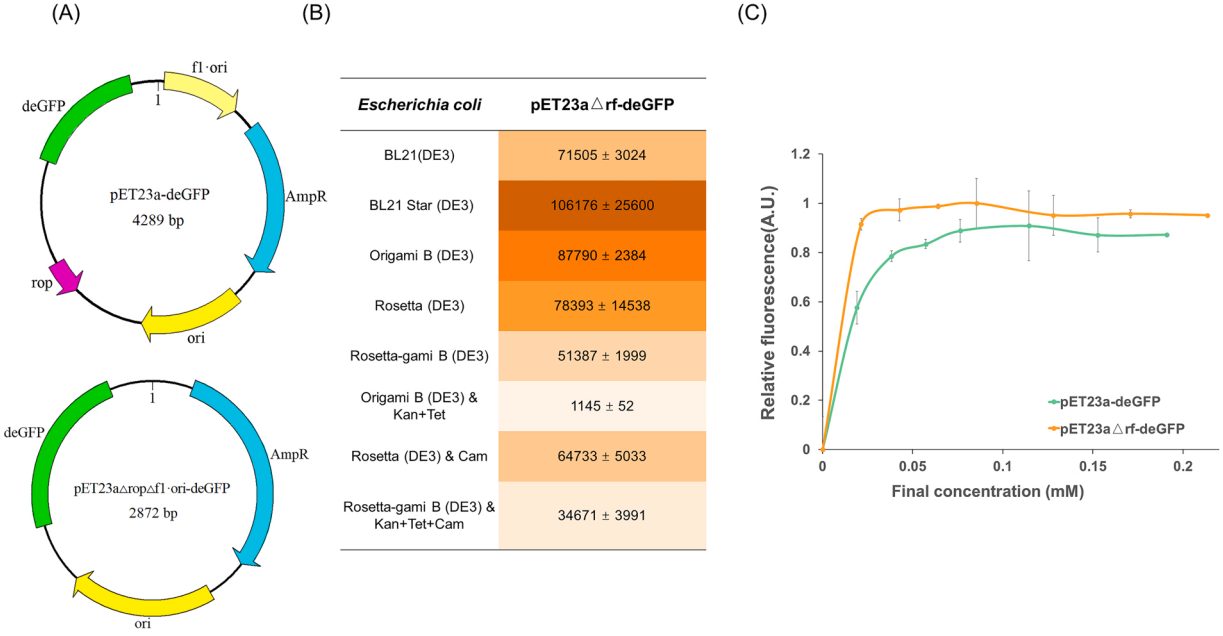


Fig. 1. The cell-free protein expression by the plasmid with different sizes. (A) The schematics of two plasmids with different sizes: pET23a-deGFP (containing deGFP sequence, f1origin (f1ori), ampicillin resistance, compressor of prime (rop) and origin(ori), one each) and pET23a△rf-deGFP (knockout of f1 ori and rop compared to pET23a-deGFP). (B) The fluorescence value of the expressed pET23a△rf-deGFP vector. The darker the color, the higher the fluorescence value. (C) The influences of different plasmid sizes and plasmid concentrations on the protein expression in the CFPS system (when the relative fluorescence value = 1.0, the concentration of deGFP =0.08 mg/mL).

specific reason was not yet known, and further investigation was needed.

3.3. Effects of metal ions

The biological significance of metal ions is that all organisms in the kingdom of life need metal ions as essential micronutrients. Metal ions participate in various biological reactions and are estimated to be required in one third of all proteins [30]. Protein-bound metal cations such as copper (Cu^{2+}, Cu^{+}), iron (Fe^{3+}, Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), calcium (Ca^{2+}), and zinc (Zn^{2+}) are key elements for regulating gene expression and catalyzing enzyme activities [30]. Among them, the electronic structure of the two redox states of iron, Fe^{2+} and Fe^{3+}, renders them the most versatile cofactors in biological redox reactions [31]. Mg^{2+} can play an essential role in folding, molecular recognition, and catalysis by RNA [32]. In particular, Mg^{2+} play an indispensable role in protein synthesis. In the CFPS system, Mg^{2+} not only participates in transcription and translation but also acts as activators of enzymes such as RNA polymerase and aminoacyl-tRNA synthase. However, excessive Mg^{2+} leads to premature termination of

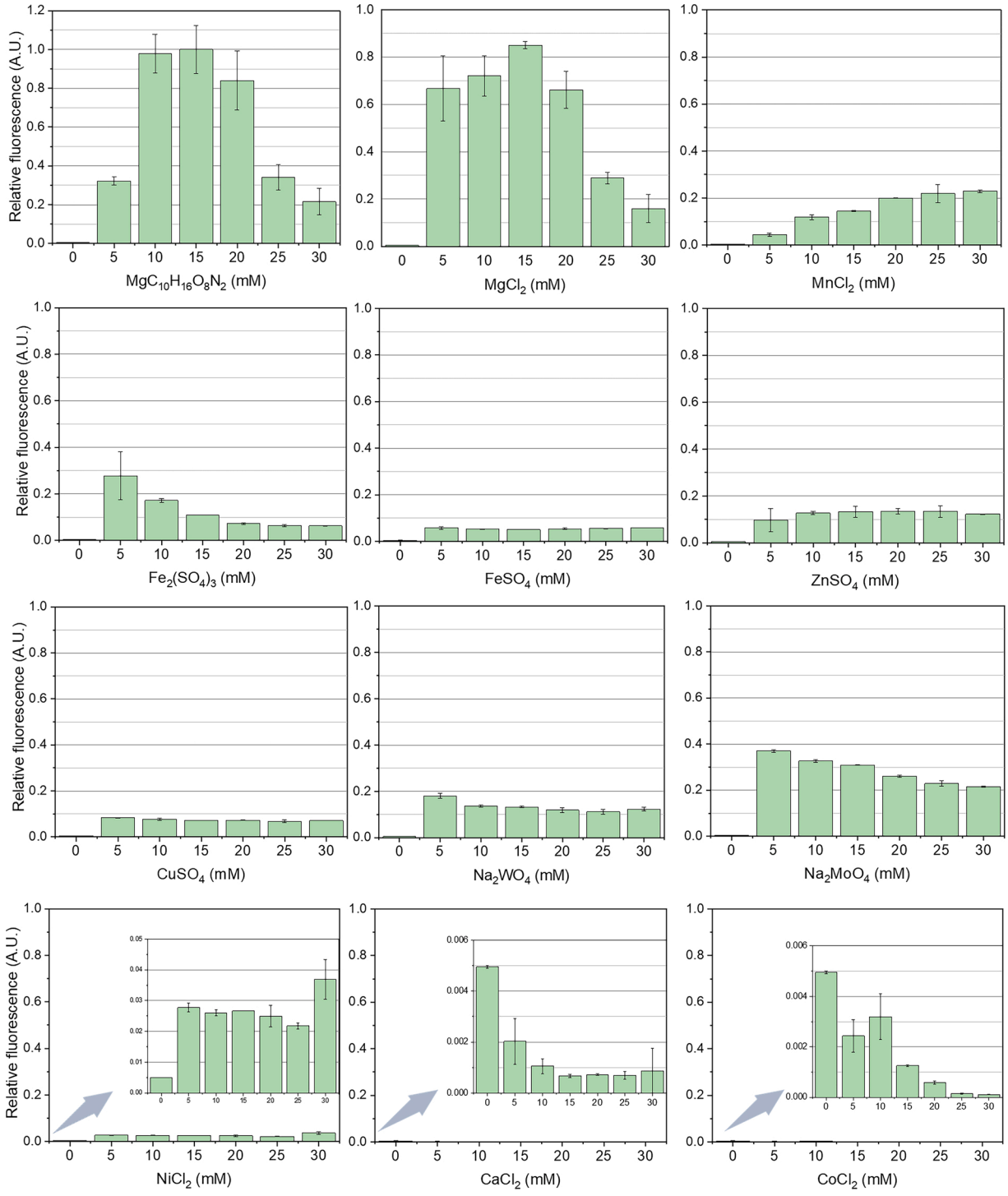


Fig. 2. The effects of metal ions on CFPS. Fifteen metal ions at different concentrations (0-30 mM) were added to the CFPS system, and the protein expression levels were explored. When the relative fluorescence value = 1.0, the concentration of deGFP =0.39 mg/mL.

protein synthesis. Consequently, the final concentration of Mg^{2+} has received widespread attention so that it is one of the most influential factors in the cell-free reaction mixture [33]. However, when it is necessary to synthesize metalloproteins in the CFPS system or enzymes that require metals as cofactors, it is not enough to focus only on the concentration of magnesium ions. Therefore, in this section, we explored the effect of different metal ions (Mg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{+}, Mn^{+}, Cu^{+}, Ni^{+}, W^{6+}, Mo^{6+}, Ca^{2+} and Co^{2+}) at different degrees on the protein expression in the CFPS platform.

The effects of different metal ions at different concentrations on protein synthesis were tested from 0 mM to 30 mM. The results showed that when BL21 Star (DE3) was selected as the extract, the addition of Mg^{2+} had the highest expression. The relative fluorescence was gradually rising when the final Mg^{2+} concentration ranged from 0 mM to 15 mM. The relative fluorescence started to decline after reaching the highest point at 15 mM. Therefore, when *E. coli* BL21 Star (DE3) was selected as the extract, the optimal concentration of Mg^{2+} was 15 mM. In contrast, when Fe^{2+}, Fe^{3+}, Zn^{+}, Mn^{+}, Cu^{+} and Ni^{+} were added to the system, the relative fluorescence value was relatively low, but the protein expression of CFPS still increased compared with not adding metal ions. In addition, two salt solutions Na_2WO_4 and Na_2MoO_4 were added to the system to test the effects of $W^{6+}$and Mo^{6+} on the CFPS system. The results showed that the addition of the two metal ions promoted the protein expression, but the increase was very limited (Fig. 2). When Ca^{2+} and Co^{2+} were added to the system, the relative fluorescence value was lower than the control. In summary, Mg^{2+} was the optimal metal ion for this system, and the maximum yield was achieved when its concentration was 15 mM. Notably, the effects of $MgCl_2$ and $MgCH_{16}O_8N_2$ on CFPS were tested here. Although the results showed a similar trend, there were differences in the protein expression level between these two. It indicates that the same metal ions were tested, but the paired anions had different effects on the protein expression. However, because the free Mg^{2+} has a complicated function of interactions with many cell-free reaction components, it is often beneficial to optimize Mg^{2+} for each lot of cell extract and cell-free reagents to obtain maximal protein synthesis. In addition, when the target protein to be synthesized is a metalloprotein or a specific metal ion is required as a cofactor, the corresponding metal ion can be added to the system. Therefore, researchers need to optimize the metal ions and the corresponding concentration for the best cell-free protein synthesis.

3.4. Effect of crowding environment

The interior of biological cells is a crowded environment. The biochemical interaction network that controls cell functions behaves very differently than the dilution environment of a typical test tube [34]. The biochemical reaction network involves the transcription, translation, and folding of proteins. One of the key distinguishing features between artificial cellular systems and living cells is the molecular density around cellular components [17]. Molecular crowding is a natural state of cells, which could cause volume exclusion effects that reduce diffusion rates and enhance the binding rates of macromolecules, leading to a fundamental impact on cellular properties [18]. In this part, different kinds of crowding agents were selected to explore the influence of agents on the efficiency of protein synthesis in the CFPS system.

Here, the effects of fifteen crowding agents (PEG200, PEG400, PEG600, PEG1000, PEG4000, PEG8000, Dextran5, Dextran500, trehalose, ethylene glycol, glycerol, carnitine, lactose, trimethylglycine and raffinose) at different concentrations were evaluated on the efficiency of protein synthesis in the CFPS system. First, the effects of different crowding agents on protein synthesis at concentrations of 0–5 % (w/v) were tested. The results showed that the relative fluorescence value decreased after adding crowding agents. Among them, the addition of Dextran5, Dextran500, carnitine and raffinose had little effect on the protein expression. PEG200, PEG400, PEG600, PEG1000, PEG4000, PEG8000, ethyleneglycol, trimethylglycine, trehalose, glycerol and lactose had a great influence on the protein expression. Among them, the addition of trehalose, glycerol and lactose inhibited the protein expression rapidly (Fig.3). The concentration range of the test was then reduced to 0 to 0.5 % (w/v). The results showed that after the concentration range was reduced, the results were similar to before. It indicated that the crowding agents actually did not greatly improve the CFPS efficiency. It might be due to the increased viscosity of some crowding agents, which increased the burden of the CFPS system and therefore reduced the efficiency of protein expression.

From the above, after optimizing the system (Fig. 4), important components of the CFPS system have changed (Table 1). By comparing five *E. coli* extracts, the BL21 Star (DE3) extract was more suitable for the expression of the model proteins (deGFP) due to its genetic features. Since the smaller the plasmid size, the less the burden on CFPS, the smaller pET23a△rf was selected as the carrier of deGFP gene. In addition, the protein expression efficiency of CFPS by metal ions was explored, and the protein expression level was highest when the metal ion was Mg^{2+} and the concentration was 15 mM. The crowding effect was explored and found that using additives to simulate the crowded environment would not improve the protein expression efficiency of the CFPS system. After optimization, the fluorescence value of deGFP increased from several thousand to one million. After the protein concentration determination, 0.535 mg/mL deGFP were obtained in the optimized CFPS system. The optimized cell-free system with better protein synthesis was obtained, which would be used in the following applications.

4. Applications

4.1. The cell-free fundamental scientific research platform

The cell-free system is of significant benefit for expressing protein complexes consisting of hetero subunits, as it allows co-translation of multiple mRNAs and forms the active protein complex. For example, active yeast tRNA methytransferase could be successfully expressed by co-translating mRNAs for protein subunits Trm8 and Trm82 [35]. Efforts to express the individual protein subunits and then reconstitute the complex, did not yield an active Trm8-Trm82 heterodimer [35]. Also, CFPS can also be used in the research of basic education, avoiding the limitations of expensive special equipment for growing, storing and transporting cells and biosafety considerations [36].

Here, the expression of the model proteins (deGFP and mCherry) with genetic sequence similarity were demonstrated in the same CFPS system. The addition of pET23a△rf-deGFP and pET23a△rf-mCherry followed the proportion from 0:10 to 10:0. All the other reaction components were shown in Table 1. It showed that the transcription and translation were performed normally. However, the expression of two different fluorescent proteins did not affect each other, which were reflected in the relative fluorescence and visible colors. As their proportions changed, not only the fluorescence values of the two fluorescent proteins changed but also visible colors changed from green to red (Fig. 5A). Thus, the expression of the target protein was methodical, even if multiple transcripts and translations were performed simultaneously. These results provided a reliable basis for fundamental research in the field of biochemistry.

In addition, the reaction system only needed to simply mix several substances together, and the results could be observed through the change of fluorescence color and intensity. Therefore, our system also provides a platform for basic education, avoiding the inherent limitations, so that untrained operators can experience synthetic biology research in such an easy-to-operate way.

4.2. The cell-free primary screening platform

Speeding up the design-build-test (DBT) cycles is a fundamental challenge that biochemical engineering faces. Right now, *in vivo*

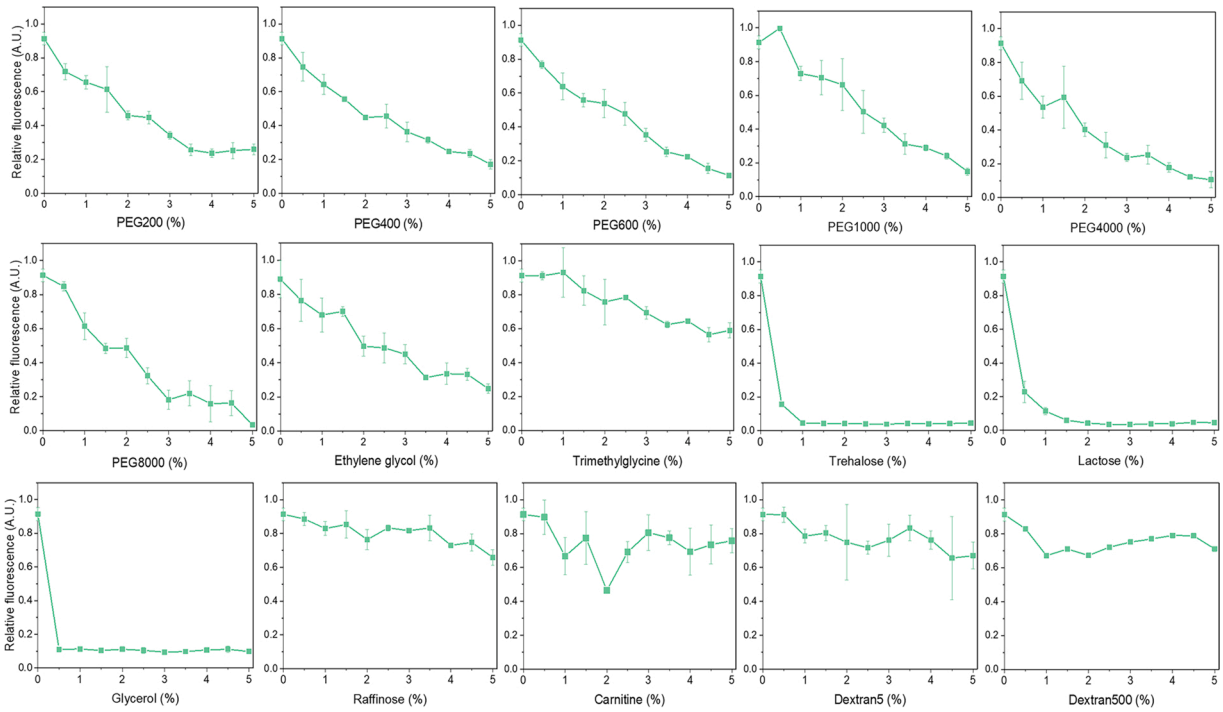


Fig. 3. The effects of crowding agents on CFPS. Sixteen crowding agents at different concentrations (0–5 %, w/v) were added to the CFPS system, and the protein expression levels were explored. When relative fluorescence value = 1.0, the concentration of deGFP =0.43 mg/mL.

approaches take, on average, 3∼4 months to complete this cycle. In cell-free systems, the design cycle is not limited by how fast cells reproduce. Rather than, it can be faster, potentially approaching the limit of synthesizing the components (DNA, RNA, and proteins). Cell-free systems may therefore speed up the design cycle for engineering by more than 10-fold relative to *in vivo* approaches. In addition, CFPS could be used as their own biomanufacturing platforms, or as feedback in the design of *in vivo* platforms [37]. At the same time, the active products can be monitored at any time.

In order to explore whether CFPS has the potential to become a preliminary screening platform, a novel thermostable alcohol dehydrogenase (ADH) was selected and expressed *in vitro*. The enzyme has the activity toward aromatic secondary alcohols from the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 (TkADH) and exhibits extreme thermostability as well as high resistance to organic solvents [38]. Using the cell-free reaction mixture (Fig. 5B), the transcription and translation of TkADH were performed. The results indicated that the enzyme could be expressed, and enzyme activity could be quickly detected in the protein synthesis processes. The level of enzyme activity changed with the concentration of DNA (Fig. 5C). The higher the concentration of DNA, the higher the enzyme activity. In addition, the level of enzyme activity could be quickly confirmed by the color depth (Fig. 5B). In summary, the use of CFPS could not only express functional enzymes but also quickly and visually screen high-activity enzymes. The results provided a reliable basis for the cell-free preliminary screening platform.

4.3. The portable biomolecular synthesis platform

Using living cells, molecular foundries for the biosynthesis of drugs, therapeutic proteins and other commodities required specialized equipment and refrigeration for their production and distribution. The traditional cell-free technology also has to store the main components, such as cell extracts and energy systems, below the freezing point in a bulky aqueous solution [39]. However, the delivery of these technologies to the field and low-resource areas face challenges. Therefore, the portable freeze-dried cell-free (FD-CF) system has been developed [40, 41]. The FD-CF system provides the means for on-site, on-demand manufacturing of therapeutics, biomolecular biosensors, biocatalysts, and high-throughput protein synthesis [42,43]. The flexible system is based on reaction pellets composed of freeze-dried cell-free transcription and translation machinery, which can be easily hydrated and utilized for biosynthesis through the addition of DNA encoding the desired output (Fig. 5D).

The preservation time, water loss rate, and activity changes of FD-CF components at different temperatures were investigated. The reaction components (Table 1) prepared without the plasmid and water were needed to be stored at −80 °C for 1 h. And then, they were freeze-dried into pellets by an FDU-1100 Freeze dryer (EYELA, Tokyo, Japan) and stored at −80 °C. Next, took samples every day and placed them at 4 °C or room temperature. Finally, proteins could be synthesized by simply adding water and template DNA at room temperature. The results showed that the water loss rate of the system was between 90 % and 92 % (Fig. 5E). Using pET23a△rf-deGFP as the DNA template, the preservation conditions of the reaction mixture freeze-dried powders were tested in 25 days. It could preserve 20−30 % activity for one week at room temperature and 5 % activity after 25 days. More than 50 % of the activity could be preserved after 2 weeks at 4 °C and up to 30 % after 25 days (Fig. 5F). The results showed that our system could be stored for about one week at room temperature, 25 days or more at 4 ℃. The activity at room temperature was less than 4 ℃, which may be due to the latter being better to maintaining the activity of components. In addition, at 25 ℃, the freeze-dried components were more likely to be contaminated, in which bacteria might degrade the activity of the extract. The water loss rate in the FD-CF system reaches 97 %, which reduced the storage capacity. Above all, the FD-CF system could be stored at high density, simplifying transportation conditions and avoiding storage at −80 °C, and most importantly, simplifying the reaction process. It made it possible for on-site treatment and on-site rapid biosensor detection.

In order to better prove that our FD-CF system can become a portable therapeutic protein expression platform, the S1 region and the receptor-

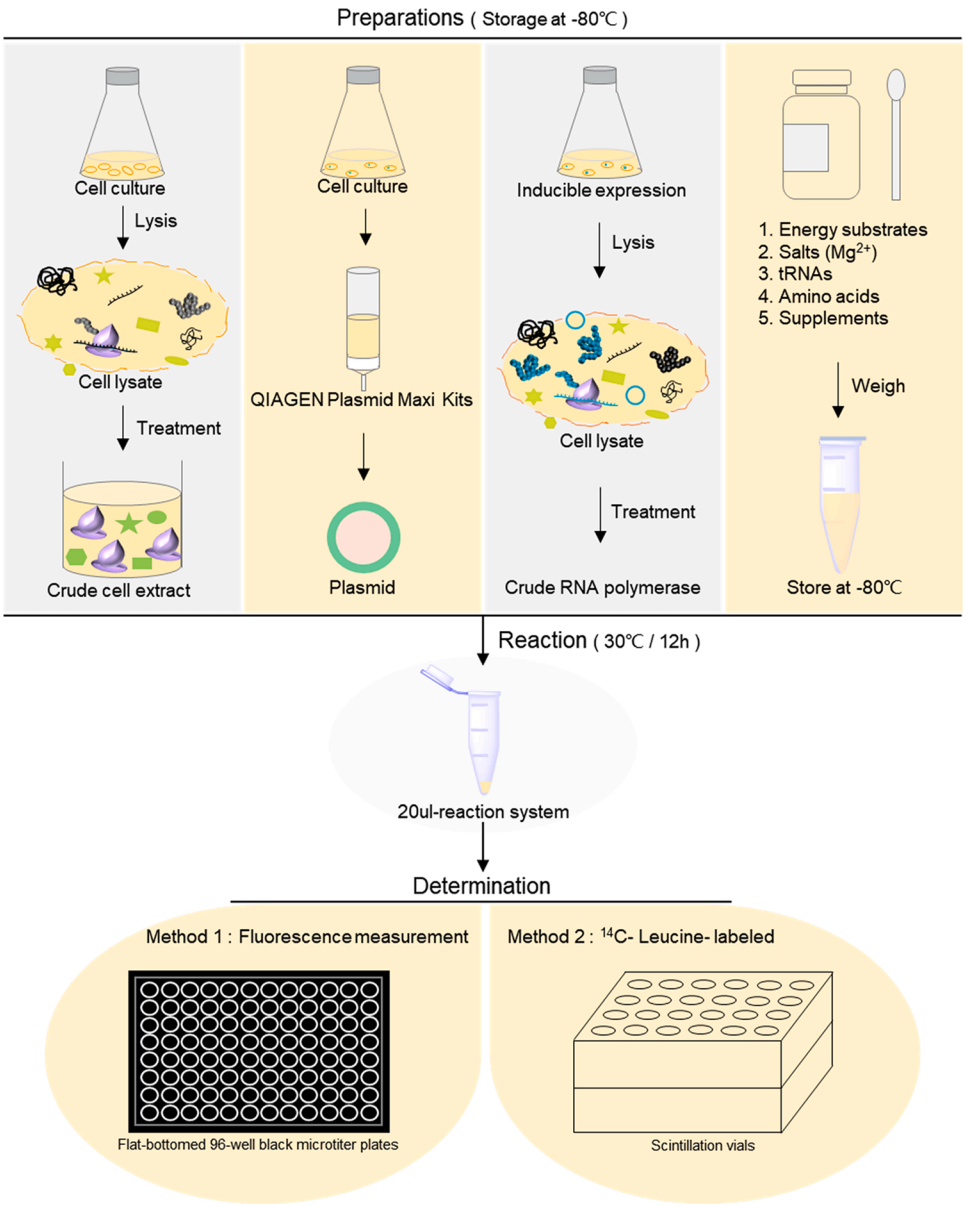
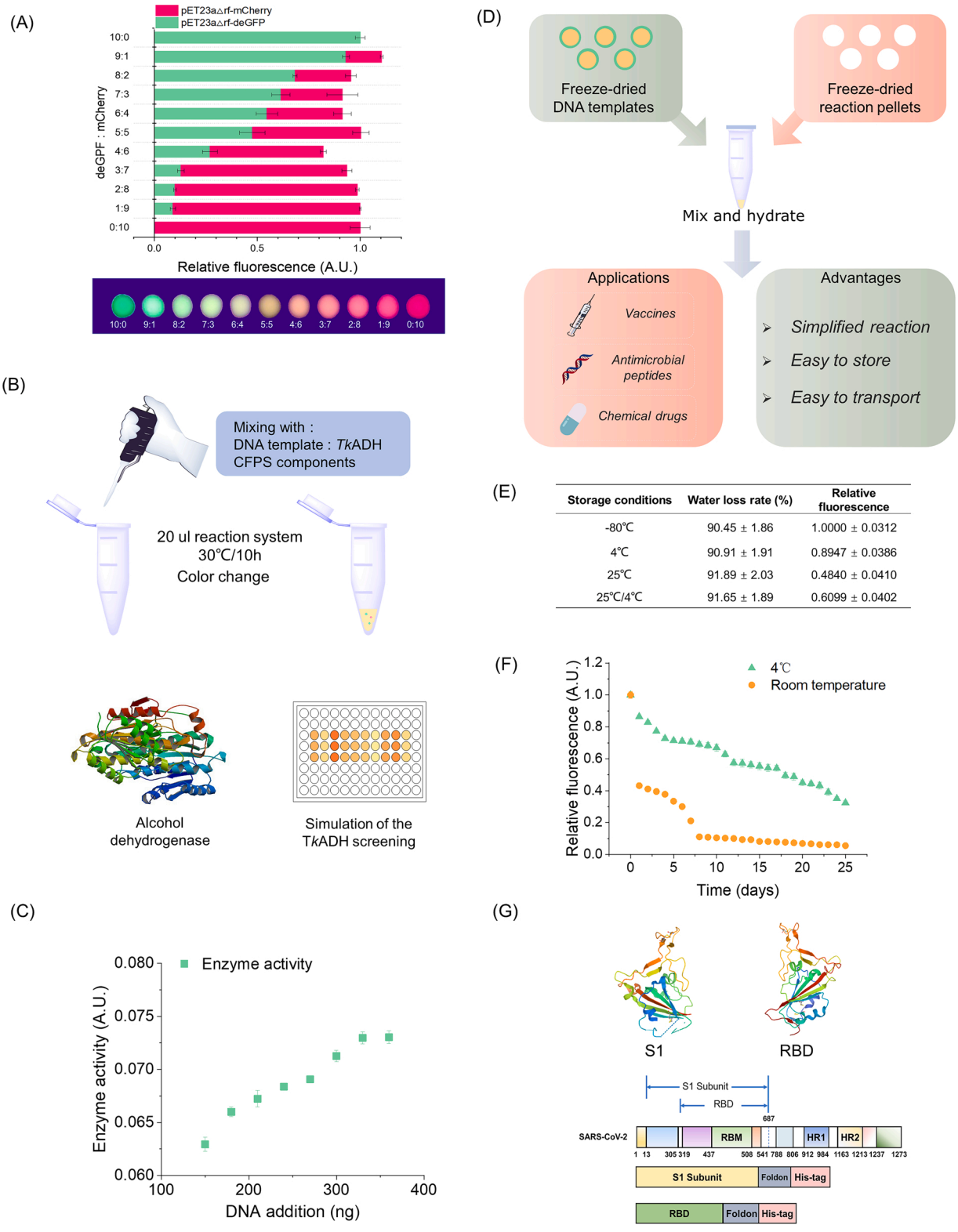


Fig. 4. The optimization of the CFPS system, including crude extract preparation, plasmid sizes, and energy substrates. The deGFP was expressed as the model protein.

binding domain (RBD) of the SARS-CoV-2 surface spike (S) protein were expressed with the system. S protein is a structural protein of SARS-CoV-2, which plays the most important role in the virus attachment, fusion and entry. It is the target of antibodies, entry inhibitors and vaccine development [44,45]. The S1 subunit of the S protein contains the RBD region, responsible for recognizing and binding to cell receptors. The studies on the synthesis of S1 protein or RBD protein could help the development of SARS-CoV-2 therapeutics [46]. Therefore, we selected the S1 subunit and the RBD region in the S protein as the target protein to be expressed. The optimized freeze-dried CFPS system was used to express both of them. As shown in the results, S1 protein and RBD could be successfully expressed (Fig. S6). The protein yield of S1 protein and RBD protein could reach about 21.7 μg/mL and 108.3 μg/mL, respectively. The results showed that our FD-CF system could quickly synthesize therapeutic proteins with only the addition of expression templates and water, which might solve significant practical limitations in the production and distribution of therapeutics and molecular tools, both to the developed and developing world.

5. Conclusion

CFPS has overcome the limitations of traditional cell-based synthesis and has become an emerging platform for *in vitro* protein synthesis due to its advantages of not limited by cell growth and allowing various formats. To improve the efficiency of CFPS, the cell-free system was optimized in this study. By comparing five *E. coli* extracts, the BL21 Star (DE3) extract was more suitable for the expression of the model proteins (deGFP) due to its genetic features. Since the smaller the plasmid size, the less the burden on CFPS, the smaller pET23a△rf was selected as the expression vector. Besides, the effects of metal ions on the CFPS were explored. When the metal ion was Mg^{2+}, and the concentration was 15 mM, the protein expression efficiency was the highest. The crowding effect was also explored, and it was found that using additives to simulate the crowded environment could not greatly improve the CFPS efficiency. After the system optimization, the efficiency of the CFPS has been significantly improved. On this basis, CFPS has been expanded at different application levels, and a platform for basic scientific research, preliminary screening, and portable biomolecule synthesis have been



Storage conditions	Water loss rate (%)	Relative fluorescence
-80℃	90.45 ± 1.86	1.0000 ± 0.0312
4℃	90.91 ± 1.91	0.8947 ± 0.0386
25℃	91.89 ± 2.03	0.4840 ± 0.0410
25℃/4℃	91.65 ± 1.89	0.6099 ± 0.0402

Fig. 5. The CFPS system as a platform for basic scientific research, preliminary screening, and portable biomolecule synthesis. (A) The coexpression of two model proteins. Green is deGFP expression, and red is mCherry expression. The visual color changes showed the expression of deGFP and mCherry with different plasmid proportions from 10:0 to 0:10. (B) A schematic diagram showing the simulation of the thermostable alcohol dehydrogenase TkADH screening. (C) The CFPS of the enzyme TkADH and its activity detection. (D) A schematic diagram showing the freeze-dried, cell-free (FD-CF) system. (E) The water loss rate of the FD-CF system with different storage conditions and the corresponding protein expression ability. 25℃/4℃ represents repeated freeze-thaw twice between 25℃ and 4℃. When the relative fluorescence value = 1.0, the concentration of deGFP =0.16 mg/mL. (F) The protein expression ability of the FD-CF system after stored for different days at 4℃ or room temperature. When the relative fluorescence value = 1.0, the concentration of deGFP =0.04 mg/mL. (G) Protein structure diagrams and sequence design diagrams of S1 protein and RBD protein (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

established. All these features promote that the CFPS can have broader prospects in the fields of basic science, drug screening, and on-demand biomanufacturing.

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CRediT authorship contribution statement

Nan Jiang: Methodology, Validation, Formal analysis, Data curation, Visualization, Writing - original draft. **Xuanwei Ding:** Methodology, Validation, Formal analysis, Data curation, Visualization, Writing - original draft. **Yuan Lu:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2020.107830.

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