Protein Synthesis Directly from PCR: Progress and Applications of Cell-Free Protein Synthesis with Linear DNA

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Abbreviations: CFPS, cell-free protein synthesis; GPCR, G-protein coupled receptor; HSQC, heteronuclear single quantum coherence spectroscopy; LET, linear DNA expression template; NMR, nuclear magnetic resonance spectroscopy; TIR, translation initiation region

Abstract

A rapid, versatile method of protein expression and screening can greatly facilitate the future development of therapeutic biologics, proteomic drug targets and biocatalysts. An attractive candidate is cell-free protein synthesis (CFPS), a cell-lysate-based *in vitro* expression system, which can utilize linear DNA as expression templates, bypassing time-consuming cloning steps of plasmid-based methods. Traditionally, such linear DNA expression templates (LET) have been vulnerable to degradation by nucleases present in the cell lysate, leading to lower yields. This challenge has been significantly addressed in the recent past, propelling LET-based CFPS as a useful tool for studying, screening and engineering proteins in a high-throughput manner. Currently, LET-based CFPS has promise in fields such as functional proteomics, protein microarrays, and the optimization of complex biological systems.

Introduction

Deeper scientific insight and innovative bioengineering, buoyed by growing genomic data, has enriched and widened the application space for proteins. Protein-based biologics have largely driven the recent growth in the biopharmaceutical industry [1, 2], and represent the majority of recent top ten grossing drugs [3]. Yet the potential of proteins is far from being fully realized. Our understanding of protein behavior lags behind an abundance of sequence information, encumbering the development of new therapeutic targets and agents throughout, from discovery to production. This is in great part due to the sheer complexity of proteins, whose activity can change widely with post-translational modifications and environmental factors, including interactions with other proteins. Consequently, a protein's physiological as well as pharmacological role is often multiplex. Correspondingly, a protein's optimal condition for expression and correct folding is also diverse and complex. Therefore, there is an acute need to quickly screen protein libraries across multiple variables, using a rapid, parallel, robust protein expression platform.

Cell-Free Protein Synthesis

Cell-free protein synthesis (CFPS) represents an ideal staging ground for a high-throughput protein expression. CFPS exploits cellular gene-expression machinery outside of the cell, as it is prepared either from a purified cell lysate, where cellular debris has been removed via centrifugation, or from a rational combination of individually purified components. Then, DNA, energy generating agents, supporting small molecules and cofactors are added to this system to form an open and flexible protein synthesis reaction.

The lack of cell imbues CFPS with several distinct advantages over *in vivo* methods: CFPS systems can (1) channel all available resources towards expressing the gene of interest, (2) bypass cell culturing steps of *in vivo* production, accelerating production, (3) function with either circular or linear DNA, such as PCR products, (4) tolerate cytotoxic or water-insoluble elements, (5) flexibly accommodate various supplemental components for optimal expression, and (6) permit lyophilization for facile storage and portability [4-7]. These characteristics have attracted much attention to CFPS as a powerful and versatile protein expression platform, as described in several articulate reviews [4, 8-18].

These flexible properties enable CFPS to express a wide variety of protein-based products, including antibodies [17, 19, 20], biocatalysts [21], microarrays [22, 23], virus-like particles [24-26], cytotoxic oncotherapeutics [6], growth factors [27], and fusion proteins [28]. CFPS has been adapted to work in batch, continuous exchange, and flow configurations. Notably, CFPS has been implemented towards high-throughput applications, including microfluidics- [29], 96 wellplate- and femtoliter-sized formats [30].

Linear-DNA-based Cell-Free Protein Synthesis

A unique advantage of CFPS over *in vivo* systems is its ability to directly utilize linear DNA templates (LET), such as PCR products. While traditional plasmid template preparation takes days, linear templates can be produced via PCR in hours, significantly simplifying the workflow of LET-based CFPS compared to other methods (Figure 1). This advantage has been less exploited due to linear DNA's increased vulnerability to native nucleases remaining in the cell-free environment, which results in a shorter template lifespan and lower reaction yields. However, steady technological advances in the past decade have started to mitigate these difficulties, as discussed below. This has advanced LET-based CFPS toward becoming an effective tool for high-throughput production and screening of proteins.

A library of LETs can be quickly produced in parallel from complex templates such as genomic DNA using gene-specific primers [31, 32]. Such primers often have overhanging sequences that contain expression regulatory elements necessary for transcription and translation. These primers are also easily customizable with protein purification tags [33] and stability- or expression-enhancing sequences [34, 35]. A DNA mutant library can be created by random or site-specific mutation [36-38] via PCR, dramatically reducing template preparation time for applications requiring multiple consecutive cycles of protein production and screening.

Strategies for Yield Increase in LET-based CFPS

Despite the shorter template preparation time, LET-based CFPS often suffers from lower product yield because linear DNA are more susceptible to native nucleases. For example, in *Escherichia coli* systems exonuclease V or RecBCD DNase complex, a product of the *recBCD* gene, has been shown to degrade linear DNA in a cell-free environment [39, 40]. The past decade has seen significant growth in yields of LET-driven CFPS, propelling it as a powerful tool for high-throughput production and screening of protein products (Figure 2). For example, over the course of a decade the yield of antibody fragments in

1 LET-based CFPS has increased more than 100-fold, approaching 10^3 $\mu\text{g/ml}$ yields [38, 41]. Such
2 increased yields have enabled more complicated downstream assays and processes, broadening the
3 application space for LET-based CFPS.

4 The clear trend of yield increase is partially due to a spectacular growth of cell-free systems in general,
5 fueled by a better understanding of inner metabolic networks [42, 43], methodological optimizations of
6 reaction conditions [44-46] and extract preparation methods [38, 47]. Comparable efforts to specifically
7 improve LET-based CFPS to inhibit nuclease attack against LET have also enjoyed success. Such efforts
8 have been often disconnected by disparate fields and motivations, which have made their evaluation and
9 comparison difficult. The following summarizes these efforts.

11 Genomic Alteration

12 In the case of *E. coli*-based cell-free systems, efforts have been made to target the RecBCD complex by
13 deleting the genes for its individual subunits. Unfortunately, because native exonucleases often play a
14 critical role in intracellular recombination processes, cell growth is retarded. For example, while a $\Delta recB$
15 strain lacks the dominant nuclease RecB, its growth is severely retarded [48], thus limiting the strain's
16 CFPS potential. In contrast, a $\Delta recD$ mutation did not affect growth, but the strain did not significantly
17 improve LET survivability either [49]. To overcome this problem, the recBCD operon was replaced with
18 the bacteriophage λ *red* recombination system, for which an improved *in vivo* recombination activity with
19 linear DNA has already been demonstrated [50]. The resulting extract did not suffer from slow growth
20 and improved protein expression seven-fold to near plasmid levels [49].

21 In one interesting study, nuclease components RecD and PNPase were modified to have a streptavidin-
22 binding sequence without affecting cell growth. As part of extract preparation, these nucleases were
23 selectively removed via specific affinity to streptavidin. The study reports plasmid-level yield [51], but
24 this process does increase the time and labor cost of extract preparation.

Inhibition of Nucleases

As an alternative to genomic engineering, molecular inhibitors can be used to target native exonucleases. These inhibitors' activities need to be specific enough to target nuclease but not inhibit other molecules necessary for CFPS. For example, the Gam protein from bacteriophage λ has been identified as an effective inhibitor of RecBCD nuclease. A truncated version of this protein, GamS, has been employed to successfully inhibit nuclease activity in CFPS and achieve close to plasmid-level yields [38, 52, 53]. The GamS protein can be stored with DMSO as a cryoprotectant which is non-toxic to CFPS activity [53]. Small molecule inhibitors have also been used to target RecBCD in CFPS with moderate success [54].

One simple and straightforward solution is a lower temperature, which has been explored in general plasmid-based CFPS with success, especially in context where “nonstandard components” are added [55, 56]. Specifically for LET-based systems, nuclease activity is decreased at lower temperatures, resulting in increased yields [57].

Alteration of Template Geometry

Lastly, template preparation can include steps to improve survivability in LET-CFPS. For example, T7 terminator sequence, poly(G) sequence, mini-hairpin sequences and other noncoding sequences have been explored to improve yield [35, 53]. One creative study addressed the solubility limit of LETs and their vulnerability to nuclease degradation simultaneously by crosslinking LETs into a hydrogel. This hydrogel sustained a CFPS reaction that produced up to 5 mg/mL of functional protein [58].

Future Directions

The last decade has seen exciting improvements to yields of LET-based CFPS. Further improvements can be accelerated by methodologically reproducing, synergizing and innovating on past advances. A standardized reporting method and protein (such as Green Fluorescent Protein) would help facilitate systematic comparison and improvement.

While the majority of LET-based CFPS studies have been largely focused on the *E. coli* system, other reports have shown that wheat germ [59, 60] and yeast [34] cell-free systems may produce higher yields from linear DNA. These initial results encourage further research into the utility and behavior of LET in eukaryotic cell-free systems, which better accommodate certain types of complex protein expression, assembly, and post-translational modifications not found in prokaryotes. The wheat-germ system, in particular, is a popular eukaryotic CFPS platform that is commercially available, highly productive, and relatively robust. Despite its time-consuming extract preparation process, it has been widely used for vaccine development [61], structural genomics, proteomics and other studies [62, 63]. The yeast system enjoys a relatively low cost and high stability. These and other CFPS systems – such as rabbit reticulocyte, insect cells and human systems – remain potentially fruitful frontiers for LET-based expression.

Another relevant alternative CFPS system is the PURE system, which is constructed from individually purified components essential for protein synthesis [64]. This means that the PURE system has reduced concentrations of residual nucleases, making it an ideal platform for LET-based CFPS. This advantage was recently demonstrated by the production of functional membrane proteins SecYEG translocon and F₀F₁-ATP synthetase in PURE CFPS using LETs and supplemental liposomes [65]. The process of LET preparation, protein expression and activity screening was accomplished in one day, thus displaying fitness for high-throughput applications.

Applications

Current scientific and engineering endeavors tackle complex and difficult tasks such as drug development, biocatalyst discovery and synthetic gene circuit design. Such efforts are guided by “bounded rationality”, relying on both scientific insight and trial-and-error testing. Both approaches depend upon the ability to make and evaluate protein in a simple and timely fashion. This renders LET-based CFPS as an appealing tool for high-throughput screening applications. It has already begun to prove its usefulness in various arenas, as demonstrated by the following examples.

Cloning-Independent Functional Genomics

Recent advances in sequencing technology have created a wealth of genomic information which wait to be translated to deeper insights into protein functions and protein-protein interactions. In order to help keep pace with the swell of sequence data, high-throughput protein synthesis methods must analyze complex protein behaviors in tandem with computational tools. By doing this, CFPS would accelerate the discovery of novel therapeutics, drug targets and industrially interesting enzymes. Traditionally, such functional genomics studies have required the subcloning of cDNA into expression plasmid vectors, a process which can be tedious and time-intensive. This process can be streamlined by amplifying genes from genomic DNA or mRNA, and directly introducing them into *E. coli* CFPS. A pioneering study utilized this method to express 52 gene targets in parallel in CFPS, supplemented with a cocktail of cofactors and metal ions to yield adequate amounts of products for calorimetric assays without purification [31]. The supplemental cocktail often increased the activity of enzymes at the expense of their solubility, suggesting the need for further optimizations. Overall, the method bypassed time-consuming tasks such as cloning and protein purification, paving the way for rapid, high-throughput protein screening.

1 Since then, LET-based CFPS has been used in a variety of different functional genomics studies. In
2 particular, LET-based wheat-germ CFPS has been explored as a promising tool for malarial vaccine
3 development [61, 66]. Malaria, caused by the protozoans of the *Plasmodium* genus which are transmitted
4 by mosquito vectors, has seen a recent resurgence due to rising multidrug- and insecticide-resistance. A
5 malarial vaccine would dramatically augment current strategies to combat the disease, which rely mainly
6 on drugs and insecticides. However, vaccine discovery is hampered by a complex life cycle, high
7 antigenic diversity, immune evasion and difficult functional expression of antigens *in vivo*, necessitating
8 an expression method which can rapidly produce large varieties of eukaryotic proteins. The wheat-germ
9 CFPS system can fulfill this role compellingly, as it can be fashioned to quickly produce small amounts
10 of proteins from an LET-based cDNA library created via PCR techniques [61, 67]. The resulting products
11 could be functionally assessed or immunoscreened to identify promising candidates for subsequent
12 screenings. LET-based wheat-germ CFPS was able to express 478 of 567 genes from the *P. falciparum*
13 genome, demonstrating its potential for malarial vaccine discovery [60]. This method, along with other
14 CFPS systems, was employed to express 46 malarial antigens for a longitudinal cohort study which
15 provided critical insights into natural human immunization against malaria, and identified new vaccine
16 and biomarker candidates [54].

17 In another study, LET-based CFPS was used to engineer a bacterial “endoplasmic reticulum” prototype
18 by identifying chaperones effective for immunoglobulin folding in prokaryote systems. A bacterial
19 “endoplasmic reticulum” could enable the rapid antibody production in prokaryotic systems while
20 maintaining the eukaryotic ability to perform complex folding and post-translational modifications.
21 Towards this goal, LET-based CFPS was used to express and screen 20 different candidate chaperones.
22 The study was able to identify two chaperones which improved trastuzumab folding and assembly 10 to
23 100 fold, and shed light on protein-chaperone interactions for immunoglobulins [68]. Future studies could
24 continue to identify enzymes needed to mimic natural folding and modification pathways during
25 industrial antibody production.

The LET-based CFPS method could also be applied towards investigating the functions of unknown proteins for applications in biocatalyst discovery and development. Recent advances in computational tools, such as sequence searching of metagenomes and databases, can accelerate transaminase discovery, but need to be accompanied by adaptable methods of experimental confirmation. A proof-of-concept study used LET-based CFPS to identify new transaminases, which are used to produce chiral amines, an important type of intermediate compound in many pharmaceuticals syntheses [69, 70]. In the experiment, 9 putative transaminases were expressed in LET-based CFPS along with 2 known transaminases as controls, and screened against 16 potential substrates in a colorimetry assay [71]. Notably, PCR products were amplified directly from bacterial colonies, shortening the entire workflow into hours. In the future, LET-based CFPS could be developed into a powerful experimental companion to computational approaches, especially if it can be utilized to screen more detailed parameters, such as biocatalyst substrate specificity, enantioselectivity and reaction equilibrium.

Protein Microarrays

Protein microarrays are a powerful assaying method that can simultaneously screen many proteins' activity and interactions, with potential impacts in functional proteomics studies, medical diagnostics, biomarker discovery [72] and personalized medicine [73]. Despite this, protein microarrays have yet to replicate the success of DNA microarrays due to several persisting technical challenges – proteins are often time-consuming to express, purify and capture onto surfaces, and they can be difficult to store long-term without degradation. LET-based CFPS offers an attractive solution to these problems, as it enables *in situ* protein synthesis and direct surface attachment in a streamlined and just-in-time manner, eliminating the need for protein storage[22].

Several methods exist for creating protein microarrays using LET-based CFPS. In the protein *in situ* array (PISA) method, LETs are supplied in solution with the CFPS extract, which is applied directly onto the

1 chip, either by droplet or microfluidics [74]. The protein products are expressed *in situ* and in parallel,
2 then captured by preexisting purification tags. The PISA method has been demonstrated notably with
3 antibody fragments, and in miniaturized formats (40 nL) [22, 33, 75].

4 In the DNA array to protein array (DAPA) method, protein microarrays are “printed” from DNA arrays
5 [76, 77]. In this method, a DNA array chip is placed face-to-face onto a Ni-NTA-coated chip. CFPS
6 extract is applied at the interface between the two chips, where polyhistadine-tagged protein products are
7 captured on the Ni-NTA surface, forming *in situ* a protein array with well-defined and reproducible spot
8 profiles. The DNA array is then removed and can be reused for subsequent protein microarray production.

9 Protein microarrays produced by LET-based CFPS have already been utilized in enzyme screening [78],
10 protein-protein interaction studies [79-81], and biosensors [74]. The unique synergy of LET-based CFPS
11 and protein microarrays has become a highly active area of study, with numerous reviews dedicated to the
12 subject [23, 82-84].

14 **Difficult-to-Express Proteins**

15 Not bound by the limits of the cell, LET-based CFPS can express proteins which are difficult to produce
16 *in vivo* – many of which are therapeutically relevant. For example, onconase, a toxic ribonuclease derived
17 from the oocytes of the Northern Leopard Frog, is a potential cancer therapeutic [85-87]. However,
18 oocyte culture and harvest procedures, as well as recombinant recombinant expression methods, involve
19 extremely labor-intensive purification or refolding steps. Recently, active and soluble onconase was
20 successfully expressed in CFPS at 1.8 mg/mL by continuously supplementing the reaction with tRNAs at
21 optimized intervals to counter the ribonuclease activity of nascent onconase products [6]. This illustrates
22 that LET-based CFPS can be highly customized to efficiently produce proteins that are otherwise difficult
23 to express.

1 As another example, membrane proteins, including successful drug targets such as G-protein coupled
2 receptors (GPCR), are notoriously difficult to express *in vivo* at high titers because of their high
3 hydrophobicity. Cell-free systems can tolerate cytotoxic expression levels of membrane proteins, all the
4 while being open to the addition of detergents or nanodiscs to stabilize nascent membrane proteins [88],
5 and have demonstrated efficient expression of membrane proteins [16, 89], including GPCR [88, 90].
6 Near plasmid-level yields of GPCR have been reported using LET in both crude extract and PURE
7 systems [65, 91]. This enables LET-based CFPS to be an effective platform for expressing and studying
8 membrane proteins.

9 For example, LET-based CFPS can address problems in NMR studies of membrane proteins, which are
10 often challenging due to broad resonance lines that lead to congested spectra where residue assignment is
11 difficult. Various site- and residue-specific labeling techniques are simple but powerful ways to overcome
12 this problem. CFPS systems allow for rapid expression of the isotopically labeled proteins for direct
13 spectroscopic analysis without chromatographic purification [92, 93]. In addition, CFPS systems' lack of
14 metabolic conversion between amino acids enables amino acid-selective isotope labeling of proteins [94].
15 The small reaction volume of cell-free systems means that expensive isotope-labeled amino acids are used
16 sparingly [95]. Most important, single-site amino acid labeling requires rapid, parallel protein expression,
17 which lends itself naturally to LET-driven CFPS [91, 96, 97]. In one notable study, an NMR spectrum of
18 dengue virus protease complex was obtained in less than 24 hours – which included PCR production of
19 LETs, 7-hour CFPS, and an overnight dialysis [97]. The protein complex of choice would have been
20 difficult to produce *in vivo* due to its high rate of degradation, but was readily obtained using CFPS,
21 highlighting the robustness of cell-free systems. Sixteen valine residues were systematically targeted via
22 site-specific, parallel mutation into $^{15}\text{N}/^{13}\text{C}$ -labeled isoleucine. The accelerated production of these
23 labeled variants was made possible by the LET-based CFPS platforms in the experiment. As only
24 isoleucine was isotope-labeled, the missing peaks from the HSQC spectrum of a mutant protein led to
25 residue identification in the wild-type protein.

Only a small number of GPCR are currently targeted by marketed drugs, while a significant number of all human GPCR elucidated by the Human Genome Project are “orphaned”. These facts suggest that this field represents a fertile application space for LET-CFPS in the future.

Optimizing Complex Systems

LET-based CFPS enables rapid iterations of screenings to optimize complex engineered biological systems across multiple variables. For example, protein-based biologics have complicated and diverse physicochemical properties and stability characteristics that need to be accommodated during production. In particular, soluble antibody expression and assembly depends on successful disulfide bond formation and the proper interaction between chaperones, light chains and heavy chains at favorable ratios. Therefore, *in vitro* production of antibodies needs to optimize (1) the redox conditions for disulfide bond formation, and (2) the relative expression levels of light and heavy chains [98-100]. The relative expression levels of the two chains can be coordinated by fine-tuning the interaction between the ribosomal S1 protein and 5' UTR RNA via sequence mutations in the translation initiation region (TIR). This interaction is not yet well understood, necessitating combinatorial screening of a library of TIR with single point mutations. LET-based CFPS was employed to screen a library of LETs with varying TIR, and optimize the antibody fragment production across multiple glutathione buffers [38]. Highly functional antibody fragments such as single-chain variable fragments have been synthesized in LET-based CFPS [19, 101]. Recently, the synthesis of a fully assembled, full-length antibody, trastuzumab has also been realized in CFPS [38], suggesting that the future horizon of LET-based CFPS could extend to the rapid synthesis and screening of whole antibodies.

Another application for LET-based CFPS is synthetic biological circuits, where standardized regulatory elements are assembled in modular fashion to give rise to logic gate-like behavior. Currently, synthetic biological circuits are often prototyped through time-consuming reiterations between *in silico* design and revision and *in vivo* assembly and testing. Consequently, the design and assembly of sophisticated large-scale circuits can demand an enormous number of man-hours, necessitating a more rapid expression

1 system for shorter testing cycles. LET-based CFPS is an ideal candidate for this role, as it allows for
2 circuit assembly and testing in a well-characterized, sufficient approximation of the *in vivo* environment
3 of *E. coli*, while bypassing time-intensive cloning and propagation steps. A proof-of-concept study
4 demonstrated this potential by comparing genetic switch behavior across LET and plasmid-based circuits
5 using 12 commonly used promoters [53]. The study found that LET- and plasmid-based circuits were
6 comparable to *in vivo* at most concentrations. Future studies could carry out comparable experiments with
7 more complex circuits – such as oscillators, logic gates, translational regulators and chaotic systems,
8 which already have been constructed in CFPS – and thus be able to program more complex and intricate
9 computing behavior [102-104].

11 Conclusions

12 Proteins carry significant scientific and practical interest as potential therapeutic biologics, drug targets
13 and biocatalysts. Due to the complex nature of proteins, there is a growing need to make and evaluate
14 proteins in a simple and timely fashion. LET-based CFPS provides an open and versatile platform for
15 rapid and parallel protein synthesis. LET-based CFPS has been applied in functional and structural
16 studies, *in situ* protein microarray production, high-throughput screening and pre-production bioprocess
17 optimization. These achievements strongly suggest that the unique advantages of LET-based CFPS will
18 play a spearheading role in novel biologics discovery, future personalized medicine, and other protein-
19 related fields.

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Figure Legends

Figure 1: LET-based CFPS simplifies and accelerates protein synthesis workflows for high-throughput screening applications (A). A major technical challenge of LET-based CFPS is the degradation of LET by native nucleases (B). Significant strides have been made to overcome this challenge. The components of LET-based CFPS can be quickly assembled and customized as a versatile tool for many application fields (C).

Figure 2: Growth in yields of LET-based CFPS has been achieved thanks to technological innovations, as illustrated by a survey of over 30 reported experiments [19, 20, 22, 31, 35, 38, 41, 49, 51, 52, 54, 57, 71, 91, 97, 101, 105, 106]. Green Fluorescent Protein and antibody fragments are highlighted in green and red, respectively.

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Figure 1 -

Figure 2

Figure - Graphical Abstract

Linear-DNA-based Cell Free System