Part IV

Early Applications of Synthetic Biology: Pathways, Therapies, and Cell-Free Synthesis

15

Cell-Free Protein Synthesis: An Emerging Technology for Understanding, Harnessing, and Expanding the Capabilities of Biological Systems

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15.1 Introduction

Cell-free protein synthesis (CFPS) systems have transformed our ability to understand, harness, and expand the capabilities of biological systems. In the groundbreaking experiments of Nirenberg and Matthaei in 1961, CFPS played an essential role in the discovery of the genetic code [1]. More recently, a technical renaissance has revitalized CFPS systems to help meet increasing demands for simple and efficient protein synthesis. Moving forward, this renaissance is enabling new processes never seen in nature, such as noncanonical amino acid (ncAA) incorporation and man-made genetic circuits.

The driving force behind this development has been the unprecedented freedom of design to modify and control biological systems that is unattainable with *in vivo* approaches [2–6]. The ability to "open the hood" of the cell and treat biology as a set of chemical reactions leads to many advantages for using cell-free systems, highlighted in Figure 15.1. First, the open reaction environment allows the user to directly influence the biochemical systems of interest (e.g., protein synthesis, metabolism, etc.). As a result, new components (natural and nonnatural) can be added or synthesized and can be maintained at precise concentrations, while the chemical environment is monitored and sampled. Second, since the reaction is not "living," cellular objectives, such as growth, can be bypassed. As is desirable in chemical transformations, cell-free systems separate catalyst synthesis (cell growth) from catalyst utilization (protein production), circumventing a major challenge afflicting *in vivo* engineering efforts. This is featured in Figure 15.2. Without living cells, timelines for process and product development can be faster and scale-up can be easier [4]. Although the CFPS technology offers many exciting advantages, challenges remain that provide

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CFPS gives an unprecedented freedom of design to modify and control biology

Open reaction environment

Control added components precisely

Monitor and sample reaction environment

Bypass cellular objectives

Separate catalyst synthesis from catalyst utilization

Direct resources toward the exclusive production of one product

Accelerate timelines from DNA to protein

Supplement and produce toxic molecules

Scale linearly from μl to 100 l (expansion factor of 10^6)

Figure 15.1 Advantages for cell-free biology. By bypassing cellular objectives and opening the reaction environment, cell-free protein synthesis allows for increased freedom of design as a result of the benefits highlighted here.

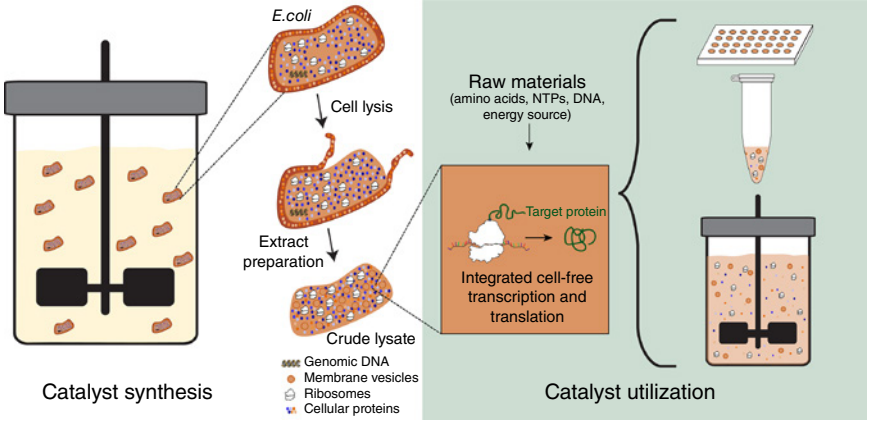

Figure 15.2 A new paradigm for cell-free biomanufacturing. Cell-free protein synthesis is able to separate catalyst synthesis (cell growth) from catalyst utilization (protein synthesis). This allows resources to be funneled toward the product of interest in ways not possible *in vivo*.

opportunity for improvement. For example, many emerging cell-free platforms are not yet commercially available, and thus their broad impact is limited. In addition, cell lysis procedures can be difficult to standardize, leading to different extract performance across labs. Further, complex posttranslational modifications (PTMs) (e.g., human glycosylation) are still limited or not yet shown. Finally, CFPS costs exceed *in vivo* methods for comparable organisms, which limit the scale for most academic labs. Despite these challenges, the benefits of CFPS are inspiring new applications from the synthesis of pharmaceutical proteins to the understanding of synthetic gene circuits [7].

This review highlights achievements of the existing systems for crude extract-based protein synthesis. We begin with an overview of the state-of-the-art systems from different organisms. Then, we discuss their capabilities for protein production, highlighting applications that greatly benefit from the open environment and lack of cell viability of CFPS. Finally, we describe benefits for high-throughput applications and offer some commentary about the future growth of the field.

15.2 Background/Current Status

Crude extract-based CFPS harnesses the cell's native translational machinery to produce proteins in a process that, instead of occurring in a live cell, becomes more like a chemical reaction. The crude extract contains the translational machinery, which consists of ribosomes, aminoacyl-tRNA synthetases, initiation factors, elongation factors, chaperones, and so on. In addition to the translational machinery, other enzymes exist in the extract: some are beneficial (e.g., those for recycling nucleotides or energy metabolism) and some are detrimental (e.g., those using CFPS substrates nonproductively). In combined transcription–translation reactions, the crude extract is added to a solution containing buffer, amino acids, nucleotides, RNA polymerase, a secondary energy source (for regenerating adenosine triphosphate (ATP)), salts, and other molecules for maintaining the environment (e.g., dithiothreitol for a reducing environment or spermidine and putrescine for mimicking the cytoplasm). Thus far, when compared with the use of purified enzyme translation systems, such as the PURE system developed by Ueda and colleagues [8], as well as New England Biolabs [9, 10], crude cell lysates offer significantly lower system catalyst costs and much greater system capabilities (e.g., cofactor regeneration, proteins produced per ribosome, and long-lived biocatalytic activity) [2, 11]. The primary crude extract-based platforms and trends will be discussed.

15.2.1 Platforms

15.2.1.1 Prokaryotic Platforms

E. coli Extract The well-established *E. coli* system provides high protein yields (up to $2.3\,g\,l^{-1}$) [12], as can be seen in Figure 15.3. The system has benefitted from its highly active metabolic activity, as well as the low-cost and scalability of fermentable cells for extract preparation [11]. Notably, the dilute cell-free system has decreased translation elongation rates compared with *in vivo* (~10-fold lower), which improves the expression of mammalian proteins [2]. While perhaps unexpected, it should also be noted that this platform has even had success synthesizing some complex, and even disulfide-bonded proteins [18, 19]. Additionally, well-developed genetic tools to make modifications to the source strain have been critical for developing synthetic genomes that upon cell lysis lead to improved protein production capabilities by removing negative effectors [20]. So far, a limitation of this system is its inability to produce PTMs, such as glycosylation. While PTMs could be enabled through the site-specific introduction of ncAAs (see Section 15.4.1), for example, the inability to introduce PTMs has driven interest in developing eukaryotic platforms.

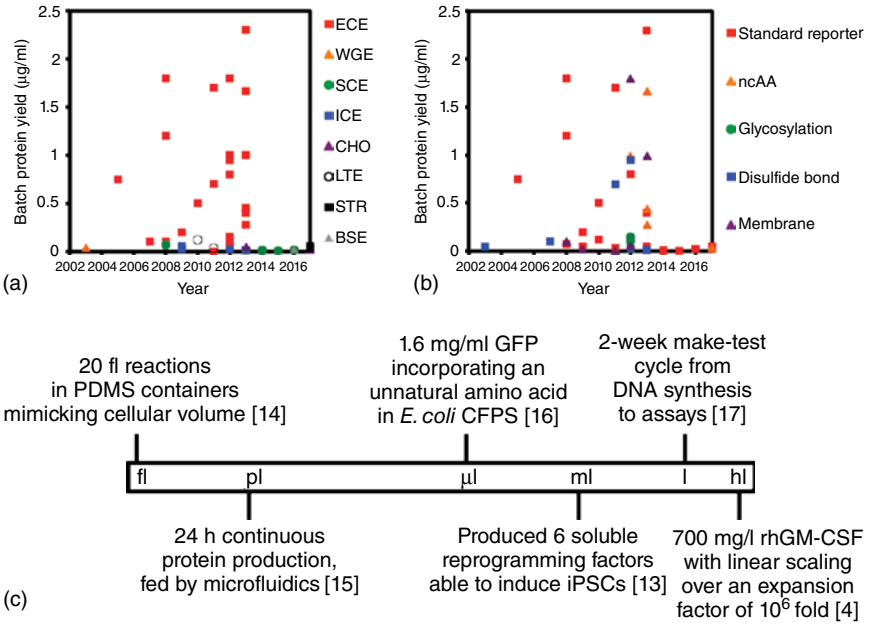

Figure 15.3 Historical trends for different CFPS systems. Batch protein yields for the papers cited in this review are arranged by platform (a) and product type (b). In addition, cell-free protein synthesis has seen successes at a variety of volumes (c). ECE, *E. coli* extract; WGE, wheat germ extract; SCE, *S. cerevisiae* extract; ICE, insect cell extract; CHO, Chinese hamster ovary cell extract; LTE, *L. tarentolae* extract; STR, *Streptomyces* extract; BSE, *B. subtilis* extract; PDMS, polydimethylsiloxane; GFP, green fluorescent protein; iPSCs, induced pluripotent stem cells; rhGM-CSF, recombinant human granulocyte macrophage colony-stimulating factor.

Other Prokaryotic Platforms More recently, alternative prokaryotic platforms have emerged. These platforms have been based on *Bacillus subtilis* [21] and several *Streptomyces* strains: *Streptomyces coelicolor* [22], *Streptomyces lividans* [22], and *Streptomyces venezuelae* [23]. However, the goals around these production systems are more specialized. The *B. subtilis* platform was intended for promoter prototyping and genetic circuits with the hope of translating this to *in vivo* protein expression for metabolic engineering. Alternatively, the *Streptomyces* platform was intended for expression of GC-rich proteins, particularly for expressing and studying natural product gene clusters.

15.2.1.2 Eukaryotic Platforms

In contrast to the *E. coli* CFPS platforms, eukaryotic systems often produce complex proteins with higher percentages of soluble yields. However, they are hampered by comparatively low overall yields (e.g., an order of magnitude in standard batch reactions for similar model proteins) and costly scale-up. While wheat germ extract (WGE) has been the historical eukaryotic system of choice, several promising platforms for industrial use are also now emerging, which include extracts from *Saccharomyces cerevisiae*, insect cells, Chinese hamster ovary (CHO) cells, and *Leishmania tarentolae*, all of which are fermentable, providing possibilities for simple scale-up.

Wheat Germ Extract The WGE system has been the most productive eukaryotic system thus far, producing over 13 000 human proteins in one study [24]. The WGE platform is able to achieve several endogenous PTMs. However, there are aspects of the platform that are not amenable to large-scale protein production. For example, batch yields are typically low (~1–10 $\mu g\,ml^{-1}$ luciferase) [25], the extract preparation is complex, and genetic modifications are challenging. That said, the semicontinuous format has been shown to produce 9.7 $g\,l^{-1}$ green fluorescent protein (GFP) [26]. This is remarkable, enabling the system to be a workhorse for crystallography, NMR, and structural biology studies.

Yeast Extract Pioneered by the work of Iizuka and colleagues, several methods have been used for producing extracts from the yeast *S. cerevisiae*, which is another enticing option for a eukaryotic platform [27]. Like *E. coli*, it is easily grown in a fermenter. Also, the entire genome has been sequenced, and there is a wealth of biological tools, allowing for possible modifications to be made to improve protein production, which was important in the development of the *E. coli* platform.

One method, developed by Wang and colleagues, starts by removing the outer membrane of the cell wall using lyticase, producing a protoplast. Then the protoplast is lysed with a 25-gauge needle. While this method is likely to maintain cellular compartments, the lyticase treatment is expensive on an industrial scale [28].

Other efforts have strived to be more viable as an economical and scalable system. These methods include the use of high-pressure homogenization for cell lysis, combined transcription/translation without need for mRNA capping [29], and a focus on technically simple extract preparation methods [25]. This new method was able to produce $7.69 \pm 0.53\,\mu g\,ml^{-1}$ active luciferase, giving it a fourfold improvement in relative product yield ($\mu g\ \$\ reagent\ cost^{-1}$) over the protoplast method. At this time, it is uncertain whether this approach retains cellular compartments after extract preparation, yet this is a very interesting question. Additionally, using a semicontinuous reaction format to feed limiting substrates (creatine phosphate, nucleotide triphosphates, and perhaps aspartic acid) while removing toxic by-products (inorganic phosphate) led to product yields of $17.0 \pm 3.8\,\mu g\,ml^{-1}$ [30]. Other recent work with the system has explored alternative energy sources [31], fermentation conditions [32], 5′ mRNA leader sequences [33], and gene knockouts [34]. Despite recent work in this system, yields need to be further improved. To do so, a better understanding of the metabolism of the lysate is necessary. Also, elimination of background, nonproductive translation would allow for more efficient use of reactants toward the protein of interest.

Insect Cell Extract Insect cell extract (ICE) systems are another promising platform for eukaryotic CFPS. This approach uses ovary cells of *Spodoptera frugiperda*, the fall armyworm, an industrial *in vivo* protein expression system [35]. Typical yields for the ICE system are ~45 $\mu g\,ml^{-1}$ luciferase [25]. Using mechanical lysis and mild treatment of the extract, a process developed by Kubick and colleagues is able to retain microsomal vesicles of the endoplasmic

reticulum (ER) within the extract [36]. These vesicles are important for trafficking proteins into the ER for membrane insertion and PTMs. The Kubick lab has exploited this by producing membrane proteins, which are able to co-translationally insert into the lipid-enclosed vesicles for stability, as well as glycosylated proteins, both of which will be described later [36, 37]. In addition to glycosylated and membrane proteins, the ICE system has also been demonstrated to incorporate ncAAs using a plasmid developed by the Schultz lab for use in *S. cerevisiae* [38].

Chinese Hamster Ovary Cell Extract CHO cells are widely used industrially for the expression of human recombinant proteins [38]. A benefit is their ability to achieve mammalian PTMs, which remains a challenge. Using the same extract preparation method as ICE, the Kubick lab has begun to develop a highly efficient and high-yielding CHO cell extract. To achieve glycosylation and produce membrane proteins, the reaction mixture can be enriched with microsomal vesicles, yielding 30–50 $\mu g\,ml^{-1}$ of the protein of interest (e.g., luciferase) [38, 39]. This platform offers exciting opportunities for developing advanced process development pipelines for discovering and assaying protein therapeutics, which can be directly translated *in vivo*.

Leishmania tarentolae *Extract* *L. tarentolae*, a lizard parasite, is a fermentable protozoan that was chosen for CFPS. The *in vivo* expression system is able to produce disulfide bonds and glycosylation, and the cells are easy to genetically modify [40, 41]. For extract preparation, a nitrogen cavitation method is used for lysis [42]. A key for the system is that the native mRNA all has the same "splice leader" sequence, allowing for inhibition of endogenous mRNA using an oligonucleotide [40]. This prevents background translation, allowing resources to be directed to synthesis of the protein of interest, producing 50 $\mu g\,ml^{-1}$ GFP. Using the *L. tarentolae* platform, Mureev and colleagues were able to develop species-independent translational sequences (SITS), which allowed for translation in not only *L. tarentolae* platform but also *E. coli* and several eukaryotic cell-free platforms, presumably by a cap-independent pathway [40]. It is expected that this system will aid in expressing proteins from parasitic genomes to test their functions and annotate parasitic genomes, including that of *L. tarentolae* [43].

15.2.2 Trends

Several trends can be observed in the development of the aforementioned cell-free platforms. First, the recent development of several eukaryotic CFPS platforms highlights the enthusiasm and growth of the field.

Second, yields continue to increase for CFPS, with a majority of products expressed in the *E. coli* platform as seen in Figure 15.3a, which catalogs the proteins expressed from manuscripts covered by this review. These improvements have occurred as a result of improved soluble yields for the *E. coli* platform and increased overall yields for the eukaryotic platforms. One method that has been useful in the *E. coli* system was the use of fusion partners to aid

aggregation-prone proteins [13, 44]. Also, moving from glucose to starch as an inexpensive energy source allowed for better pH maintenance, increasing soluble enhanced GFP from 10% to 25% in a study by Kim and colleagues [45], as well as by Caschera and Noireaux [12]. The manuscript by Caschera and Noireaux achieved the highest batch CFPS yield to date or 2.3 g l^{-1} superfolder GFP. The increased yields and decreased cost have enabled the use of freeze-dried lysates for solving cold chain problems with on-demand synthesis of proteins for therapeutics [46, 47] and diagnostics [48, 49]. In contrast to prokaryotic systems, eukaryotic systems generally produce a higher soluble portion but are working toward increasing overall yields cost effectively. So far, this has typically involved reducing background translation, although there are many exciting opportunities for strain engineering. A target goal in the upcoming years is to enable eukaryotic batch CFPS yields of greater than 0.5 mg ml^{-1}, which is chosen because it is about an order of magnitude higher than current levels.

Third, there is also an effort to reduce cost for CFPS. This has been done by moving toward lower cost energy sources, as well as streamlining the process. Instead of fueling the reactions with substrates containing high-energy phosphate bond donors, such as creatine phosphate or phosphoenolpyruvate, *E. coli* reactions have been shown to use glucose and starch as well as nucleoside monophosphates in lieu of triphosphates, greatly reducing cost [12, 45, 50]. So far, eukaryotic systems have not been able to activate cost-effective energy metabolism from non-phosphorylated energy substrates, which will be critical for any industrial-scale applications. Toward more robust and consistent extract preparation methods, extract protocols have been streamlined [51–53]. Another method has combined the small molecules in the reaction into a premix, used T7 polymerase from a crude lysate without purification, and reduced extract preparation by two steps [54].

Fourth, over the last decade, efforts to synthesize complex proteins have intensified. Figure 15.3b, which organizes the values from Figure 15.3a by product, highlights the shift from production of standard reporter proteins, such as luciferase and GFP, toward products containing ncAAs, glycosylation, and disulfide bonds as well as membrane proteins. We expect this trend to continue, particularly given the freedom of design in adjusting cell-free components by the direct addition of new components.

Finally, we note that cell-free platforms have been able to span 17 orders of magnitude in terms of reaction volumes (Figure 15.3c). Notably, the *E. coli* system has been shown to scale linearly from 250 µl reactions to 100 l, an expansion factor of 10^6, producing 700 mg ml^{-1} to enable manufacturing scale synthesis of soluble human granulocyte macrophage colony-stimulating factor (GM-CSF) with two disulfide bonds [4]. In the other direction, there has recently been a move toward smaller, microbe-mimicking reaction sizes [14, 15]. These efforts are useful for high-throughput applications and breadboarding of genetic circuits, both of which will be described later. To learn more about economical scale-up of cell-free systems, see reviews by Swartz [2] and by Carlson *et al.* [6].

The improvements in yields and cost, as well as scalability, give CFPS great utility. Examples of its applications are highlighted in the next section.

15.3 Products

CFPS allows the opportunity to not only produce proteins that standard methods are able to produce but to also solve expression problems with proteins that are notoriously difficult to synthesize *in vivo*. Examples of such products are described in the following section.

15.3.1 Noncanonical Amino Acids

Site-specific incorporation of ncAAs into proteins opens many doors for the production of proteins with new structures, functions, and properties. For such applications, cell-free systems have an advantage over *in vivo* systems because of their open environment and lack of need for cell viability. Indeed, recent efforts by Albayrak and Swartz [55], as well as Jewett and colleagues (unpublished), have shown the ability to synthesize greater yields of protein in batch CFPS reactions as compared with the *in vivo* approach. The benefit appears to come from the fact that the orthogonal translation systems can be toxic to the cell. Moreover, the ncAA can be added directly to the reaction mixture, instead of relying on cellular uptake, and ncAAs can be used that would otherwise be toxic to cells. This technology has been used in cell-free systems to polymerize proteins [16], conjugate human erythropoietin to a fluorophore in ICE [38], and modify the oncoprotein c-Ha-Ras in the WGE [56], along with many others.

The most common method for ncAA incorporation is through amber suppression, which inserts the ncAA at the location of the amber stop codon (UAG) in the reading frame of the gene of interest. With the addition of an orthogonal tRNA, orthogonal aminoacyl-synthetase, and ncAA, the UAG can be incorporated at a specific location in the gene, allowing for the template-encoded addition of the ncAA, as seen in Figure 15.4. This method has been extended to insert a second amino acid using the ochre stop codon (UAA) in combination with the amber codon for the incorporation of two unique ncAAs in a CFPS reaction [57]. Recent advancements from Albayrak and colleagues allow for the synthesis for the orthogonal tRNA (o-tRNA) during the protein synthesis reaction, improving scale-up possibilities [55]. One problem that plagues amber suppression both *in vivo* and *in vitro* is competition between the o-tRNA and release factor 1 (RF1). One solution to this problem is to use a different system for incorporation, using a four-nucleotide codon [58]. Further, cell-free systems open the possibility of expanding the genetic code by introducing additional Watson–Crick base pairs [59] and hijacking sense codons [60]. Since cell viability is no longer an issue, other options remove the problem with RF1 by either adding an aptamer to inhibit it [58] or tagging RF1 and removing it prior to protein synthesis [58, 61]. Looking forward, the development of an RF1 deletion strain as a chassis for CFPS will open new avenues for using cell-free synthetic biology for synthetic chemistry [62].

15.3.2 Glycosylation

For any protein synthesis technology, glycosylation cannot be ignored. It is estimated that over 50% of human proteins are glycosylated [63]. For pure chemical

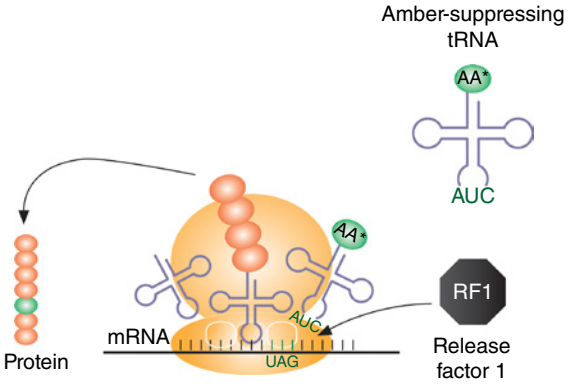

Figure 15.4 The production of proteins containing ncAAs is a frontier of CFPS. Amber suppression, shown here, is the most common method for ncAA incorporation in CFPS platforms but is hampered by competition between the amber-suppressing tRNA and release factor 1 (RF1). Several methods have been developed to prevent this competition. Also, new strains lacking RF1 should address this issue.

synthesis, the stereochemistry of sugars is challenging to make consistently [64], and for *in vivo* protein production, one must use mammalian cells, which are significantly more challenging and more expensive to culture than *E. coli*. This motivates a need for a fast, accurate method for producing glycoproteins using CFPS systems.

Initial work on the production of glycoproteins in CFPS was reported in 1978 by adding canine pancreas microsomes, containing glycosylation machinery, to a WGE reaction [65]. More recently, Guarino and colleagues chose to use the *E. coli* cell-free platform for synthesizing glycoproteins by adding the *Campylobacter jejuni* glycosylation machinery [66]. Since *E. coli* has no native glycosylation machinery, there was no mixture of glycosylation products. Also, due to the open environment of the system, the substrates could be directly added to the reaction to achieve *N*-linked glycosylation. Alternatively, the ICE system is able to maintain microsomes due to the method of lysate production [36]. These microsomes allow for *N*-linked glycosylation, as well as aid in the production of membrane proteins, described later. The CHO cell system had similar results to the ICE system [39]. While efforts to make glycoproteins are underway, there are still two drawbacks: no system is yet able to produce human glycosylation patterns and efforts to achieve O-linked glycosylation are limited. Addressing these limitations will open new avenues for studying and engineering glycosylation. For example, our ability to study and control glycosylation outside the restrictive confines of a cell will help answer fundamental questions such as how glycan attachment affects protein folding and stability. Answers to these questions could lead to general rules for predicting the structural consequences of site-specific protein glycosylation and, in turn, rules for designing modified proteins with advantageous properties.

15.3.3 Antibodies

Antibodies and their variants, typically tackled by *in vivo* recombinant protein methods, have recently gained much attention largely due to their high specificity [67]. However, *in vivo* methods, particularly in prokaryotic cells, can be a challenge when producing high concentrations of antibodies due to their aggregation, leading to insolubility [68]. Yin and colleagues faced this challenge when producing full-length antibodies in the *E. coli* extract (ECE) platform. Notably, they observed that the heavy chain (HC) was more prone to aggregation and needed the light chain (LC) for soluble co-expression [17]. This was an easy problem to solve with the open reaction environment of CFPS. They first expressed the LC plasmid for 1 h and then added the plasmid for the HC to start its translation. This strategy produced 300 mg l^{-1} aglycosylated trastuzumab in reactions ranging from 60 µl to 4 l at greater than 95% solubility. Martin *et al.* were able to then translate this lesson in plasmid timing, as well as oxidizing conditions and chaperone addition, to the CHO CFPS platform for the expression of >100 mg l^{-1} active, intact mAb [69]. In addition to the full-length antibody, antigen-binding fragments [19] and single-chain variable fragments [18, 70, 71] have been produced in a variety of cell-free systems. In fact, notable work by Kanter and colleagues created fusion proteins of a tumor-derived scFv with GM-CSF (a cytokine) or nine amino acids from interleukin-1ß, which improved potency of the scFv by increasing immune system stimulation for cancer therapy [18]. These advances demonstrate the merits of CFPS systems as a potentially powerful antibody production technology. However, cell-free antibody production still struggles from a lack of human glycosylation, which could be achievable in the future through the aforementioned glycosylation methods or ncAA incorporation and coupling of the oligosaccharides.

15.3.4 Membrane Proteins

Membrane proteins are an excellent application for CFPS. Chemical synthesis of membrane proteins can take 1–2 weeks [72], while *in vivo* methods struggle with obtaining high yields, minimizing degradation, and maintaining cell viability [73]. Cell-free systems speed up the process to a matter of hours with decreased proteolysis and no need to maintain living cells. Indeed, CFPS of membrane proteins has received considerable attention in recent years. For example, it has aided in the determination of protein structures, via NMR and crystallography, which were previously impossible, such as ATP synthase and G protein-coupled receptors (GPCRs) [74–76]. The challenge is finding a suitable substitute for the lipid bilayer. As seen in Figure 15.5, these substitutions include the use of detergents (in micelles or bicelles) [74, 77, 78], liposomes [74, 75, 77, 78], nanodiscs [76, 79, 80], tethered bilayer lipid membranes (tBLMs) [81, 82], and microsomal vesicles [36, 37].

One option is to produce the protein, precipitate it, and then solubilize it in detergents or liposomes; however, this does not allow for ideal structure and function studies because it is not an accurate membrane mimic [83]. Further, some detergents cannot be added to the reaction in high concentrations

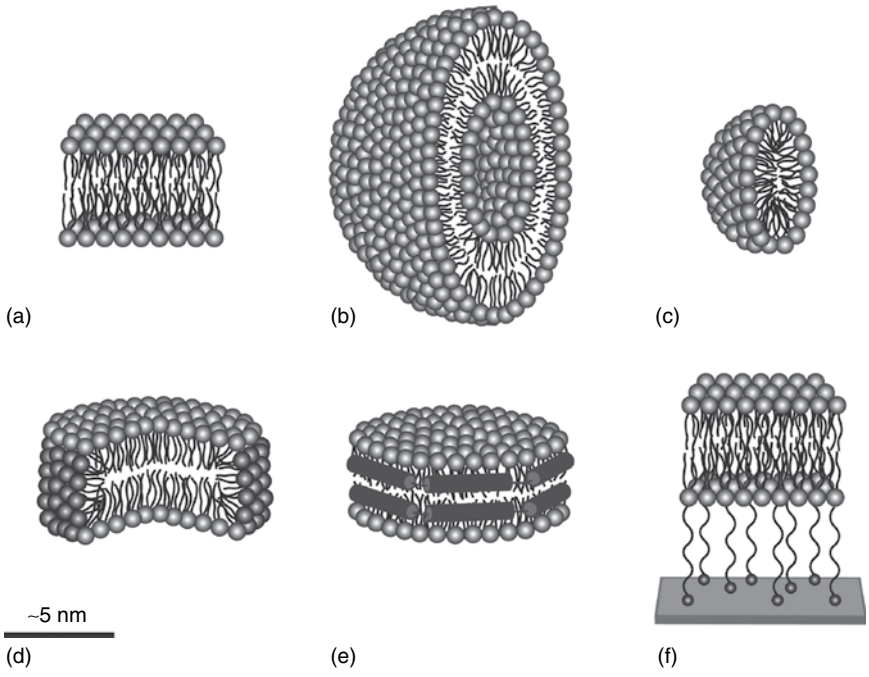

Figure 15.5 CFPS is a useful approach for the production of membrane proteins. Several methods have been implemented to mimic the cell membrane in cell-free protein synthesis: (a) lipid bilayer, (b) liposome, (c) micelle, (d) bicelle, (e) nanodisc, and (f) tethered bilayer lipid membrane.

because they inhibit transcription and translation [75, 83]. These methods also do not take advantage of the open reaction environment of cell-free systems. Unlike cells, where it is impossible to add chemicals directly to the protein as it is synthesized, CFPS allows for co-translation into liposomes, nanodiscs, tBLMs, or microsomes, all of which can be added exogenously to the reaction. Nanodiscs, consisting of a lipid bilayer surrounded by a protein scaffold, were found to be a better mimic of the lipid bilayer and thus obtained higher yields of soluble membrane proteins when compared with detergents and liposomes [80]. In fact, a functional GPCR, a highly studied but difficult to produce protein, was first produced in soluble form using nanodiscs in a cell-free reaction [76]. Another useful aspect of nanodiscs is the ability to co-express the nanodisc protein scaffold and membrane protein in the cell-free reaction, reducing the number of production and purification steps necessary [79]. For deeper structural and functional studies, the tBLMs use self-assembly to attach a membrane to a gold surface. The protein can then be co-translationally inserted into the membrane and immediately studied using surface plasmon-enhanced fluorescence spectroscopy (SPFS) and imaging surface plasmon resonance (iSPR), fluorescence polarization (FP) [84]. Similar to the tBLMs, CFPS has also been used in conjunction with a phospholipid bilayer supported on quartz crystal microbalances for direct characterization of membrane proteins as they

are expressed [85]. CFPS of membrane proteins promises to help unravel the function and structure of many potential drug targets.

15.4 High-Throughput Applications

Processes that take days or weeks to design, prepare, and execute *in vivo* can often be done more rapidly in a cell-free system. The use of polymerase chain reaction (PCR) templates significantly speeds up the process, since no time-consuming cloning steps are needed. Also, since the cell-free system is simpler and easier to control than cells, it allows for direct manipulation of reaction environments, as well as optimization of the reaction conditions. These characteristics are highlighted in the following examples of high-throughput protein synthesis for both production and screening as well as genetic circuit designing and testing.

15.4.1 Protein Production and Screening

While chemistry has been able to produce small molecule libraries for easy screening, the ability to produce proteins for similar procedures has been challenging. However, with cell-free systems, there is no need to transform cells with plasmids, produce the protein, and then lyse the cells. Instead, a PCR template or plasmid can be added to a small reaction mixture in a plate, the protein can be produced, and then the various proteins on the plate can be screened *in situ*, all in a matter of hours [86]. For example, Karim and Jewett expressed several enzymes in a CFPS reaction for prototyping metabolic pathways in *E. coli* lysates in order to quickly arrive upon the best combination of enzymes for the production of butanol [87]. Since CFPS reactions are at a small scale, microfluidics can also be used to supply small molecules [88] or when the number of reactions becomes too large, liquid handling can easily be automated [89]. One of the most impressive examples of using CFPS for high-throughput protein production is the human protein factory [24]. In this study, the authors expressed 13,364 human proteins using the WGE platform and then compiled the protein expression information in an online database [24, 90].

In addition to producing proteins from standard plasmids and PCR products, it is possible to produce protein arrays from DNA arrays. Since DNA arrays are much easier and more stable than protein arrays, He and colleagues developed a method to "stamp" the proteins on a new array by putting a DNA array plate face down on a second plate with the CFPS reaction mixture between the plates [91]. After the proteins were produced, they associated with the surface of the new plate. Stoevesandt and colleagues demonstrated the utility of this method when they produced an array of 116 distinct proteins [92]. In addition to its ease, it was found that one DNA array was able to produce at least 20 new protein arrays [91]. Protein arrays are beginning to enable an improved toolbox, and a faster process to probe different aspects of protein function and their role in enzyme screening will continue to grow in the upcoming years.

15.4.2 Genetic Circuit Optimization

There is currently a need for "breadboarding" of *in vivo* biological circuits in order to accelerate the design–build–test loops associated with synthetic biology studies. Biological circuits rely on regulation and control of protein products and can take a long time to assemble *in vivo*, so a system is needed that will function similarly to the cell with faster results and greater flexibility for manipulation: a great application for CFPS platforms. The combinatorial nature of testing the variations of the circuits also lends itself to high-throughput methods. Also, the open environment of the CFPS reaction allows for more control for these studies, since the initial concentrations of mRNA and protein as well as the exact reaction size can be directly manipulated. Methods have been developed to characterize parts (e.g., promoters, ribosome binding sites, terminators, and spacing), as well as multienzyme systems, such that they function predictably both *in vitro* and *in vivo* [21, 23, 93–96]. In one such example, Chappell and colleagues recognized that ribosome binding sites correlated directly when using PCR products *in vitro*, but promoters did not [94]. Thus, they used a USER–ligase method to circularize PCR products, the results of which were able to correlate between both platforms while keeping production time short by avoiding the need for a plasmid typically obtained by cell growth. In addition to characterization, cell-free systems have been used to test new options for circuit proteins, such as endogenous sigma factors, to supplement the common LacI and TetR proteins [7]. Aiding in the high-throughput area, reactions at the nanoliter, picoliter, and femtoliter scales are being explored as a method to better approximate the volume of a cell. This involves using microfluidics to feed small molecules to the reaction [15, 97], which diffuse well due to the small volume, as well as studying noise in gene expression [14], which could aid in the future design of gene circuits. To learn more about *in vitro* genetic circuits, see a review by Hockenberry [98].

15.5 Future of the Field

CFPS is emerging as a disruptive technology. It has promising applications for rapid, high-throughput screening and production of enzymes and personalized medicines, membrane proteins, and proteins containing ncAAs. Other applications include efforts to construct fully synthetic ribosomes *in vitro* [99] as well as artificial cells [7, 100]. Equally important, CFPS is expected to help address the increasing discrepancy between genome sequence data and their translation products. The Sargasso Sea expedition alone, for example, generated 1.2 million new genes, many with unknown function [101]. This concept has already been proven by the expression of the entire T7 bacteriophage genome [102] as well as nanoassemblies of T4 bacteriophage structural proteins [103]. Unfortunately, current cell-based technologies for heterologous protein expression have been unable to meet the rapidly expanding need for affordable, simple, and efficient protein production because they (i) can be slow (requiring time-consuming cloning strategies), (ii) can require laborious protein purification procedures,

and (iii) can lack robustness and predictability due to several reasons: the complexity, the host-dependent gene expression and protein folding/function, the necessity of product export from the cell membrane for improved production, and the toxicity of high levels of expressed proteins to the host. CFPS can address many of these limitations to help complement existing technologies, but there are remaining immediate challenges. For example, the field is limited by its ability to produce posttranslationally modified proteins at high titers, particularly those with human patterns. Moreover, we still do not have the protein equivalent of PCR. Further, inefficiencies in site-specific incorporation of ncAAs limits innovation. By addressing these challenges, we anticipate that cell-free systems will continue to penetrate and be recognized for value by industry. Given the capability to modify and control cell-free systems, CFPS holds promise to be a powerful tool for systems biology, for synthetic biology, and as a protein production technology in years to come.

Definitions

Cell-free protein synthesis is the process of translating proteins in lysates
In vitro is the processes performed outside of their biological context, e.g. protein synthesis occurring outside the cell
Noncanonical amino acid is any amino acid outside the 20 canonical amino acids
Glycosylation is the addition of sugar moieties to proteins
Antibody is the protein of the immune system that recognizes and neutralizes pathogens
Membrane protein is the protein that is associated with or integrated into a cellular membrane
High-throughput is the capability of being performed many times in parallel
Protein screening is the process of testing one or more proteins or protein variants in one or more contexts to determine properties of the protein(s) or optimize
Genetic circuit is the engineered use of DNA sequences to control biological reactions and programs

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