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Review

Cell-Free Gene Expression: Methods and Applications

Andrew C. Hunt,[¶] Blake J. Rasor,[¶] Kosuke Seki, Holly M. Ekas, Katherine F. Warfel, Ashty S. Karim, and Michael C. Jewett*

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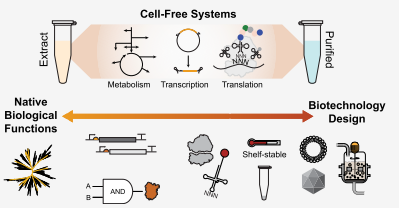
ABSTRACT: Cell-free gene expression (CFE) systems empower synthetic biologists to build biological molecules and processes outside of living intact cells. The foundational principle is that precise, complex biomolecular transformations can be conducted in purified enzyme or crude cell lysate systems. This concept circumvents mechanisms that have evolved to facilitate species survival, bypasses limitations on molecular transport across the cell wall, and provides a significant departure from traditional, cell-based processes that rely on microscopic cellular "reactors." In addition, cell-free systems are inherently distributable through freeze-drying, which allows simple distribution before rehydration at the point-of-use. Furthermore, as cell-free systems are nonliving, they provide built-in safeguards for biocontainment without the constraints attendant on genetically modified organisms. These features have led to a significant increase in the development and use of CFE systems over the past two decades. Here, we discuss recent advances in CFE systems and highlight how they are transforming efforts to build cells, control genetic networks, and manufacture biobased products.



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1. INTRODUCTION

Cell-free biology, the activation of biological processes without the use of intact living cells,[1] provides a versatile approach for understanding, building, and harnessing biological systems. Cell-free biology offers several advantages over living systems by bypassing cell growth and viability constraints, enabling customization of biosynthesis conditions for a single product, and offering unprecedented flexibility in designing and controlling biological systems. The scope of the term "cell-free systems" is broad, as it has been applied to many biological samples or mixtures that do not contain whole cells. Here we focus on cell-free gene expression (CFE), the process of producing proteins using transcription and translation machinery extracted from cells and carried out in a controlled, nonliving environment.

Cell-free studies have played a crucial role in the elucidation of biological mechanisms since the late 19th and early 20th century.[2] These foundational investigations include the observation of fermentation in extract from yeast cells,[3,4] the subsequent reconstitution of glycolysis with purified enzymes,[5] the determination of the cell cycle using eukaryotic cell extracts,[6] and the discovery of the genetic code.[7,8] The latter led to the development of CFE systems, which form the core of many modern cell-free applications by activating transcription and/or translation machinery in purified systems or crude cell extracts to execute biologically encoded genetic programs.

CFE was originally developed as a simplified method to study protein biosynthesis.[2] From 1948 to 1960, several researchers used crude cell extracts (comprising the soluble components of lysed cells, often mixed with exogenous reagents) to assess the incorporation of radiolabeled amino acids into proteins, studying the mechanism of peptide bond formation without much knowledge of translation beyond the essentiality of ATP, GTP, and tRNAs.[9−12] A turning point came in 1961, when Nirenberg and Matthaei uncovered the dependence of protein synthesis on RNA templates in extract from *Escherichia coli*.[7] Several years of investigation uncovered the entire set of RNA triplets encoding each amino acid[8] in addition to the canonical start and stop codons.[13] This pioneering work enabled the translation of proteins in a wide variety of cell extracts beyond *E. coli*, including rabbit reticulocytes and wheat germ, but all initial systems relied on RNA templates with reactions lasting on the order of minutes.[13]

In the 1970s, Zubay demonstrated CFE from DNA templates,[14] thereby recapitulating combined transcription and translation outside living cells, enabling longer lasting reactions and incorporating genetic regulatory elements into CFE; yet, these reactions produced only trace amounts of protein measured by radioactive counts. The limitations were addressed in part using viral RNA polymerases in the 1980s-90s,[15,16] and with improvements in reaction conditions,[17] metabolism activation,[18,19] and extract preparation developed in the late 1990s and early 2000s.[2,20] Modern *E. coli* CFE systems now routinely reach gram per liter yields of protein in batch reactions.[21−24] Innovations in CFE systems derived from wheat germ cells also led to g/L yields as early as 2000,[25] though in continuous exchange systems as we will discuss later. While most of these studies utilized reporter proteins such as chloramphenicol acetyltransferase or green fluorescent protein (GFP), the simultaneous optimization of CFE in *E. coli* extract and the development of a fully purified transcription and translation platform (PURE: Protein synthesis Using Recombinant Elements) in the early 2000s[26] led to an explosion of research applications enabled by rapid protein synthesis *in vitro*. Common uses of CFE now include the production of toxic or post-translationally modified proteins, making medicines at the point-of-use, the incorporation of non-canonical amino acids into proteins, the screening of genetic parts and enzymes, and the generation of synthetic biological systems (e.g., artificial cells or engineered ribosomes).

The primary objective of this review is to provide a detailed roadmap of the ways in which CFE systems are manufactured and how they have been applied to diverse problems in science and engineering. This review focuses largely on CFE systems that involve both transcription and translation. There have been many other excellent reviews in this space,[1,27−31] and we aimed to update and collate this information here. We start by providing an overview of the different types of CFE systems and the different components required for activity (Section 2). We focus on *E. coli* derived CFE systems as they are the most well-developed and widely used. We then detail the diverse applications of CFE in order of increasing biological complexity (Section 3), from the expression of single genes up to the construction of synthetic cells.

2. METHODS FOR CELL-FREE GENE EXPRESSION

Cell-free gene expression (CFE), also referred to as *in vitro* transcription and translation (IVTT or TX-TL)[21,32] or cell-free protein synthesis (CFPS),[1] is the activation of transcription and translation from cellular components derived from either crude cellular extracts of various organisms[28] (e.g., *E. coli*, yeast, Chinese Hamster Ovary (CHO) cells, etc.) or from purified components as in the PURE system.[26] CFE reactions are carried out by combining these components with a DNA (combined transcription and translation) or RNA (translation only) template and a cocktail of other molecules (e.g., NTPs, amino acids) required to activate and sustain transcription and translation.[33] Run in batch mode, the highest yielding CFE reactions can reach up to 4 mg/mL[21] and >20 h[22] of protein synthesis, while semicontinuous reactions (i.e., semipermeable membrane exchange of small molecules) can reach yields of 8 mg/mL[21] and 14 days[34] of sustained protein synthesis. CFE reactions are scalable, with batch reaction volumes ranging from femtoliters in microfluidic systems[35,36] to >100 L in

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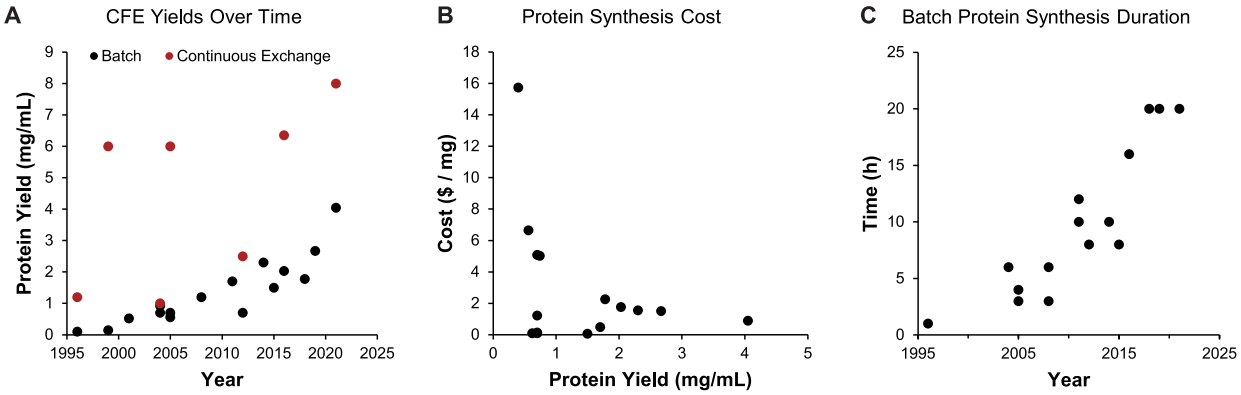

Figure 1. Summary of key metrics for *E. coli* CFE over time. (A) Optimization of CFE using *E. coli* extract has led to high yields of several grams protein per liter of reaction in both batch and continuous modes. (B) Cost per mg of synthesized protein has decreased with time. Note that costs plotted here include only the the nucleoside tri- or mono-phosphates and carbon/energy sources. (C) The longevity of batch reactions has been extended to ∼20 h.

Table 1. Cost and Productivity of CFE Formulations over Time[a]

Year	Yield (mg/mL)	Type	Time (h)	Protein	Cost* ($/L Rxn)	$/mg Protein	Reference
1996	0.4	Batch	1	CAT	6290	15.72	Kim et al., 1996[38]
2004	0.7	Batch	6	CAT	845	1.21	Jewett and Swartz, 2004[18]
2005	0.56	Batch	4	CAT	3716	6.64	Voloshin and Swartz, 2005[39]
2005	0.7	Batch	3	CAT	58	0.09	Calhoun and Swartz, 2005[40]
2008	0.74	Batch	3	CAT	3716	5.02	Jewett et al., 2008[19]
	0.62	Batch	6	CAT	58	0.09	
2011	0.7	Batch	10	GM-CSF	96	0.14	Zawada et al., 2011[37]
2011	1.7	Batch	12	CAT	811	0.48	Kim et al., 2011[41]
2012	0.7	Batch	8	GFP	3553	5.08	Shin and Noireaux, 2012[42]
2014	2.3	Batch	10	GFP	3555	1.55	Caschera and Noireaux, 2014[24]
2015	1.5	Batch	8	Antibody	75	0.05	Cai et al., 2015[43]
2016	2.03	Batch	16	GFP	3555	1.75	Garamella et al., 2016[44]
2018	1.78	Batch	20	GFP	4006	2.25	Martin et al., 2018[22]
2019	2.67	Batch	20	GFP	4006	1.50	Des Soye et al., 2019[23]
2021	4.05	Batch	20	GFP	3592	0.89	Garenne et al., 2021[21]
2023	1	Batch	20	GFP	90	0.09	Warfel et al., 2023[45]
1996	1.2	CE	14	CAT			Kim and Choi, 1996[17]
1999	6	CE	20	CAT			Kigawa et al., 1999[46]
2004	1	CE	100	GFP			Noireaux and Libchaber, 2004[47]
2005	6	CE	20	GPCR			Klammt et al., 2005[48]
2012	2.5	CE	20	GFP			Shin and Noireaux, 2012[42]
2016	6.35	CE	24	GFP			Garamella et al., 2016[44]
2021	8	CE	20	GFP			Garenne et al., 2021[21]

[a]Selected publications from 1996-2023 highlight the trends in CFE metrics over decades of optimization in batch and continuous exchange (CE) formats. These data are graphically displayed in Figure 1. *Note that the reaction cost estimate includes only the nucleotides and carbon/energy sources, as the primary determinants of low-cost CFE are using NMPs and non-phosphorylated carbon substrates. Costs were not determined for continuous exchange reactions due to the large and variable volumes of feeding solution. Reporter proteins include chloramphenicol acetyltransferase (CAT), granulocyte-macrophage colony-stimulating factor (GM-CSF), green fluorescent protein (GFP), antibodies, and a G-protein coupled receptor (GPCR).

traditional large-scale bioreactors.[37] Trends in CFE systems over the past 20 years are summarized in Figure 1 and Table 1.

In the subsections that follow, we discuss extract-based CFE systems, extract preparation methods, CFE reaction components and energy systems, purified CFE systems, CFE reaction formats and scale, monitoring CFE reactions, high-throughput experimentation for CFE, and modeling CFE systems. In Section 3, we will cover applications.

2.1. Extract-Based CFE Systems

To produce proteins of interest, CFE systems harness an ensemble of catalytic components necessary for transcription, protein biosynthesis, protein folding, and energy regeneration from crude cell extracts. Crude cell extracts from a broad range of organisms have been employed. While early studies focused on *E. coli*,[14] rabbit reticulocyte,[49] and wheat germ systems,[50] extracts from a more diverse array of organisms are now used to exploit differences in post-translational modifications, metabolism, temperature optima, and other properties.[20,28] Here, we provide a brief overview of prokaryotic (Table 2) and eukaryotic (Table 3) CFE platforms.

2.1.1. Prokaryotic Cell-Free Systems. *E. coli* cell-free systems are the most robust and well-characterized CFE platforms.[1,30] Over the last 30 years, extract preparation and

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Table 2. Example Prokaryotic CFE Systems[a]

Source Organism	Synthesized Protein (μg/mL)	Reaction Format	Time (h)	Reference
Bacillus megaterium	134	Combined batch	4	Moore et al., 2018[51]
Bacillus subtilis	21	Combined batch	2.5	Kelwick et al., 2016[52]
	561 ± 8.7	Combined batch	13	Zhang et al., 2021[53]
Clostridium autoethanogeum	236 ± 24	Combined batch	3	Krüger et al., 2020[54]
Corynebacterium glutamicum	385 ± 7	Combined batch	13	Zhang et al., 2021[53]
Cutibacterium acnes	85	Combined batch	24	Fábrega et al., 2021[55]
Escherichia coli	8000	Combined continuous	24	Garenne et al., 2021[21]
	4050	Combined batch	20	
Escherichia fergusonii	50	Combined batch	8	Yim et al., 2019[56]
Klebsiella oxytoca, *Pantoea agglomerans*	4 150			
Pseudomonas fluorescens	95	Combined batch	10	Nakashima et al., 2004[57]
Pseudomonas putida	198 ± 6	Combined batch	4	Wang et al., 2018[58]
Streptomyces coelicolor	30	Combined batch	3	Li et al., 2017[59]
Streptomyces lividans	516 ± 25	Combined batch	8	Xu et al., 2022[60]
	400	Combined continuous	48	Xu et al., 2020[61]
Streptomyces venezuelae	266	Combined batch	3	Moore et al., 2021[62]
Thermococcus kodakaraensis	115	Batch translation	0.25	Endoh et al., 2007[63]
Vibrio natriegens	1600 ± 50	Combined batch	20	Des Soye et al., 2018[64]
Yersinia pestis	30	Combined batch	16	McDonald et al., 2021[65]

[a]Organisms are listed alphabetically. Synthesized protein is reported as mean ± standard deviation when error was enumerated in the original publication.

Table 3. Example Eukaryotic CFE Systems[a]

Source Organism	Synthesized Protein (μg/mL)	Reaction Format	Time (h)	Reference
Bombyx mori	70	Batch translation	n.r.	Ito, 2021[66]
CHO	982 ± 30	Combined continuous	48	Thoring et al., 2021[67]
	50.2	Batch	2	Brödel et al., 2017[68]
HeLa	50	Continuous translation	36	Mikami et al., 2006[69]
Leishmania tarentolae	300	Batch translation	1.5	Mureev et al., 2009[70]
Nicotiana tabacum	3000	Combined batch	48	Das Gupta et al., 2023[71]
Pichia pastoris	116	Combined batch	4	Spice et al., 2020[72]
Rabbit reticulocyte	40	Batch translation	1.5	Mureev et al., 2009[70]
Saccharomyces cerevisiae	20 ± 1.3	Combined batch	5	Schoborg et al., 2016[73]
	17.0 ± 3.8	Combined continuous	10	Schoborg et al., 2014[74]
Spodoptera frugiperda	40	Combined batch	1.5	Merk et al., 2015[75]
	700	Combined continuous	72	
Trichoplusia ni	71	Batch translation	6	Ezure et al., 2006[76]
Wheat germ	5000	Continuous translation	18	Beebe et al., 2011[77]
	1600	Batch	n.r.	Harbers, 2014[78]

[a]Organisms are listed alphabetically. Synthesized protein is reported as mean ± standard deviation when error was enumerated in the original publication. n.r. = not reported.

CFE protocols have been optimized by several groups to increase protein production to > g/L quantities (Figure 1, Table 2). These works have shown that requirements for maximal cell-free expression include adequate substrate supply, a homeostatic environment, and the removal or avoidance of inhibitory byproducts. These efforts have also taught us that crude extract cell-free systems should be thought of as a complex set of biochemical reactions rather than a "black box". Current efforts are underway to understand the molecular composition of *E. coli* extracts and their impact on activity.[79−83] For example, it is now known that proteomic shifts in extract preparations occur due to stress response,[81] altered media formulations,[84] engineered strains,[85] as well as culture formats and lysis methods.[86] Similarly, metabolomic analyses have characterized molecular changes in CFE reactions due to variations in extract source strain growth media and harvest conditions,[87] the impact of metabolites on CFE,[88] metabolic flux over time in differentially lysed extracts,[89] and batch-to-batch variability.[90]

While *E. coli* extracts are used most predominantly, other bacterial CFE systems have recently emerged. These systems provide advantages for specific applications, such as genetic part prototyping or unique metabolism,[51,54,56,58,91] but they have been less optimized and typically produce less protein than *E. coli*-based systems (Table 2). CFE systems from *Vibrio natriegens* are one exception. With little optimization, a *V. natriegens*-based CFE system was able to produce up to ∼400 μg/mL GFP from endogenous promoters[92] and up to ∼1,600 μg/mL using T7 RNA polymerase[64,93] with plasmid DNA templates. While bacterial CFE systems are routinely used for their protein biosynthesis activity and lower costs, they can lack chaperones and foldases that facilitate folding of eukaryotic proteins and in addition lack machinery for post-translational modifications. Such machinery can be added to the system, as we will discuss later in Section 3.1.

2.1.2. Eukaryotic Cell-Free Systems. Eukaryotic CFE systems generally have more laborious extract preparation procedures and are more costly than bacterial CFE systems due to slower growth rates, more specialized media components, and phosphorylated energy substrates. However, they can have advantages for expressing complex proteins, such as those with post-translational modifications. Rabbit reticulocyte and wheat germ based systems have been the most widely used eukaryotic CFE systems.[28]

Wheat germ extract is the most widespread CFE platform other than *E. coli*,[28] and it has facilitated structural biology,[94] synthetic biology,[95] and pharmaceutical[96] studies since its initial development for scientific research.[78,97] This platform benefits from commercial availability, demonstrated scalability, and batch protein yields greater than 1 g/L.[77,78]

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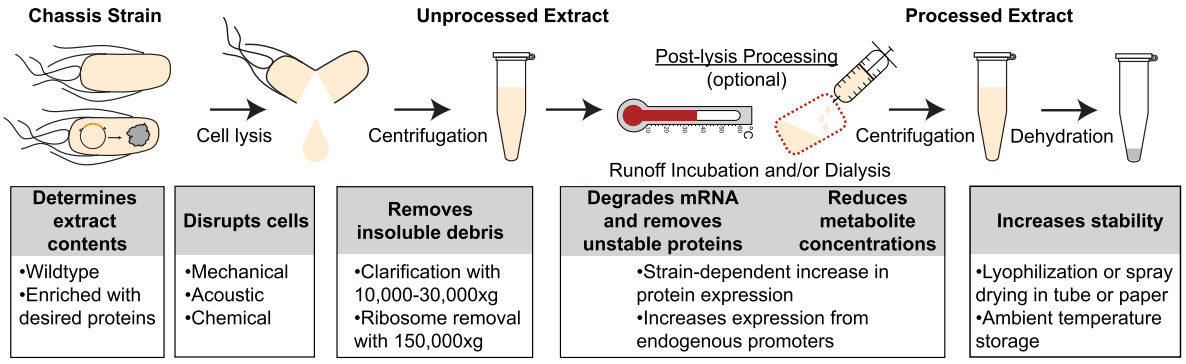

Figure 2. Cell extract preparation workflow. All protocols incorporate growth and lysis of cells, with variation in the density and lysis method. The soluble fraction of lysate is referred to as extract, and this may be further processed with a runoff incubation, dialysis, or lyophilization for strain- or application-dependent increases in activity.

More recently, a high-yielding plant CFE platform derived from *Nicotiana tabacum* (tobacco) BY-2 cell cultures has been developed, which can be harvested and lysed much more rapidly than wheat germ.[98] Tobacco BY-2 extract can express more than 3 g/L of protein[71] and has successfully produced transmembrane, disulfide bonded, and glycosylated proteins,[99] and has been scaled to 1 L reactions.[100] Less optimized sources of translationally active plant extracts have come from other components of tobacco,[101,102] *Arabidopsis thaliana* callus,[103] *Pisum sativum* chloroplasts,[104] *Zea mays* seeds,[105] and leaves from *Z. mays* and *Beta vulgaris*.[106]

Chinese hamster ovary (CHO) cells have also been used for eukaryotic CFE systems due to their abundance in commercial synthesis of biologics, such as antibodies. CHO CFE debuted in 1981 for translation of mRNA, and the platform has since been optimized for combined transcription and translation with batch yields ∼50 μg/mL[68] and semicontinuous yields ∼1 g/L[67] with the capacity to produce functional antibodies.[107,108] Less productive platforms include HeLa,[69] hybridoma,[109] and historical systems like PC12[110] and mouse[111] or rat liver[9] cell extracts. These CFE systems can facilitate high-throughput prototyping at small scales prior to application in cultures of therapeutic-producing cell lines.[112]

While other eukaryotic systems, such as those derived from fungi,[74,113,114] trypanosomes,[115] and insects[75,76] are also being developed, the field is still working to develop strategies to reduce costs, increase yields, and efficiently produce proteins with humanized glycosylation patterns.

2.2. Cell Extract Preparation Methods

The preparation of cellular extracts is integral to efficient protein biosynthesis *in vitro*. The workflow for producing extracts generally consists of: (i) growing and harvesting cell cultures, (ii) lysing cells, and (iii) processing cell extracts. However, the broad applications for CFE and adoption by various research laboratories have resulted in subtle differences within each step of extract preparation. In this section, we detail methods to produce extracts and highlight certain steps which may be necessary for certain CFE applications. Throughout this section and those that follow, we focus on bacterial CFE systems derived from *E. coli*.

2.2.1. Extract Preparation from *E. coli*. Cell extracts can be prepared using a variety of *E. coli* strains and processing steps to enhance performance in a desired application (Figure 2). State-of-the-art CFE workflows commonly use engineered derivatives of the BL21 strain, which are typically high-yielding due to enhanced mRNA stability and decreased protease activity. Specific applications may call for other strains that are described in relevant sections and/or reviewed previously.[20] For example, using engineered *E. coli* strains optimized for disulfide bond formation within the cytoplasm is useful for synthesizing full-length antibodies within CFE.[116] In addition, *E. coli* extracts can be made from derivatives of the genomically recoded C.321ΔA strain to incorporate noncanonical amino acids (ncAAs) into proteins more efficiently.[117]

Once the strain is selected, it is then grown and harvested. Typically, freshly isolated colonies from agar plates are used, although glycerol stocks are an alternative.[118,119] Cells are generally grown using common media formulations like Luria–Bertani (LB) broth, 2x Yeast Extract Tryptone (2x YT), 2x Yeast Extract Tryptone Phosphate (2x YTP), and 2x Yeast Extract Tryptone Phosphate Glucose (2x YTPG). Protein synthesis yields could be improved by using 2x YTPG media relative to LB media from 37% to 500% in conjunction with CFE conditions that mimicked the *E. coli* cytoplasm.[120,121] In addition, the inclusion of glucose in the growth media is hypothesized to upregulate genes involved in central metabolism, leading to improved protein synthesis capabilities while lowering phosphatase activity.[19,121] Glucose, however, was found to inhibit translation from endogenous bacterial promoters.[122] This suggests that the choice of media should be dictated by the final application for CFE.

Protein expression during cell growth can enrich heterologous proteins of interest within the cell extract. These enriched extracts are useful when a protein of interest is necessary for a downstream application or when it simplifies the reaction setup. Both constitutive and inducible expression systems can be used, but inducible systems are often preferred, particularly when the protein of interest is deleterious to cell growth. In one example, the T7 RNA polymerase-promoter system is used and is induced from the *lac* operon in BL21 (DE3)-based strains to circumvent the need to separately purify and supplement T7 RNA polymerase into CFE reactions. Methods using autoinducing media compositions that enable protein expression without user intervention during cell extract preparation have also been developed.[87,123,124] This reduces the labor required for extract preparation and improves the total yield of cell extract from a single preparation.

Cells are typically harvested by centrifugation during mid-to-late exponential phase. This maximizes the yield of cell extract and translation machinery components recovered while maintaining productivity.[125] Extracts made from cultures at high cell densities are less productive than using traditional

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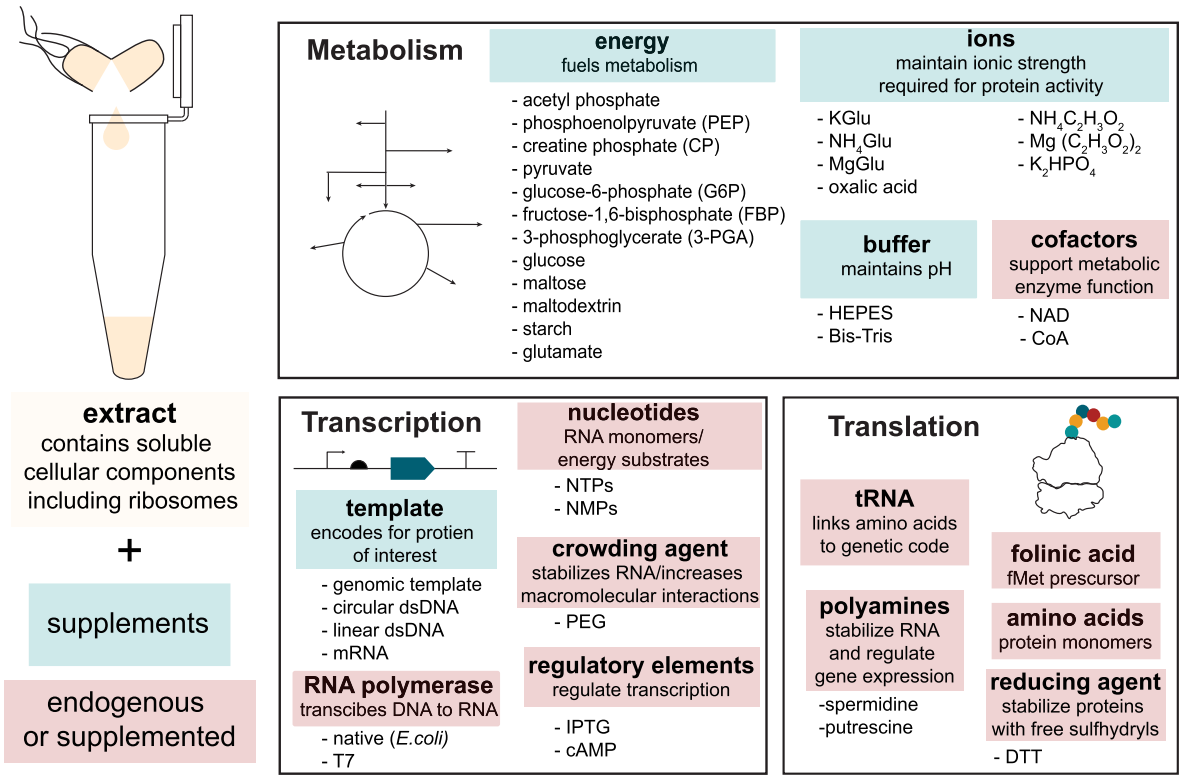

Figure 3. Typical additives to CFE systems. Components required for protein synthesis are grouped based on the three main processes activated in the reaction: metabolism, transcription, and translation. Components highlighted in blue represent factors that are supplemented while components highlighted in red are either present endogenously or can be supplemented into the cell-free reaction depending on the formulation.

extract preparation protocols.[87] This decrease in productivity is hypothesized to result from a smaller number of active ribosomes.[126] However, optimized media formulations with increased buffering capacity can prevent acidification of the cell culture and maintain extract productivity from high cell density cultures, resulting in four times the volume of extract without losing extract productivity.[87] After harvesting, the cells can be processed into extract immediately or stored at −80 °C until a more convenient time.

Crude cell extracts are prepared by lysing cells, which to date has been achieved using homogenization,[127−130] sonication,[125,131] bead-beating,[131,132] or enzymatic lysis.[133,134] Cell lysis is the step most likely to differ between laboratories that have adopted CFE. Homogenization and sonication are most widely used for extract preparation and historically have produced the highest-yielding extracts. However, the choice of lysis method depends on variables such as the volume of cells, the available instrumentation, the presence of membrane vesicles,[135] and the *E. coli* strain used. For example, processing large volumes of cells benefits from using homogenization to reduce the time invested during cell lysis.

After lysis, crude cell extracts are processed to remove cellular debris by centrifugation. Historically, extracts have been centrifuged at 30,000*g*, but slower centrifugation speeds (12,000*g*) have been found to improve extract productivity, which could be due to the increased number of inverted membrane vesicles that house enzymes involved in ATP regeneration.[19,133,135] Lower centrifugation harvesting is also useful in applications requiring expression of membrane proteins.[135] After centrifugation, extracts may be used immediately, aliquoted and flash frozen for later use, or further processed. Further processing can include dialysis, which removes small molecule inhibitors of transcription and translation, or a runoff reaction, which is hypothesized to provide ribosomes time to finish translating native transcripts as endogenous mRNA is degraded.[136] The runoff step also reduces the abundance of several proteins that precipitate out of solution.[137] Interestingly, these processing steps appear to be most important for transcription from native promoters and do not typically affect extract productivity when using T7 RNA polymerase in BL21 Star (DE3) strains.[122,129] Hypotheses around these impacts include the depletion of cold shock-like proteins during runoff that otherwise hinder gene expression and/or the depletion of unknown inhibitory metabolites during dialysis.[122,137] Although more extensive analysis is required to understand the complex changes in extract-based systems during runoff and dialysis, it is clear that these post-lysis processing steps can be important for reconstituting activity in specific applications of CFE.

2.2.2. Lyophilization of Cell Extract. Creating shelf stable CFE reactions using lyophilization is a growing area of research. This may have promising applications in on-demand production of therapeutics,[138−141] biosensors[142,143] at the point-of-use, and educational kits.[144,145] Compared to fresh reactions that lose activity after storage at room temperature over a month, lyophilized CFE reactions retain protein synthesis activity in equivalent conditions over several months.[140,146] Extract stability after lyophilization can be enhanced with additives. CFE reactions that are lyophilized with sugars like trehalose and lactose, for example, retained more protein synthesis activity compared to those lyophilized without additives after 2 weeks.[147,148] Lyophilized CFE systems have been reported to maintain activity out to 90 days,[140] and the longevity of lyophilized CFE reactions past

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that are still being actively explored. Additives are particularly appealing in combination with the observation that sugars such as maltodextrin can be used as alternative, cheaper energy sources in CFE.[40,45]

2.2.3. Cell Extract Preparation from Other Organisms. While the previous two subsections focused on *E. coli* extracts, extracts have also been prepared and characterized from over 40 different organisms. Extract preparation for these organisms differ from *E. coli*-based methods on a case-by-case basis. For example, active extracts from the fast-growing marine bacterium *Vibrio natriegens* has improved protein synthesis yields from cells grown in brain-heart infusion broth compared to the more common 2x YTPG medium.[64,92,93] Additionally, compared to homogenization pressures used to lyse *E. coli* (~20,000 psig), the homogenization pressure to make extracts from *Streptomyces lividans* B-12275 were optimal at 12,000 psig.[59] For eukaryotes such as *S. cerevisiae*, parameters such as OD of the harvest, RNase treatment, and others differ significantly from *E. coli* methods.[149] Other differences in extract preparation methods for non-*E. coli* organisms have been reviewed elsewhere.[20]

2.3. Reaction Components and Energy Systems

In this section, we describe the components most frequently found in cell-free reaction mixtures to activate metabolism, transcription, and translation (Figure 3). We focus on formulations developed for contemporary *E. coli*-based systems; a more detailed historical perspective has been reviewed elsewhere.[33]

2.3.1. Energy and ATP Production. Activating metabolism in a CFE reaction is necessary to enable regeneration of ATP to fuel high-level transcription and translation. CFE formulations either activate the native metabolism of the cell extract or rely on supplemented metabolic pathways. These metabolic pathways are supported by energy inputs, cofactors, buffers, and salts in numerous formulations.

Phosphorylated energy substrates with high-energy phosphate bonds such as phosphoenolpyruvate (PEP),[150,151] creatine phosphate,[26,152,153] or acetyl phosphate[154] were traditionally used as CFE substrates in combination with their respective kinases to generate ATP. Unfortunately, these expensive phosphorylated substrates typically account for the majority of reaction cost, which has driven innovation toward more economical alternatives.[155−157] Additionally, phosphorylated substrates are degraded by phosphorylases present in the extract, destabilize reaction pH,[18] and cause the accumulation of inorganic phosphate that can sequester magnesium ions necessary for protein synthesis.[158,159]

To alleviate these limitations, other energy substrates have been investigated in *E. coli*-based systems. For example, ATP generation from pyruvate can be activated without additional enzymes by supplementing reactions with nicotinamide adenine dinucleotide (NAD) and coenzyme A (CoA).[151] Other glycolytic intermediates like glucose-6-phosphate (G6P),[151] fructose-1,6-bisphosphate (FBP),[155] and 3-phosphoglycerate (3-PGA)[42,160] can also be used as energy sources for CFE. Low-cost primary energy sources such as glucose,[40,156] maltose,[161] maltodextrin,[148,161,162] and soluble starch[41] can be fed into glycolysis, but require the addition of a phosphate source (e.g., potassium phosphate[40,41] or hexametaphosphate (HMP)[161]).

The discovery that early glycolytic intermediates could be used to fuel CFE also resulted in new systems combining multiple energy substrates and metabolic pathways in the extract. For example, the PANOx system includes NAD and CoA in addition to PEP, which increases reaction productivity by harnessing both ATP from the dephosphorylation of PEP as well as the downstream metabolism of the resulting pyruvate.[151] In another example, the 3-PGA system has been coupled with both maltose and maltodextrin to manage the accumulation of inorganic phosphate, which was found to extend the reaction time and resulted in high yields.[163]

In addition to glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation can be activated in the cell-free context. In an effort to optimize cell-free reaction productivity by more closely mimicking the cellular environment, the PANOx-SP system removed unnatural components such as buffer and polyethylene glycol (PEG), adjusted salt concentration, and added in the polyamines spermidine and putrescine.[18] While this did not make a large difference in protein synthesis levels when PEP was used as an energy substrate, it dramatically improved the yields and maintained consistent pH when pyruvate was added (Cytomim system) as the energy substrate instead of PEP.[18] This system was also active in the absence of pyruvate, indicating that the Cytomim system supported other modes of energy generation.[120] Glutamate was also described to be an energy source, which can produce NADH through the TCA cycle and fuel oxidative phosphorylation enabled by the presence of a proton gradient and ATP synthase found in inverted membrane vesicles in the extract.[19] Doubling the glutamate concentration and optimizing other reagents can also result in a robust and low-cost protein synthesis system.[157]

2.3.2. Cofactors, Small Molecules, Ionic Strength, and pH. The cofactors NAD and CoA are expensive reaction components that are desirable targets for removal to decrease reaction cost. Supplementation of NAD was crucial for the metabolism of pyruvate in a traditional S30 extract formulation, and CoA further boosted protein synthesis yields.[151] Removal of NAD and CoA resulted in substantial decreases in protein synthesis yields in subsequent optimizations using glucose as an energy source.[156] However, changes to expedite extract production such as low-speed clarification steps (S12) and the absence of the dialysis step, have changed the concentration of endogenous components in the final extract.[125,130] Optimizations using glutamate,[157] FBP,[155] and maltodextrin[148] have found supplementation of NAD, CoA, or both cofactors to be unnecessary in the reaction when dialysis is not carried out.

Maintaining the overall ionic strength of the CFE environment is important to mimic the cellular environment and maintain the proper function of biological processes. Cations such as magnesium, potassium, and ammonium are traditionally added to CFE reactions to balance the charge of negative biomolecules, like nucleic acids.[33] Magnesium plays a significant role in CFE productivity as it is involved in ternary complex formation, ribosome assembly, and tRNA aminoacylation, among others, so the magnesium concentration is typically optimized for each individual extract.[14] Importantly, due to the high necessary concentration of supplemented cations, the accompanying anionic counterions, often glutamate or acetate, are present at higher concentrations in a CFE reaction than in the *E. coli* cytoplasm.[18] Formulation iterations use glutamate rather than acetate or chloride salts to better reflect the cytoplasmic environment and reduce acetate concentration.[18]

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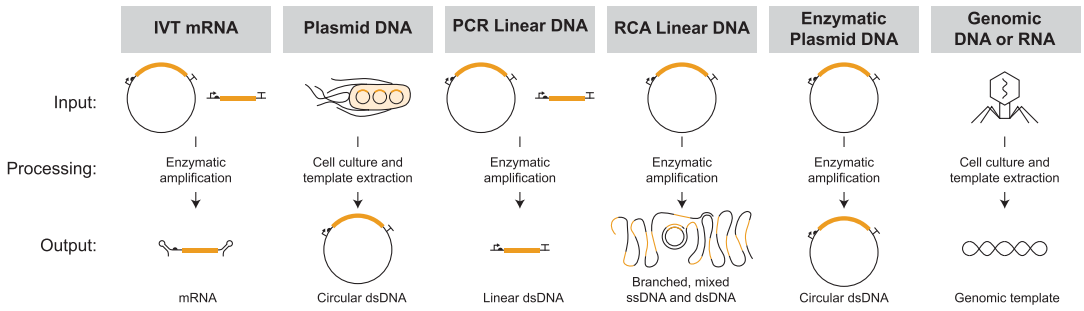

Figure 4. Templates used to drive transcription and/or translation in CFE systems. IVT: *in vitro* transcription; DNA: deoxyribonucleic acid; mRNA: messenger ribonucleic acid; PCR: polymerase chain reaction; RCA: rolling circle amplification.

Small molecules are also added to improve energy regeneration. Oxalic acid is a commonly added small molecule that inhibits PEP synthetase and avoids the unproductive conversion of pyruvate back to PEP.[164] However, oxalic acid is detrimental to protein yields when using early glycolytic intermediates such as G6P as an energy source.[151] Phosphate salts such as potassium phosphate are also important when using early glycolytic substrates such as glucose or maltodextrin for initial substrate phosphorylation.[40,41]

Maintaining appropriate pH in the reaction is important for metabolism and many other CFE processes. While the Cytomim system removed the HEPES buffer entirely,[18] certain energy sources, like glucose, have different impacts on reaction pH, typically due to the production of acidic byproducts.[40] Important considerations for buffer use in CFE include buffer concentration, pH, and pK_a/buffering range. HEPES at pH 7.2−8.2 is most commonly used; however, it was found that Bis-Tris (which has a lower pK_a than HEPES) improved protein synthesis when using glucose-based energy systems and better stabilized reaction pH.[40]

2.3.3. Transcription. RNA polymerase (RNAP) is required to transcribe the DNA encoding the gene of interest into RNA in CFE reactions. Bacteriophage polymerases such as T7 or Sp6 polymerases are often used[16,99] and can be supplemented as a purified enzyme or induced during cell growth and enriched in the extract.[128] The endogenous *E. coli* RNA polymerase and sigma factor 70 (σ^{70}) can also be leveraged for transcription in CFE, although optimal reaction formulation and extract processing conditions required for efficient expression can differ from bacteriophage RNAP systems.[122,165]

For systems that incorporate more complex transcriptional programs, the addition of exogenous regulatory elements is required. For example, IPTG[42] and cyclic adenosine monophosphate (cAMP)[33] can be supplemented to the cell-free environment to induce regulated gene expression. Transcription factors are also commonly added or enriched in the cell extract to build genetic circuits or biosensors, which is discussed further in Sections 3.5 and 3.6.

Nucleotides are critical additives for transcription. Nucleoside triphosphates (NTPs) are the most common form of nucleotide supplement; however, they are one of the most expensive additives. It has been demonstrated that the native cellular machinery in extract can phosphorylate nucleoside monophosphates (NMPs) when they are added to the reaction instead of NTPs, which is more cost-effective.[19,156,157] In fact, protein synthesis can be carried out without the supplementation of exogenous nucleotides in some formulations.[148,166]

Crowding agents can be added to CFE reactions to help stabilize molecules such as RNA and promote macromolecular interactions. These additives can impact both transcription and translation, and their identity and concentrations are an important factor to consider as the protein concentration in a CFE reaction is approximately 20 times lower than the protein concentration in the cytoplasm of *E. coli*.[18] PEG is commonly added as a non-native crowding agent, but optimal concentrations can depend on the molecular weight of PEG[38,167,168] and the desired protein product.[44] Other crowding and stabilizing agents that have been investigated in addition to PEG include alcohols, sugars, amino acids, and polyols.[167] While PEG is still used in some contemporary formulations, the Cytomim formulation notably replaced the unnatural crowding agent with the native polyamines spermidine and putrescine to stabilize RNA and other compounds,[18] which can also impact translation (see Section 2.3.4).

2.3.4. Translation. Transfer RNA (tRNA) recognizes the mRNA sequence and brings the corresponding amino acids to the ribosome to enable translation. Typically, *E. coli* tRNA is supplemented to the cell-free reaction; however, it is not an essential additive due to its native presence in the extract. Optimizations using FBP,[155] glutamate,[157] and maltodextrin[45] as energy sources have removed exogenous tRNA without a negative impact on protein synthesis, suggesting that native tRNA is present at suitable concentrations in the S12 extracts used.

Folinic acid is a necessary precursor to N-formylmethionine and has been historically supplemented due to its importance in translation initiation. However, exogenous folinic acid was determined to be unnecessary for high levels of protein synthesis in a glutamate-based CFE system.[157]

Typically, the 20 amino acids are supplemented in excess to the CFE reaction at mM concentrations. Metabolism in the extract can both consume and produce amino acids. For example, depending on the extract source strain, it has been demonstrated that aspartate, lysine, and tyrosine can be produced in the cell-free reaction,[19] so amino acid concentration could be a point of optimization or target for removal from the reaction in future studies.[157] Investigation into the necessary amino acids for a specific protein of interest could result in reduced cost for a particular product. Optimized concentrations of serine and glutamine, for example, led to increased yields in a creatine phosphate fueled system.[169]

Dithiothreitol (DTT), a reducing agent used to stabilize proteins with free sulfhydryl groups, is a common additive during extract preparation when a reducing environment is

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Table 4. Strategies for Protecting Linear DNA in CFE

Strategy	Implementation	Proposed Mechanism	Result	Organism	Reference
Extract preparation method	No runoff or dialysis	Unknown	50% of plasmid yield[a]	*E. coli*	Hunt et al.[116]
Genomic modification	A19ΔrecCBD::Plac-red-kan-ΔendA	Replacement of RecBCD with bacteriophage λ red recombination system	45−63% of plasmid yield	*E. coli*	Michel-Reydellet et al.[193]
Genomic modification	SBP tagged RecD and PNPase	removal of RecD and PNPase from extract	2−4x improved LET yield	*E. coli*	Seki et al.[199]
Genomic modification	ΔrecCBD	Knockout of RecBCD	48−78% of plasmid yield	*E. coli*	Batista et al.[200]
Nuclease inhibition	Chi6 oligos	Competitive inhibition of RecBCD	23% of plasmid yield	*E. coli*	Marshall et al.[201]
Nuclease inhibition	gamS	Protein inhibitor of RecBCD	38% of plasmid yield	*E. coli*	Sun et al.[185]
Nuclease inhibition	CID 697851, CID 1517823	Small molecule inhibitor of RecBCD	2.5−3x improved LET yield	*E. coli*	Shrestha et al.[203]
Nuclease inhibition	Tus-Ter	Protein binds linear dsDNA termini	100−146% of plasmid yield	*E. coli, V. natriegens*	Norouzi et al.[205]
Nuclease inhibition	Ku	Protein binds linear dsDNA termini	improvement in transcription yield	*E. coli* and 4 non-model organisms	Yim et al.[206]
Nuclease inhibition	ssCro	Protein binds linear dsDNA termini	2−28% of plasmid yield	*E. coli, V. natriegens*	Zhu et al.[204]
DNA modification	Terminal 5′ phosphorothioate linkages (x2)	5′ DNA more resistant to nuclease degradation	36% improved LET yield	*E. coli*	Sun et al.[185]
DNA modification	Methylation	Methylation of linear DNA potentially inhibits degradation	No significant improvement	*E. coli*	Zhu et al.[204]
Template design	Flanking DNA	5−500 bp of flanking DNA as a buffer for exonucleases	2.4−6x improved LET yield	*E. coli*	Sun et al.[185]
Template design	Nuclease Resistant Sequence	Reduced activity of nuclease on linear DNA	75% of plasmid w/o GamS and 100% of plasmid w/GamS	*E. coli*	Chen et al.[202]
Template design	Circularization	PCR-based circularization of LET	same as plasmid yield	*E. coli*	Wu et al.[207]
Template amplification method	Rolling Circle Amplification	Long concatemers serve as competitive inhibitor for exonucleases	75−80% of plasmid yield[a]	*E. coli*	Gyanendra et al.[197]

[a]Approximate result listed when the exact improvement was not reported.

desired during CFE.[125] Optimal concentrations of DTT in CFE systems generally are around 1 mM.[33,165,167]

The polyamines spermidine and putrescine are added to the Cytomim formulation to more closely mimic the cytoplasmic environment.[18] They also facilitate ribosome assembly.[170] While high concentrations of putrescine are found in *E. coli* cells, optimizations found that when adding spermidine, putrescine is not needed in the cell-free reaction.[157]

2.3.5. Expression Templates. The DNA or RNA templates used to drive CFE take a variety of forms (Figure 4). The uniting feature of these templates is that they contain the appropriate elements to activate transcription (e.g., promoter, terminator, etc.) and/or translation (e.g., ribosome binding site (RBS), Kozak sequence, open reading frame (ORF), etc.) in the CFE system of interest. Typically, synthetic constructs are built containing the gene of interest and the required regulatory elements,[171] although genomic DNA and RNA, for example phage genomes,[172] are also used as templates for CFE. For protein expression applications, it is common to use vectors lacking inducible regulatory elements (e.g., the lacO site) in order to maximize protein expression yield.[173]

Users of CFE systems are faced with the choice of using either DNA or RNA as templates to drive gene expression. Here, *in vitro* transcription (IVT) derived mRNA is commonly used to drive translation-only CFE reactions and DNA is used to drive combined, one-pot transcription and translation reactions. Several considerations can help users choose between the two templates. Combined reactions (note transcription rates and translation rates are not typically coupled/matched and thus we use the word combined) tend to be more convenient, as they require fewer steps to prepare the template and do not require the manufacture, handling, and storage of RNA. Many common CFE systems can perform high yielding, combined transcription and translation. These include the prokaryotic *E. coli*,[21,23] the protozoan *L. tarentolae*,[70,174] the insect *S. frugiperda*,[175] the plant *N. tabacum* BY-2,[71] and the mammalian CHO[107,176] CFE systems. However, in some common CFE systems (e.g., wheat germ,[78] rabbit reticulocyte[177]), mRNA is the typical template, although there are combined versions available.[178] Combined (i.e., transcription and translation) and translation-only reactions can also have different yields. In some systems, transcription and translation can have different optimum conditions (e.g., temperature, salt) or cause resource competition that reduces yields, which can be overcome with mRNA as a template.[177] However, in high yielding *E. coli* systems, IVT mRNA has been found to result in lower protein yields than its DNA counterpart.[179,180] This was hypothesized to be related to mRNA folding leading to inaccessibility of the translation start site,[179,180] and interestingly this issue was partially or completely alleviated by performing the IVT step in the presence of a ribosomal extract.[180]

Linear DNA templates, frequently referred to as linear expression templates (LETs) (reviewed elsewhere[181−183]), are particularly attractive because they circumvent the time-consuming and low-throughput steps associated with plasmid assembly, cell culture, and plasmid purification, saving days of effort and enabling throughputs much greater than cell-based workflows.[184−187] High-throughput LET approaches often involve an assembly step, to build an LET capable of supporting transcription and translation, and an amplification step, to manufacture the template in sufficient quantity to drive a CFE reaction. The assembly step typically involves assembly

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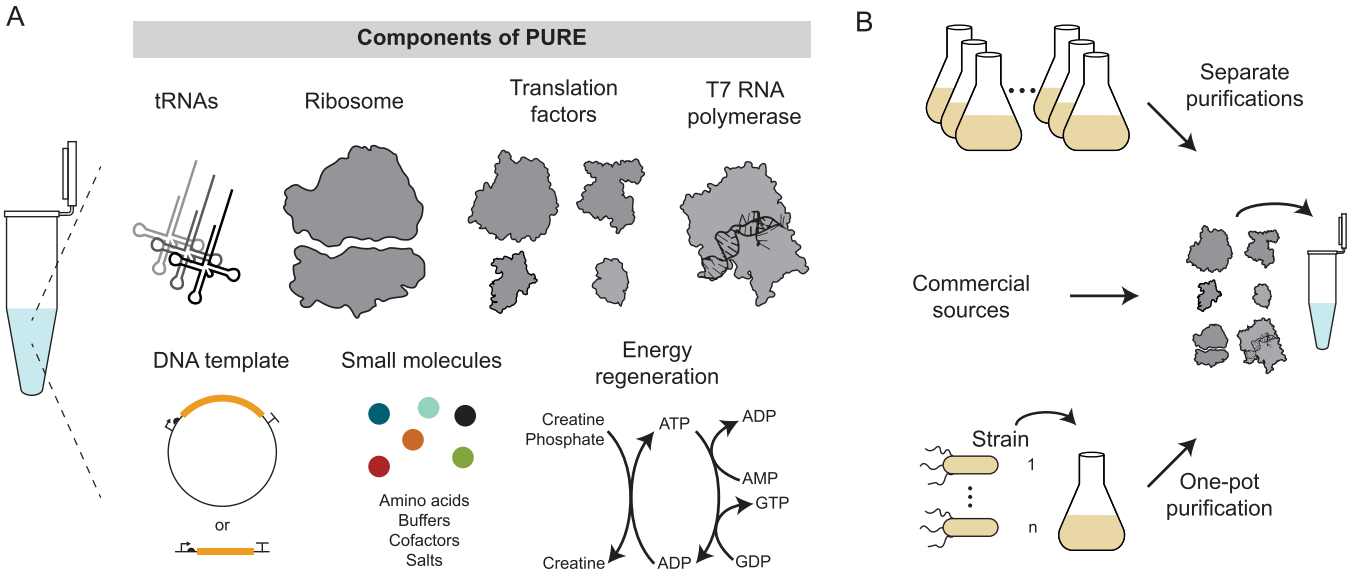

Figure 5. CFE systems using purified components. (A) Key components required for purified CFE systems. (B) Methods of obtaining purified CFE systems.

of the gene with other regulatory elements (e.g., promoter, ribosome binding site, terminator, etc.) and potentially with other components of the ORF (e.g., different protein fragments). To date, assembly has been accomplished with Gibson assembly,[185,186,188,189] Golden Gate assembly,[185] Gateway assembly,[187] or polymerase chain reaction (PCR) based assembly.[32,34,190−192] After assembly, LETs are amplified to generate sufficient quantities to drive a CFE reaction either by PCR[32,185,187,191,193−195] or rolling circle amplification (RCA).[184,187,196,197] PCR generates linear dsDNA containing only a single copy of the template and requires thermal cycling, whereas RCA leverages a circular template, exonuclease resistant primers, and a strand displacing polymerase to generate large fragments of mixed ssDNA and dsDNA containing many copies of the template in a single molecule. Because the assembly and amplification reactions are *in vitro* enzymatic reactions, they can be carried out sequentially in microplates and without cells, making them amenable to high-throughput screening applications.

A major consideration in the use of linear DNA in CFE is that crude extract-based systems contain exonucleases that degrade LETs, which can hinder protein expression. Interestingly, cell culture conditions and extract processing steps play a major role in the ability of a given CFE system to utilize LETs. For example, when using *E. coli* extracts that are not processed with runoff or dialysis, protein yields using LETs can be within 50% of that from plasmids.[116] Methods that protect LETs from degradation are frequently required. The RecBCD exonuclease complex is known as a contributor to the instability of linear DNA in *E. coli*[198] and has been the major target for inhibition in *E. coli* CFE systems. Strategies tested to inhibit RecBCD include genomic modification,[193,199,200] competitive substrate inhibitors,[201] protective DNA sequences,[202] protein inhibitors,[185] and small molecule inhibitors[203] which have yielded varying degrees of success and are summarized in Table 4. The success inhibiting the RecBCD exonuclease in *E. coli* has led to other strategies to inhibit exonucleases more broadly that may be applicable in other CFE systems. Several different DNA modifications with potential to increase nuclease resistance, including methylation[204] and chemical modification,[185,200] have been evaluated but yielded little to no improved protein expression. A more successful general strategy has been to utilize DNA binding proteins that block the terminal ends of the LET,[204−206] which in one case resulted in LET-based expression on par with that of plasmid-based expression.[205] Circularization of the linear template DNA has also been a successful strategy for protecting against degradation, yielding similar results between linear and plasmid-based templates.[207] A potential benefit of RCA-derived templates over PCR-derived templates is that they are reported to be less sensitive to degradation, likely a result of the long length of the LET product serving as its own competitive substrate inhibitor.[196]

There are several other factors to consider when choosing a template for CFE. Template "quality"—both the purity of the template and the damage accumulated by the template during purification—are known to impact CFE reactions and lead to increased variability, and thus preparation methods can have an impact on CFE results.[208,209] Toward improving template preparation outcomes, best practices for template preparation have been suggested.[210] Fortuitously, in applications utilizing linear DNA, the buffers associated with both PCR and RCA have been found in some cases to be tolerated in CFE, enabling the use of unpurified template.[116,187,188,196] Methods for quantifying the concentration of unpurified DNA templates have also been reported.[196]

Linear DNA may not be suitable for all applications. In efforts to evaluate *E. coli* σ^{70} promoters in CFE, multiple researchers have observed that the strength of a given promoter correlated better with *in vivo* results for plasmid-based CFE than for LET-based CFE.[185,211] It was hypothesized that this was related to DNA supercoiling and its impact on transcription rate.[211] Interestingly, others have found that the addition of linearized vector instead of PCR product eliminated the difference between linear and circular templates when evaluating the transcription factor TetR.[212] The authors proposed that nonspecific binding of transcription factors to the vector could be the a cause of the observed differences.[212] Thus, it is important to evaluate the suitability of linear templates for a given application before undertaking large scale

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Table 5. Comparison of PURE and Extract-Based CFE Systems

Category		Extract-Based	PURE
Reaction Composition	Redox State	Typically reducing. Can be manipulated by addition of small molecules such as glutathione. Modified redox state may benefit from genomically modified strains.	Typically reducing. Can be manipulated by addition of small molecules such as glutathione.
	Energy Regeneration	Many energy sources, including glucose, PEP, and others, can be used due to active central metabolism.	Uses creatine phosphate and creatine kinase system to regenerate ATP and cofactors.
	DNA Templates	Both plasmid and linear DNA can be used. Linear DNA may require additional protection methods depending on the CFE system.	Both plasmid and linear DNA can be used.
	Vesicles	Already present in cell extracts.[135]	Must be supplemented if necessary for specific application.[219]
	RNA Polymerase	Both T7 and *E. coli* RNA polymerase are commonly used.	T7 RNA polymerase most common. *E. coli* RNA polymerase is less active and requires additional factors.[220]
Practical Considerations	Maximum Batch Yield	>2−4 mg/mL protein.[21,23]	∼0.1 mg/mL to 1 mg/mL protein.[221,222]
	Difficulty of Making Reagents	Easy to Moderate. Extracts optionally require processing after lysis.	Difficult. Proteins and ribosomes must be purified from cell extracts.
	Commercial Availability	Widely available (e.g., myTXTL (Arbor Biosciences) and NEBxpress (NEB), among others).	Widely available (PURExpress (NEB) and PURExpress (Gene Frontiers)).
	Cost	∼ 5 $/mL homemade[1] and ∼400 to 1,000 $/mL commercial.[223,224]	∼100 $/mL homemade[225,226] and ∼ $1,000 to 1,200 $/mL commercial.[221,222]
Applications	Short Peptide Synthesis	Peptides can be unstable in extract-based systems due to low translational efficiency or proteolytic degradation[227] unless attached to a fusion protein.[228,229]	Peptides are stable in purified systems.
	Modeling	Models must be fit empirically due to unknown composition of extract components.	Well-defined composition enables bottom-up modeling
	Noncanonical Amino Acid Incorporation	Amber suppression using orthogonal translation systems is highly productive[22,23]	Genetic code reprogramming using flexizymes and traditional orthogonal translation systems[230]

experiments. While this may be a limitation for some applications for the time being, advances in cell-free plasmid replication systems may enable enzymatic plasmid amplification with the same ease as the amplification of linear DNA.[213,214]

2.4. Purified CFE Systems

While the previous subsections have covered extract-based CFE systems, *in vitro* transcription and/or translation can also be recapitulated using only purified components (Figure 5), allowing the reaction composition to be precisely defined. The state-of-the-art and widely used purified CFE system is called the PURE system.[26,215−217] PURE comprises purified transcription and translation machinery mixed with a circular or linear DNA template and energy sources to activate transcription and translation. The proteinaceous components of PURE are sourced from *E. coli* and consist of all 20 aminoacyl-tRNA synthetases (aaRS), Initiation Factors (IF) 1−3, Elongation Factors (EF) Tu, Ts, and G, Release Factors (RF) 1−3, Ribosome Recycling Factor, Methionyl-tRNA formyltransferase (MTF), T7 RNA polymerase, creatine kinase, myokinase, nucleoside-diphosphate kinase, and pyrophosphatase. The system is further supplemented with *E. coli* tRNAs and ribosomes. Finally, PURE also includes relevant small molecules essential for transcription, translation, and energy regeneration, including all 20 amino acids, 10-formyl-5,6,7,8-tetrahydrofolic acid, NTPs, and creatine phosphate. PURE is commercially available as PURExpress (NEB) and PURExfrex (Gene Frontiers), which has enabled its use by many laboratories worldwide.

The differences between CFE using PURE or cell extracts mainly arise from the presence of proteins and small molecules within the *E. coli* cytoplasm that remain after extract preparation. Functionally, this has resulted in reduced capacity in PURE reactions, relative to extracts, in terms of number of proteins produced per ribosome.[218] This must be considered as these components can affect its performance in a desired application. We have listed several general differences in Table 5.

Historically, PURE has been made by individually purifying all transcription and translation machinery from *E. coli* overexpression cultures and mixing them at defined concentrations.[26] While this affords the greatest amount of control over the reaction composition, this process is time-consuming, laborious, and expensive. There have been several efforts to simplify the preparation of PURE. Most examples focus on reducing the number of purifications required by including multiple PURE components within a single culture. This has been accomplished by installing genomic His tags onto PURE proteins within a set of *E. coli* strains,[231] grouping PURE components onto multicistronic plasmids,[232] or by coculturing all 36 *E. coli* strains required to express each PURE component within a single culture.[225,226] One challenge of these systems has been batch to batch variability, which was recently addressed by modifying promoters for more stable expression.[233] While all methods that have moved away from individual protein purification sacrifice some control over PURE's composition, they circumvent the high cost and laborious nature of producing PURE, which are two of the main barriers to its use. With further improvements, the preparation of PURE could perhaps become as simple as the preparation of extracts for *in vitro* transcription and translation.

As protein yields from PURE are lower than those from extract-based CFE systems, there have been many efforts to optimize protein yields from PURE. Combinatorial optimization of transcription and translation components has shown that increasing the concentration of translation factors increases protein synthesis capacities.[234,235] Changes to improve PURE can also be guided by computational models, which are more tractable for PURE compared to extract-based systems.[236] Another direction has focused on the addition of supplements to PURE. Similar to extract-based CFE, the

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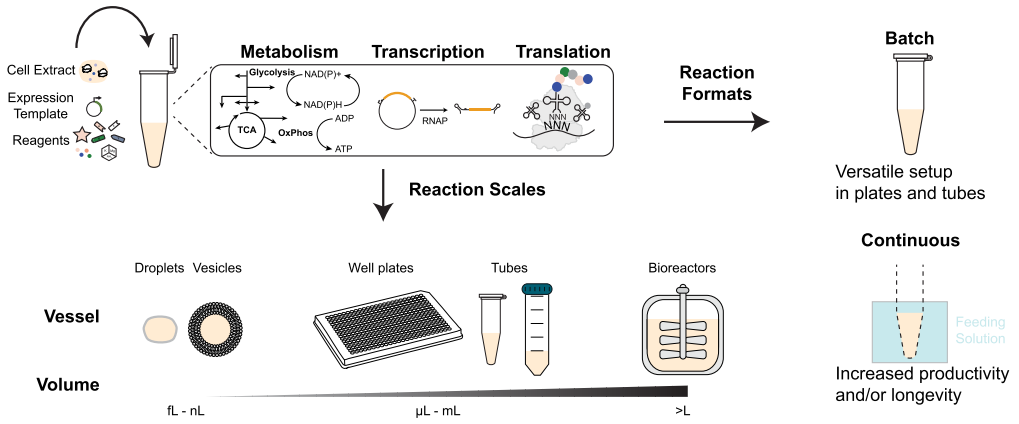

Figure 6. CFE reaction formats and scales. Cell-free reactions carry out metabolism, transcription, and/or translation as a part of gene expression. CFE is perfromed in batch or continuous modes in a range of vessel sizes and geometries.

addition of crowding agents in PURE to mimic the cellular environment has also improved protein yields.[234,237] A recent example has shown that incorporating dextran allows reduction of the proteinaceous components of PURE by up to 97.3% while maintaining protein synthesis yields, allowing PURE to be used more effectively.[238] To prevent ribosome stalling, a known issue in PURE, elongation factors, such as EF-P, GreA, and GreB, can be added to improve protein synthesis.[220] It has also been reported that the addition of ribosomal protein S1 improves PURE by increasing the number of functional ribosomes.[239] The addition of protein chaperones, such as GroEL/ES, can also be useful for proteins that are difficult to fold.[234,240] Additionally, fed-batch reactions where depleted small molecule substrates are added back into the reaction can also improve yields from PURE. Finally, PURE can be improved by using different energy regeneration systems. For example, the use of a polyphosphate kinase to regenerate ATP and GTP in place of the three kinases present in conventional PURE increases yields of active protein from ∼400 μg/mL to >500 μg/mL of sfGFP.[241] These improvements are useful for making PURE more cost-effective and efficient for protein synthesis.

2.5. CFE Reaction Formats and Scales

CFE reactions can be grouped by the function(s), reaction vessel, and format for supplying reagents (Figure 6). Reactions may be transcription-only, translation-only, or combined transcription and translation, offering varying levels of control.[1] Reactions are often executed in batch mode, where the reagents described above are combined and incubated until a limiting reagent is exhausted or inhibiting products accumulate. Batch reactions are versatile and can be performed across multiple scales including: encapsulated emulsions and vesicles at the femtoliter to nanoliter scale, tubes or well-plates at the microliter scale, thin films and test tubes at the mL scale, and bioreactors at the mL to L scale.[29,37,242] However, batch reactions have limited longevity and output due to the accumulation of metabolic byproducts and exhaustion of energy sources. These limitations are addressed in fed-batch and continuous exchange (also referred to as semicontinuous) setups in which additional template, substrates, and/or energy sources are provided during the reaction,[29] increasing reaction longevity and/or yield.[21,153] Continuous reactions (either continuous exchange[175] or continuous flow[243]) are often carried out in dialysis membranes[74] or bilayers[42] such that the CFE reaction is surrounded by a feeding solution to increase the longevity and productivity of gene expression.[28] Alternatively, semipermeable liposomes and microfluidic chips can function like dialysis cassettes to enable continuous reactions.[21,244,245] In all formats, aeration is a significant consideration for sufficient activity of oxidative phosphorylation to generate ATP for transcription and translation.[19]

Additionally, CFE reactions are amenable to incorporation within material matrices, which offer unique options for point-of-use applications and functionalized biomaterials. Key examples include clay mineral gels for studies mimicking early life,[246,247] fibrous matrices like paper for distributable biosensors,[248−250] diverse hydrogels for reaction immobilization,[251−254] and polymer encapsulation for printing sturdy structures imbued with biological functions.[255−257] Two recent reviews focus more extensively on the combination of CFE with materials.[258,259]

2.6. Monitoring and Quantifying the Results of CFE

CFE systems are complex mixtures, which can complicate quantification of the products of their reactions. Evaluating the functional activity of the expressed molecule (e.g., fluorescence, binding, catalytic activity, etc.) or system of molecules (e.g., complex assembly, metabolic pathway productivity, etc.) is frequently the goal; however, quantifying the results of transcription and translation in the system is often crucial to understanding the functional result. Here, we review methods for quantifying RNA and protein produced by CFE systems.

Incorporation of nucleotide radiolabels into RNA[74] and amino acid radiolabels into proteins[260] is the gold standard to quantify the molecules produced during the CFE reaction and does not require modification of the expressed sequences. To quantify transcription, it is common to fuse a fluorescent aptamer to the RNA of interest.[22,51,59,261,262] Similarly, protein fusions — including fluorescent proteins,[187,263,264] high affinity luciferase complementation reporters,[95,265] tetracysteine tags,[266−268] and purification or antibody epitope tags for Western blotting[95,135,141,269,270] — have been used to quantify the products of translation. Other fusions have been suggested,[271] including a fluorescent protein complementation reporter,[272] protease-cleavable fluorescent proteins,[187,264,273] and nanoluciferase.[274]

Several methods to track RNA or protein production in CFE systems have been developed that do not require modification of the expressed molecule. Transcription in the PURE CFE system has been monitored without modifying the transcript using Förster resonance energy transfer (FRET) based binary

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DNA primer probes.[275] As an alternative to radiolabels, tRNAs precharged with a noncanonical amino acid containing a trackable modification (e.g., a fluorophore or biotin) are also widely used to stochastically label the native protein sequences produced in CFE.[276] Furthermore, standard protein visualization techniques like gel electrophoresis and staining can be used to visualize protein expression from an unpurified CFE reaction provided enough product is expressed.[277]

2.7. High-Throughput Experimentation for CFE Applications

CFE systems are valuable tools for accelerating biological research. Below, we highlight technologies that are often coupled to CFE systems to enable high-throughput experimentation.

The simplicity of setting up CFE reactions has led multiple groups to develop automated systems for the manufacture and purification of proteins. Early embodiments combined template and CFE reagents with automated systems to synthesize up to milligram quantities of purified protein.[77,278−280] Systems have also been developed that leverage onboard DNA synthesis and amplification combined with CFE to manufacture purified protein directly from a digital DNA sequence within an integrated instrument.[32,191] These automated protein production systems point to a possible future where protein production is an entirely automated process with the only input from the user being the desired protein sequence, analogous to the advancements made in the DNA synthesis industry.[281] Even when implemented without automation, the process of producing 0.1−1 mg of purified protein via CFE, from generating DNA template to purifying the protein of interest, can require similar effort to completing a plasmid purification kit.[282] These methods can save researchers time and enable them to evaluate more hypotheses given a fixed amount of effort.

Liquid handling robotics can be integrated with CFE systems and used for the rapid setup of hundreds to thousands of distinct conditions using microplates. Traditional tip-based liquid handling robots have been interfaced with CFE systems largely to systematically test hundreds of distinct reaction conditions,[283−285] with recent iterations leveraging fully automated cloud laboratories.[286] Acoustic liquid handlers (e.g., the Echo instruments developed by LabCyte and now sold by Beckman Coulter) have enabled flexible 384 well reaction setup in minutes with reaction volumes as low as 0.5 μL.[287] Acoustic liquid handlers have thus far been used to optimize reaction conditions,[288] improve experimental reproducibility,[208,289] generate data for model parametrization,[51,288,290] and enable high-throughput genetic part[51,112,212] and protein[95,186] prototyping. Custom microwell systems also highlight the potential for smaller reaction volumes and higher density microwells.[291] While lower throughput than the other technologies discussed in this section, microplate and liquid handler-based screening has the significant advantage of requiring less specialized knowledge to implement and being highly adaptable to the application of interest.

Spatial segregation or compartmentalization on a surface is another common strategy for extending the capabilities of CFE systems. Several immobilization strategies using self-assembling microarrays have been implemented including the protein *in situ* array (PISA),[292,293] the multiple spotting technique (MST),[294] the nucleic acid programmable protein array (NAPPA),[295−297] the DNA array to protein array (DAPA),[298] and microintaglio printing (μIP).[299,300] These strategies generally rely on spatial segregation of the template DNAs in a DNA array and noncovalent capture of the synthesized protein to the chip to manufacture the protein array. This allows proteins to be produced *in situ* immediately prior to experimentation and without having to express and purify each individual protein, two major benefits over traditional protein microarrays. In addition to the standard fluorescence-based readouts,[301,302] self-assembling microarrays have also been analyzed using fluorescent single-walled carbon nanotubes[303] and surface plasmon resonance (SPR) sensors.[304]

Analogous to protein microarrays, several microfluidic surface-associated compartment systems containing tens to thousands of distinct compartments for CFE have been developed. These systems have been used to measure gene expression and genetic circuits, molecular interactions,[303,305−307] the self-assembly of proteins[308] and ribosomal subunits,[309] and enzymatic activities.[310] A key benefit of this approach is that the information in each chamber is linked to a known genotype and can sometimes be monitored in real-time. Furthermore, fabricated chip devices offer exquisite chemical, spatial, and temporal control over the process of gene expression and protein assembly, which has enabled the detailed investigations of a myriad of genetic circuits,[245,311−314] molecular machine assemblies,[309,315−317] and synthetic cell systems.[245,313,314] Some iterations of this technology enable millions of defined compartments,[318] although the genotype is not known until individual wells are recovered. Recent work using cell-free displayed proteins spatially segregated on Illumina sequencer flow cells has enabled the collection of large (10^4−10^6 variants) data sets of quantitative and genotype-phenotype coupled measurements of binding proteins.[319,320]

Nonsurface associated compartments are also frequently used to encapsulate CFE systems. The methods vary widely in implementation and include emulsion-based droplets, liposomes, polymersomes, and more.[29,321] These techniques can generate millions[322−325] of compartments via vortex mixing, microfluidics, or other methods depending on the compartment type. Compartments are exciting prospects for CFE-based high-throughput screening and directed evolution,[322−328] though unlike the surface-based microfluidic chambers above, they typically do not provide coupled information about the identity and activity of each condition screened. However, this is not the case for all applications. Droplet-based compartments combined with optical barcoding, the incorporation of unique combinations and concentrations of fluorophore barcodes, enabled the collection of millions of data points on phenotype-genotype coupled droplets to optimize genetic circuits.[329] Compartments have also been used to study the impacts of compartmentalization on gene expression[29,330−333] and to build toward synthetic cells[29,334,335] (Section 3.9.3).

Several display technologies that utilize CFE have been developed.[336] While cell-based display methods typically create the genotype to phenotype linkage through compartmentalization, CFE-based selection methods, like ribosome[337] and mRNA[338] display, frequently leverage a direct physical link between a displayed protein and its transcript to maintain the genotype and phenotype linkage. These methods circumvent the traditional transformation limitation with cell-based

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Table 6. Examples of Cell-Free Synthesized and Functionally Active, Complex Proteins[a]

Year	Protein	Protein Class	CFPS System	Yield (μg/mL)	Complex Elements	Reference
1997	α-Hemagglutinin scFv	Antibody	*E. coli*	8.3	folding chaperone required, disulfide bonded	Ryabova et al.[357]
2003	α-Salmonella O-antigen scFv	Antibody	Wheat germ	13	folding chaperone required, disulfide bonded	Kawasaki et al.[358]
2004	Urokinase protease	Protease	*E. coli*	40	disulfide bonded	Kim et al.[359]
2004	vtPA	Protease	*E. coli*	60	folding chaperone required, disulfide bonded	Yin et al.[360]
2005	scFv-GM-CSF fusion protein	Antibody Cytokine Fusion	*E. coli*	43	folding chaperone required, disulfide bonded	Yang et al.[361]
2005	β2AR	GPCR	*E. coli*	~1,000*	membrane bound	Ishihara et al.[362]
2006	IGF-I	Hormone	*E. coli*	400	disulfide bonded	Swartz[363]
2008	HydA1	[FeFe] Hydrogenase	*E. coli*	22	cofactors, oxygen sensitive	Boyer et al.[364]
2011	rhGM-CSF	Cytokine	*E. coli*	700	folding chaperone required, disulfide bonded	Zawada et al.[37]
2011	cIFN-α	Cytokine	*E. coli*	400	solubility issues	El-Baky et al.[365]
2011	ATP Synthase	Molecular Machine	*E. coli*	NA	folding chaperone required, membrane bound, multisubunit	Matthies et al.[366]
2011	HBc	Virus-Like Particle	*E. coli*	125	disulfide bonded, macromolecular assembly	Bundy et al.[367]
2014	VH3−7/Vk3−20 IgG	Antibody	*E. coli*	~1,500	folding chaperone required, disulfide bonded, multisubunit	Groff et al.[368]
2014	ERBB2 (HER2)	Receptor Kinase	*E. coli*	2	membrane bound	He et al.[369]
2017	α-NIP IgG	Antibody	CHO	114*	folding chaperone required, disulfide bonded, multisubunit, glycosylated	Martin et al.[107]
2017	α-SMAD2 IgG	Antibody	CHO	9*	folding chaperone required, disulfide bonded, multisubunit, glycosylated	Stech et al.[108]
2017	HuNoV VP1-GII.3	Virus-Like Particle	*E. coli*	620	macromolecular assembly	Sheng et al.[370]
2019	α-HER2 IgG	Antibody	PUREfrex	124	folding chaperone required, disulfide bonded, multisubunit	Murakami et al.[371]
2021	PD glycosylated with *F. tularensis* O antigen	Glycoprotein	*E. coli*	20	glycosylated	Stark et al.[141]
2021	PD glycosylated with *C. jejuni* glycan	Glycoprotein	*E. coli*	43	glycosylated	Hershewe et al.[135]
2023	PD glycosylated with ETEC O78 glycan	Glycoprotein	*E. coli*	~150	glycosylated	Warfel et al.[45]

[a]Abbreviations: scFv: antibody single-chain variable fragment, vtPA: variant of human tissue-type plasminogen activator, GM-CSF: granulocyte macrophage colony stimulating factor, IGF-I: insulin-like growth factor I, cIFN-α: consensus human interferon-alpha, rhGM-CSF: human granulocyte macrophage colony-stimulating factor, PD: Haemophilus influenzae protein D, IgG: Immunoglobulin G, β2AR: β2 adrenergic receptor, GPCR: G protein coupled receptor, HBc: Hepatitis B core antigen, HuNoV VP1-GII.3: Human norovirus genotype GII.3 VP1 capsid gene. *Indicates non-batch CFE reaction yield.

techniques and in principle enable library sizes of up to 10^{14} variants.[339,340] Generally, these systems have been applied to the selection of proteins that bind a desired target (Section 3.4.2), although they have also been expanded to map protein−protein interaction networks,[341−344] evaluate protein stability,[345−349] and evolve certain classes of enzymes.[350]

2.8. Modeling CFE Systems

While CFE serves as an excellent platform for both product synthesis and prototyping for *in vivo* systems, extract-based CFE systems are complex mixtures with not all parts defined. Purified CFE systems, on the other hand, are well-defined but still involve many different components forming a complex biological reaction network.[351] Models of CFE systems seek to better understand the activities and formation or degradation rates of essential components in CFE systems such as metabolic resources, inhibitory products, ribosomes, RNA polymerase, mRNA, and proteins. Due to the open reaction environment, CFE components can be tuned by varying the amount of DNA or other agents added to the system. Therefore, experiment-driven models can be constructed and validated to capture specific interactions such as production bottlenecks and predicting protein synthesis, which is reviewed elsewhere.[352]

Typical models are based on ordinary differential equations, using Michaelis−Menten and Hill-Langmuir equations to describe different components of system behavior. The granularity of the model determines how many equations are needed, with coarse grained models of protein transcription, translation, and degradation having as few as four equations[353,354] and complex models of reaction networks[351,355] requiring hundreds of equations. Models are often fit with experimental data to determine parameters, such as the parameters describing transcription and translation kinetics. While coarse-grained models are adept at broadly capturing trends in relative rates,[353] with translation identified as a key target for overcoming experimental bottlenecks,[290] discrepancies between published model-derived parameters and experimental results could be due to differences in extract-based systems between laboratories. The recent development of a toolbox for modeling CFE-based genetic circuits will lower the barrier to entry for future researchers.[356]

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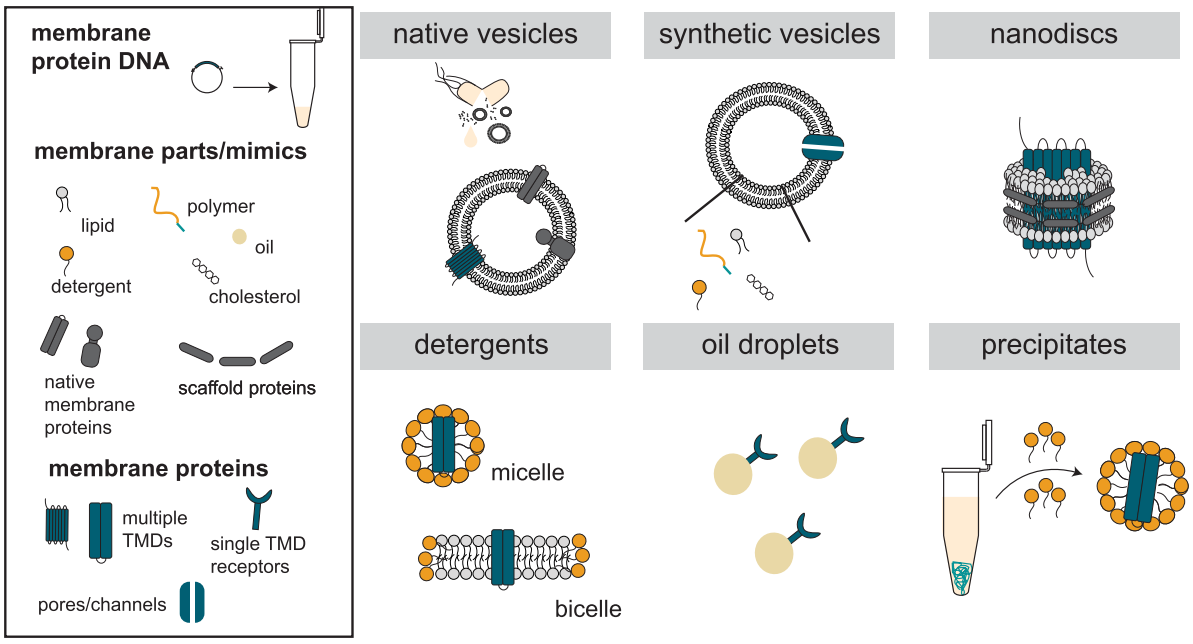

Figure 7. Components for expressing soluble membrane proteins using CFE. Membrane mimics in the form of native vesicles, synthetic vesicles, nanodiscs, detergents, and oil droplets can be supplemented to or enriched in cell-free systems to enable the soluble expression of membrane proteins with various numbers and lengths of transmembrane domains (TMDs). Alternatively, cell-free systems have been used to express insoluble membrane proteins for subsequent refolding.

3. APPLICATIONS OF CELL-FREE GENE EXPRESSION

CFE systems have been transformed by improvements in productivity, cost, scale, and complexity. Below, we highlight example application areas that take advantage of these improvements.

3.1. Expression of Proteins Using CFE

CFE systems make an ideal tool for the customized expression of proteins. To date, CFE systems have enabled the expression of disulfide bonded proteins, proteins that require folding chaperones, post-translationally modified proteins, membrane proteins, multi-subunit proteins, proteins containing oxygen sensitive cofactors, and more (Table 6). Here, we detail the efforts to use CFE to produce different types of functionally active, complex proteins.

3.1.1. Proteins Requiring Folding Chaperones. Many proteins, particularly large, multi-domain proteins, require the assistance of chaperones[372,373] and other regulatory mechanisms (e.g., modulated translation rates[374]) to properly fold. Interestingly, *E. coli* CFE systems typically have a translation rate more than an order of magnitude lower than living *E. coli*[179] (~1 amino acid/second), which is closer to that of eukaryotic translation[375] and may be beneficial for proteins that require more time for cotranslational folding.[374] However, this alone may not be sufficient to support proper folding of every complex protein in CFE systems. Furthermore, purified CFE systems by design contain no chaperones and extract-based systems may or may not have sufficient levels of endogenous chaperones to adequately support proper folding, and thus CFE systems are frequently supplemented with additional chaperones (e.g., DnaK/DnaJ/GrpE, GroEL/GroES).[357,368] In one case, a ribosome targeted fusion of the HSP 70 chaperone (BiP), which has a function analogous to that of DnaK, resulted in improved soluble yields of eukaryotic proteins expressed in an *E. coli* CFE system.[376] In another example, supplementing FkpA and SlyD prolyl isomerases as well as the SkpA deaggregase improved the yields of soluble antibody.[368] Artificial chaperone systems consisting of polysaccharide nanogels have also been shown to improve the soluble expression of proteins prone to aggregation.[377]

In addition to traditional chaperones, fusion proteins (e.g., maltose binding protein (MBP), thioredoxin (TRX), small ubiquitin-related modifier (SUMO), glutathione-S-transferase (GST)), which are thought to improve the folding and solubility of proteins, have also been utilized in CFE.[95] Interestingly, proteases like the tobacco etch virus protease are active in some CFE systems, enabling production of protein fusions and subsequent cleavage of the fusion partner in a one-pot format.[95] CFE has also been used to both express chaperones and subsequently assess their impact on folding of a target protein, a strategy that could accelerate researchers' ability to identify the chaperones required for a given protein.[378] Chaperone function and specificity can also be studied using CFE systems as has been done for the DnaK/DnaJ/GrpE chaperone system.[379]

3.1.2. Proteins Containing Disulfide Bonds. Disulfide bonds are critical elements of many proteins that serve to stabilize their structure.[380] Disulfide bonds are formed in specialized compartments or organelles—the endoplasmic reticulum (ER) in eukaryotes and the periplasm in prokaryotes—which maintain an oxidizing environment to enable spontaneous disulfide bond formation.[381] This oxidizing environment is enabled by systems of enzymes that maintain the redox potential and assist with the formation of the correct disulfide linkages.[381] Both features of these compartments can be recapitulated in a CFE systems. An oxidizing environment can be maintained through the addition of small molecules (e.g., oxidized (GSSG) and reduced (GSH) glutathione).[382] Extract-based CFE systems are also frequently treated with iodoacetamide (IAM), an alkylating reagent that inactivates the endogenous redox enzymes of the extract, to stabilize the redox environment.[359] Furthermore, the enzymes

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responsible for disulfide bond isomerization, PDI in eukaryotes[381] and DsbC in prokaryotes,[381] can be supplemented to enable the proper formation of disulfide bonds.[368]

Many different proteins containing disulfide bonds have been successfully manufactured using CFE, including human hormones,[363] cytokines,[37,365] enzymes,[359,360] and antibodies[107,108,357,358,368,371,382] (Table 6). To quantify the progress of CFE systems capable of manufacturing disulfide bonded proteins, it is instructive to track the manufacture of antibodies.[382] The first efforts to manufacture antibodies started with simpler single chain antibody variable fragments (scFvs) and yielded 8.3 μg/mL of protein.[357] Modern CFE systems enable the manufacture of full-length heterotetrametric IgG antibodies with yields from 114 μg/mL (Chinese hamster ovary cell (CHO) CFE, glycosylated)[107] to 150 μg/mL (*N. tabacum* BY-2 CFE extracts)[99] to approximately 1,500 μg/mL (*E. coli* CFE, aglycosylated).[368] These advances enable CFE not only to be a research tool for studying and prototyping disulfide bonded proteins, but also to be a viable manufacturing platform.[37,43]

3.1.3. Membrane Proteins. Despite the absence of an intact cellular membrane, CFE platforms offer ways to enable functional membrane-bound protein expression (Figure 7).[383,384] During extract preparation, native cellular membranes are disrupted at the lysis step and self-assemble to form vesicles[385] (e.g., cytoplasmic/periplasmic membrane vesicles in bacteria;[135,386] ER/golgi microsomes in eukaryotic extracts[387]). In *E. coli* CFE, functional membrane-bound proteins (e.g., oligosaccharlytransferases) have been enriched in *E. coli* extracts prior to lysis,[135,269] but synthesis of functional membrane proteins has frequently required supplementation of purified native membrane vesicles.[385,386,388] Similarly, in eukaryotic CFE platforms, cell-free membrane protein expression has been demonstrated using both supplemented (i.e., exogenous microsomes in rabbit reticulocyte extracts[389]) and enriched (i.e., endogenous microsomes in HeLa[390] and insect[387,391,392] extracts) microsomes.[383] While native vesicles preserve the membrane composition of the extract source strain they offer little control over the membranes in the final extract, and vesicle supplementation requires lengthy purifications.[386]

Synthetic membrane vesicles or liposomes can be supplemented to CFE reactions as alternatives to native membranes and enable precise control over membrane properties. Examples include aquaporin Z[393] and ATP synthase in 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes.[366] By changing properties of the lipids such as saturation, acyl chain length, charge, and curvature, or incorporating additives (e.g., polymers, cholesterol), membrane properties (e.g., elasticity) and membrane protein expression efficiency can be modulated.[384] This was shown to improve folding of a mechanosensitive channel protein (MscL) in PURE.[394] The hydrophobic matching of the protein and the lipid membrane thickness is also an important factor for expression and insertion into the membrane.[395,396] Synthetic vesicles in PURE have also been used to systematically stop and start translation to study membrane protein folding and the effects of different lipid compositions.[397,398] Due to the control over vesicle formation, membrane vesicles can also be used to encapsulate CFE reactions to make artificial cells (Section 3.9.3). Biomolecular condensates provide an alternative platform for synthetic cell formation, linking the chemical and cellular stages of living systems.[399]

Nanodiscs—noncovalent nanoscale assemblies composed of a lipid bilayer surrounded by amphiphilic scaffold proteins[400]—have also been used to synthesize diverse membrane proteins including oligosaccharyltransferases (OSTs),[270,401] the methyltransferase Opi3,[402] the antigenic vaccine carrier pore protein PorA,[141] and the chemokine GPCR CCR5.[384,403] In nanodiscs, both sides of the lipid membrane are exposed to the CFE reaction, which enables the study of both extracellular ligand binding and cytosolic activity of proteins without concern for orientation in the membrane.[383] However, the design of nanodiscs (e.g., size) is more constrained than synthetic vesicles due to the need for membrane scaffold proteins. Nanodiscs remain an attractive membrane mimic as they are commercially available and can be affinity tagged for purification of an associated membrane protein.[383,404]

Other synthetic components have been used to express functional membrane proteins in CFE. For example, detergents—typically used to resolubilize protein aggregates by forming micelles with hydrophobic centers—have been supplemented into CFE for soluble membrane protein expression.[270,362,405,406] Alternatively, precipitate-forming CFE takes advantage of the rapid and high levels of insoluble membrane protein expression followed by solubilization with detergents or reconstituted in membranes.[407,408] A major disadvantage of detergents is their disruption of membranes and incompatibility with some downstream applications.[383,409] Oil droplets have also been added to purified CFE to enable single pass transmembrane protein expression and assessment of surface receptor activity.[410] Work enabling *in situ* lipid synthesis could enable production of lipids and membrane mimics in CFE itself toward more autonomous expression systems.[411,412]

3.1.4. Glycosylated Proteins. Protein glycosylation—the enzymatic process that attaches oligosaccharides to amino acid side chains in proteins—is found in all domains of life[413] and is of interest for a variety of therapeutic applications.[414,277] For example, *N*-linked protein glycosylation is present on many therapeutically relevant proteins, such as antibodies.[415,416] Glycosylation occurs in the endoplasmic reticulum (ER) of eukaryotes where a multisubunit enzyme called an OST is responsible for the transfer of a lipid-linked oligosaccharide (LLO) to a protein at a specific amino acid residue recognized by the enzyme.[415] Studying and manufacturing glycoproteins in cells, especially with homogeneous glycans, remains difficult, and CFE offers a new strategy to make such products.

Eukaryotic organisms possess post-translational glycosylation systems that have allowed eukaryotic extracts to be used to study glycosylation mechanisms[417,418] and produce glycoproteins (e.g., antibodies,[108] erythropoietin (EPO)[419]).[28,420] Specifically, microsomes of ER and Golgi components can successfully glycosylate proteins containing signal sequences for translocation.[177] *N*-linked glycoproteins have been synthesized in multiple eukaryotic CFE systems including CHO, insect,[391] human,[68] hybridoma,[109] and tobacco.[99] Streamlined microsome enrichment protocols[391] and semi-continuous reaction formats[108,421] have increased glycoprotein yields.

While many prokaryotic organisms do not have endogenous glycosylation pathways,[422] glycoengineering strategies have emerged to build glycoproteins in bacterial CFE systems. For example, *E. coli* CFE systems, which do not have the capability to glycosylate proteins natively,[177] have been engineered to glycosylate proteins via two pathways: (i) OST-dependent -

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Table 7. Examples of Functionally Active, Toxic Proteins and Peptides Manufactured Using Various CFE Systems

Year	Toxic Protein(s)	Toxic Protein Origin	CFPS System	Reference
2001	Cecropin P1	*Ascaris suum*	*E. coli*	Martemyanov et al.[264]
2021	Hemolysin BL	*Bacillus cereus*	*Chinese hamster ovary (CHO)*	Ramm et al.[443]
1974	Colicin E1	*E. coli*	*E. coli*	Eichenlaub[444]
2010	α-Hemolysin	*E. coli*	*E. coli*	Chalmeau et al.[445]
2018	Colicins Ia, E1, and E2	*E. coli*	*E. coli*	Jin et al.[446]
2022	Heat-labile enterotoxin (LT)	*E. coli*	*Chinese hamster ovary (CHO) and Spodoptera frugiperda (Sf21)*	Ramm et al.[447]
1998	Enterocins L50A and L50B	*Enterococcus faecium*	*E. coli*	Cintas et al.[448]
2021	U2-sicaritoxin-Sdo1a (venom component)	Hexophtalma dolichocephala	*PURE, E. coli, and Spodoptera frugiperda (Sf21)*	Lüddecke et al.[442]
2011	Pierisin-1b	*Pieris rapae*	*Spodoptera frugiperda*	Orth et al.[449]
2016	Exotoxin A	*Pseudomonas aeruginosa*	*E. coli*	Krinsky et al.[450]
2011	A2	*Qubevirus durum*	*E. coli*	Smith et al.[451]
2016	Onconase	*Rana pipiens*	*E. coli*	Salehi et al.[140]
2022	Cholera toxin (Ctx)	*Vibrio cholerae*	*Chinese hamster ovary (CHO) and Spodoptera frugiperda (Sf21)*	Ramm et al.[447]
2013	Thermostable direct hemolysins	*Vibrio parahemolyticus*	*E. coli*	Bechlars et al.[452]
2017	Thermostable direct hemolysins	*Vibrio parahemolyticus*	*E. coli*	Dondapati et al.[453]

which uses an en bloc transfer mechanism and (ii) OST-independent—which harnesses individual glycosyltransferases to transfer each sugar and to build glycan structures from the bottom up. The first instance of cell-free glycoprotein synthesis was achieved with the supplementation of the well-characterized OST from *Campylobacter jejuni* (CjOST)[423] and LLO[424] from *C. jejuni* (Cj) to a CFE reaction to glycosylate a cell-free synthesized protein containing a glycosylation tag.[425] A one-pot cell-free glycoprotein synthesis platform was then developed that expresses the membrane-bound glycosylation machinery in the extract source strain prior to extract preparation.[269] By enriching exogenous glycosylation systems (e.g., CjOST and LLOs) in *E. coli* extracts or expressing them with CFE,[270,401] glycoproteins can be produced.[135,269,277,426] OST immobilization can also enable continuous glycoprotein synthesis in microfluidic devices.[427] CjOST and homologues have been used to transfer the human N-linked trimannose core, *C. jejuni* and *C. lari* glycans, as well as various O-antigens from pathogenic bacteria.[141,269] O-linked glycosylation has been shown for the *C. jejuni* glycan as well as human cancer-related mucin-type T and Tn antigens.[135,428] CFE has also been used to rapidly synthesize low-cost and thermostable[45,135] protective antibacterial conjugate vaccines against *Francisella tularensis* from lyophilized reactions.[141]

OST-independent glycosylation in *E. coli* CFE can enable high-throughput screening and building of glycan biosynthesis pathways.[277,429] These methods have also been used for high-throughput screening of glycosylation tag sequence specificity[429] and site preferences of *N*- and *O*- linked glycosyltransferases from bacteria and humans.[430] This method also enabled the screening and identification of orthogonal enzymes capable of multisite glycosylation of target proteins.[431] Another study on the modular expression and mixing of different glycosyltransferases and activated sugar donors in the CFE resulted in 20+ new glycan structures with possible applications for therapeutics, biomaterials, and antitoxins.[277]

3.1.5. Proteins With Other Post-Translational Modifications. Additional post-translational modifications (PTMs) are enabled in extracts by native biological machinery present in eukaryotic extract source strains. Phosphorylation,[432,433] lipidation,[434,435] *N*-acetylation,[436,437] ubiquitination,[438] as well as signal peptide processing,[391] have all been observed in cell-free systems derived from insect or rabbit reticulocyte cells. ER microsomes are required for many of these modifications.[28,177] While *E. coli* extracts are not typically used for making PTMs, a prenyltransferase-enriched *E. coli* extract enabled higher yields of prenylated protein than reported in insect cell-free systems.[439] Alternatively, ncAAs can be leveraged to add PTMs to proteins, such as the incorporation of L-phosphoserine into proteins cotranslationally in *E. coli* cell-free systems[440] (Section 3.2).

3.1.6. Toxic Proteins and Peptides. The lack of a cell viability constraint makes CFE a promising alternative to manufacture proteins that are toxic to cells when expressed.[441] As a result, CFE has been used for decades to study the activities of peptide and protein based toxins, and with advancements in reaction productivities, it is also now being considered as a viable manufacturing route for potential therapeutic molecules that are not possible to be produced in traditional expression systems.[140] To date, many functionally active proteins with diverse toxic effects from organisms ranging from bacteriophages to eukaryotes have been expressed using both eukaryotic and prokaryotic CFE systems (Table 7). Even for toxic proteins with activities that impact transcription or translation, it may be possible to express the protein in CFE. For example, onconase—a nuclease that degrades tRNAs and has potential as a cancer therapeutic—was successfully manufactured in *E. coli* based CFE by supplementing additional tRNAs over the course of the reaction.[140] It is important to note that not all CFE systems are suitable for a given target protein, particularly for small peptides,[442] and thus it may be important to evaluate multiple different CFE systems for the production of the desired molecule.

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3.2. Incorporation of Noncanonical Amino Acids into Proteins

Noncanonical amino acid (ncAA) incorporation provides a powerful opportunity to decorate proteins with user-defined chemistries. These chemistries are diverse,[454,455] ranging from modifications of the side chain to changes that can alter the underlying polypeptide backbone, and they provide unique opportunities to study protein structure and expand protein function.[456−458] The interface between ncAA incorporation and CFE provides several advantages. For instance, users can suppress codons in ways that would be technically difficult to accomplish in cells or use ncAAs that would interfere with cell viability. In addition, the open reaction environment allows for both the addition and removal of components that facilitate efficient ncAA incorporation, such as the inclusion of engineered aaRSs and tRNA pairs and the usage of ncAAs that do not easily cross the cell membrane. Thus, CFE provides an efficient reaction format for ncAA incorporation into peptides and proteins (Figure 8).

There are several ways to incorporate ncAAs *in vitro*, including global suppression and site-specific incorporation. Global suppression, or residue-specific incorporation, takes advantage of natural aaRS promiscuity to incorporate ncAAs in place of canonical amino acids.[459] By replacing a canonical amino acid with a close structural analog within a CFE reaction, an aaRS can acylate its cognate tRNAs with the structural analog to enable global suppression. This methodology efficiently recodes multiple sense codons for a ncAA but is limited by aaRS substrate specificity and requires residue-wide substitution of the ncAA. In site-specific incorporation, a codon is reprogrammed to encode a ncAA through a variety of techniques such as chemical modification of the acylated amino acid on the tRNA,[460−462] chemoenzymatic ligation of an acylated adenosine onto a 3′ truncated tRNA,[463−465] enzymatic acylation using aaRSs,[466] and flexizyme-mediated methods.[467] Of these methods, enzymatic acylation using aaRSs and flexizyme-mediated methods have become the most widespread. aaRSs are much better catalysts, enabling higher yields of acylated tRNA, but have limited substrate range.[466] On the other hand, because flexizymes recognize the leaving group of an activated ncAA and the near-universally conserved 3′-CCA of a tRNA, they enable nearly limitless customization of the genetic code at the cost of the catalytic performance.[467] Both options, however, enable more specific engineering of the genetic code compared to global suppression systems.

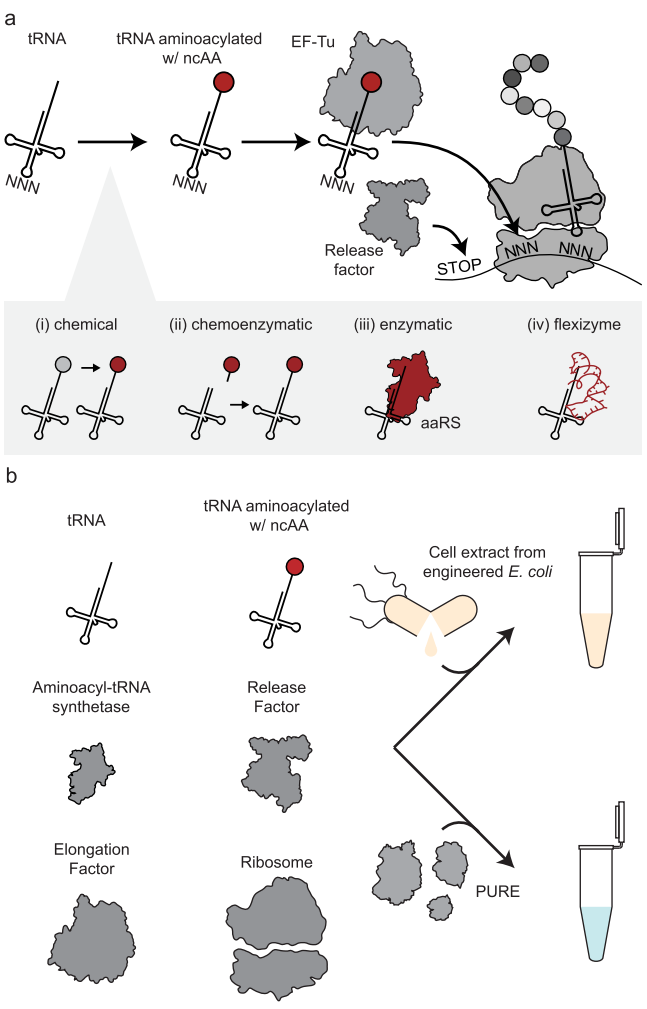

Figure 8. Incorporation of ncAAs into proteins using CFE. (A) Process for ncAA incorporation using CFE. (B) Important components involved in ncAA incorporation in CFE.

3.2.1. Incorporation of Noncanonical Amino Acids Using PURE. ncAA incorporation within the PURE system benefits from the flexibility in adding, removing, and titrating purified translation components. Many examples have used the Flexible *in vitro* Translation system (FIT), which couples flexizyme-based tRNA aminoacylation with PURE.[468] In FIT, tRNAs charged with flexizymes are added into custom PURE reactions where competing translation components, such as amino acids or aaRSs, are omitted. This enables radical reprogramming of the genetic code. Additionally, FIT can be supplemented with EF-Tu and EF-P, in conjunction with tRNAs engineered to recruit those translation factors, to enable the ribosomal incorporation of backbone-modified ncAAs, such as D-, β-, cyclic β, cyclic γ, aminobenzoic acid, α-aminoxy, α-hydrazino, and thioamide-containing ncAAs.[469−475] aaRSs can also be used in PURE to enable genetic code expansion. Orthogonal, amber-suppressing pyrrolysyl-tRNA synthetases and tRNA pairs like those from *Methanosarcina mazei* and *Candidatus Methanomethylophilus alvus* are widely used.[476,477] Genetic code reprogramming by substituting endogenous aaRSs for more promiscuous mutants, such as PheRS A294G, LeuRS D345A, and editing deficient ValRS T222P, has also been reported.[478,479]

One application for the diversity of ncAAs compatible with PURE-based systems is peptide drug discovery. A method called Random nonstandard Peptide Integrated Discovery (RaPID) couples FIT with mRNA display to identify ncAA-containing peptides that have potent binding toward a protein of interest. ncAAs in RaPID can serve many purposes, such as a cyclization handle to form peptide macrocycles,[511] as chemical warheads or drug delivery vehicles,[512,513] or merely as a source of additional diversity in peptide libraries. Often, the ncAAs are indispensable for the function of interest. Several excellent reviews provide in-depth discussions about peptides discovered in RaPID.[230,514,515] In addition, ncAA incorporation can also facilitate the discovery of peptide drugs that mimic ribosomally synthesized and post-translationally modified peptides (RiPPs).[516] One benefit is the ability to enable chemistries that mimic those typically installed post-translationally by enzymes. For example, azoles and dehydroalanines can be installed into peptides by chemical modification of ncAAs, which expands the chemical diversity of peptides that can be

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ribosomally synthesized.[517,518] These unnatural peptides can be modified with RiPP biosynthetic enzymes to further diversify peptide chemistries. The ncAAs incorporated through these methods thus may enable the discovery of novel peptide drugs.

PURE uniquely enables the study of genetic code architecture, as *in vivo* changes to the genetic code are largely lethal. For example, reconstitution of PURE with a minimized set of tRNAs, aaRSs, and amino acids, can vacate codon boxes to create a genetic code with only NNS (S = G or C) codons.[519] PURE can be further simplified by using only 21 tRNAs, thus creating a minimal 20 codon genetic code.[520] This should enable the newly vacated codons to be reassigned for ncAAs. In another example, the codons for serine, leucine, and alanine were swapped to create genetic codes that could only correctly decode functional proteins using a set of mis-acylated tRNAs.[521] PURE might also enable studies on nonspecific decoding of codons through wobble-base pairing, which might highlight how new genetic codes might be used for more accurate ncAA incorporation.[522]

PURE provides an environment within which translation factors can be studied and engineered. PURE and flexizyme-charged tRNAs were used to elucidate identity elements for EF-P binding in Pro-tRNA^{Pro}.[481,487] This study systematically dissected how the amino acid and tRNA sequence affect EF-P recruitment to alleviate ribosome stalling at proline codons. Similarly, studies have identified tRNA identity elements that serve to recruit EF-Tu, which helps to transport ncAA-charged tRNAs to the ribosome.[485,488] Other engineered translation components in addition to tRNAs can be used within PURE. Ribosomes that recognize tRNAs containing non −CCA 3′ ends have been engineered to enable two orthogonal genetic codes to function in parallel, allowing for two different peptides to be synthesized from the same DNA template.[523] These technologies might allow for the *in vitro* engineering of ribosomes within PURE to polymerize nonpeptidyl backbones.

PURE has proven itself as a uniquely powerful technology that enables research into areas that are inaccessible by lysate-based CFE or cell-based methods. However, it may not be appropriate for all applications in this space. For example, average protein yields from PURE are typically 5−10x less than crude-extract based systems.[21,23] This may limit the ability to synthesize proteins at the scales required for characterization studies. Additionally, because each translation component in PURE must be purified, PURE is significantly more expensive and labor intensive than extract-based approaches ($1,000 per mL reaction compared to $4.67 per mL in batch mode, respectively).[1] Despite these disadvantages, PURE is positioned to enhance our understanding of how translation can be engineered for ncAA incorporation.

3.2.2. Incorporation of Noncanonical Amino Acids Using Extract-Based CFE. Cell extracts provide unique features for ncAA incorporation into proteins. They are higher yielding and simpler to prepare than PURE, but the presence of cytoplasmic components (e.g., proteases; nucleases; endogenous aaRSs, tRNAs, and amino acids) can be detrimental for ncAA incorporation into proteins (Table 8). Recent progress has shown that cells can be engineered to produce extracts that are more effective for ncAA incorporation into proteins. For example, to combat premature protein truncation by Release Factor 1 (RF1), which decreases amber codon suppression efficiency,[497,524] extracts can be prepared from cells either containing a thermosensitive RF1[524] or lacking RF1 completely.[525,526] Two *E. coli* derivatives where RF1 is removed from the genome, the C.321Δ*A* and B.95Δ*A* strains, can be used for extract preparation. Further genome engineering of C.321Δ*A* to remove negative effectors of CFE (e.g., proteases, nucleases) and to introduce a T7 RNA Polymerase yielded the 759.T7 strain, which can incorporate two *p*-acetylphenylalanine (pAcF) residues into a single sfGFP at yields >2 g/L and 40 instances of pAcF into an elastin-like polypeptide in a batch reaction.[23,117] Extracts made from B.95Δ*A* are also highly productive, showing >3 g/L into 1TAG-sfGFP in a continuous-exchange format. These engineered extracts are thus highly useful tools for ncAA incorporation in CFE.[527]

CFE reaction formulations themselves can also be customized for efficient ncAA incorporation. First, translation components such as the aaRS and tRNA can be coexpressed in CFE along with the protein of interest.[491,502,528] This strategy diverts resources away from synthesizing the protein of interest but can be useful for applications such as aaRS or tRNA engineering. Second, heterologous translation components can be enriched in cell extracts by overexpression in cells prior to lysis.[489] This is particularly useful with components that are difficult to purify, such as the commonly used *M. mazei* and *M. barkeri* pylRSs. Finally, endogenous translation components can be removed from cell extracts. For example, endogenous *E. coli* tRNAs can be inactivated or depleted using chromatog-

Table 8. *In Vitro* Translation Reactions Can Be Customized by the Addition and Removal of Translation Components to Facilitate ncAA Incorporation[a]

Translation Component	PURE CFE	Exctract-Based CFE
tRNAs	+ tRNA^{fMet}[478,480−482]	+ tRNA^{Pyl}[489,490]
	+ tRNA^{AsnE2} [483,484] variants of tRNA^{AsnE2}[485]	+ tRNA$^{M.jannashii\ Tyr}$[491]
	+ tRNA^{GluE2}[486]	- RNase digestion of tRNAs[492,493]
	+ tRNA^{Pro1E2}[487,488]	- Chromatographic depletion[494,495]
	+ tRNA^{Phe}	-Inactivation using antisense oligonucleotides[496]
	+ tRNA^{Leu}	
	+ tRNA^{Pyl}	
aaRS	+ PheRS A294G *E. coli*[478]	+ *M. jannaschii* TyrRS variants[117,497−500]
	+ LeuRS D345A *E. coli*[478]	+ *M. alvus* PylRS and variants thereof[501]
	+ ValRS T222P *E. coli*[479]	+ *M. mazei* PylRS and variants[489,490,502]
	+ PylRS *M. mazei*[476,477]	
	+ PylRS *M. alvus*[476,477]	
	- Endogenous aaRSs through exclusion in PURE dependent on choice of codon and amino acid.[468]	
Elongation Factors	+ EF-Tu and variants[503−505]	+ EF-Tu and variants[440,506]
	+ EF-P[470,488]	
Release Factors	- RF-1[26]	- Inhibition of RF-1
		- Degradation of RF-1[507]
Ribosome	+ 040329[508]	- Depletion of ribosomes through ultracentrifugation[509]
		+ *in vitro* constructed ribosomes support ncAA incorporation[510]

[a]Supplemented components are indicated with a (+) and withheld or removed components are indicated with a (−).

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raphy,[494,495] enzymatic digestions,[492,493] and antisense oligonucleotides,[496] and endogenous amino acids can be dialyzed away to enable the use of global incorporation strategies,[529,530] notably for the incorporation of isotopically labeled amino acids as spectroscopic probes.[46,531] These examples, as well as others (Table 8), show strategies in CFE to improve, simplify, or expand the range of ncAA incorporation.

ncAA-containing proteins made using CFE have many applications. For example, CFE has been used to commercially synthesize antibody-drug conjugates that incorporate ncAAs such as *p*-azidophenylalanine and *p*-azidomethylphenylalanine[532] that serve as click chemistry handles. ncAAs may also be incorporated into enzymes, enabling the studies on how novel chemistries could improve enzyme catalysis and introduce enzyme regulation strategies.[533,534] Conversely, the incorporation of ncAA mimics of post-translational modifications (PTMs) can inform the function of PTMs.[535] A CFE system engineered for efficient phosphoserine incorporation, for example, was used to study the role of phosphorylation patterns on human MEK1 kinase activity.[440] In addition, ncAAs can serve as biophysical probes for spectroscopic studies, including fluorinated ncAAs for ^{19}F NMR[536] and cobalt-binding ncAAs as paramagnetic labels,[537] and as fluorescent probes to detect changes in local environment[538,539] and to detect long-range conformation changes using FRET.[540−542] Thus, CFE has enabled the incorporation of many ncAAs that serve numerous applications.

Although both PURE and extract-based CFE systems have their merits for ncAA incorporation into proteins, the trade-off between yield, cost, genetic code reprogramming capability, and efficiency must be carefully considered.

3.3. Scale-Up for Decentralized Protein Manufacturing

CFE systems are poised to enable a new approach to decentralized manufacturing.[141,543,544] To enable this vision, cell-free systems are scaled beyond their typical μL laboratory-scale volumes. Over the past 15 years, technological breakthroughs have shown that CFE reactions are linearly scalable between the μL and 100 L scales, enabling high yields of proteins.[37,545] For example, in some initial studies, reaction volumes of 2 mL, 50 mL, and 1 L all produced human insulin-like growth factor-1 (IGF-1) at the same rate in stirred tank reactors.[546] Beyond protein synthesis, cell-free reactions that require *in vitro* enzymatic activity but not protein synthesis[547] have been scaled to 20,000-L reactors to produce myo-inositol with heat-purified enzymes.[548]

A few key works highlight important considerations for scalable, cell-free protein production.[37,71] First, cost remains a major consideration for large scale reactions. The primary cost determinants are lysate preparation, carbon and energy sources in the reaction, DNA, and additives such as CoA, NAD, and NTPs.[1,45,157] As discussed in Section 2.3.1, high-cost phosphorylated energy substrates such as PEP can be replaced by more economical substrates such as glutamate,[157] which harness oxidative phosphorylation and native metabolism of the cell extract. Second, extract processing must also be optimized for efficient large-scale production. Source strain modifications can improve yields by stabilization of extract components such as amino acids.[549] To increase process throughput, disk stack centrifugation has been used to process hundreds of liters of cells for extract production and can be combined with increased dilution of the cell suspension before lysis to improve extract clarification.[37] Extract processing can also be streamlined by removal of the dialysis step,[128,130] however it should be noted that dialysis has been identified as important for applications involving transcription from native promoters using RNA polymerases present in the extract.[122] Third, transport limitations must be considered as oxygen is required for reaction formulations relying on extract metabolism for energy regeneration. One scale-up issue is that CFE reactions can foam when agitated in traditional bioreactors. The addition of antifoam agents has been optimized to reduce foaming without negative impacts on yields.[546] While most optimization has focused on bacterial systems, recent work in a *N. tabacum* BY-2 CFE system highlights that scale up with eukaryotic systems is also feasible.[71]

Amenability to lyophilization[550] and reaction modularity make CFE an ideal approach for decentralized biomanufacturing. For example, point-of-care diagnostics[142,143,250,551] and therapeutics[139−141] can readily be distributed when lyophilized without cold-chain storage. Further, the Bio-MOD (biologically derived medicines on demand) platform—an integrated suitcase-sized device for small-scale GMP production and purification of therapeutic proteins such as GCSF and erythropoietin—was developed using CFE from lyophilized CHO cell extracts.[543] Thermostability of CFE reactions can be improved through the addition of lyoprotectant additives or strategic separated storage of reaction components;[45,138,147,148,262,552] these strategies have resulted in the cell-free production of pyocin,[138] nanobodies,[553] a protein-based vaccine (DT),[553] conjugate vaccines,[45,554] and a cancer therapeutic (ErA) following storage above ambient conditions.[552]

Numerous works have sought to decrease cost of CFE reaction components while maintaining high protein yields.[41,43,45,155,156] However, reported cost analyses of CFE reactions at the laboratory scale often do not account for costs associated with bulk reagent purchase, labor, or capital equipment.[45] Looking forward, we anticipate that continued investment in biomanufacturing leveraging CFE systems will enable additional large scale economic analyses[555] and the more accurate prediction of cell-free protein production costs at a variety of scales. Beyond cost, we anticipate that innovations in purification and downstream quality control processes from lyophilized cell-free expression reactions will enable scaled batch production and distribution of protein products at the point-of-need.

3.4. CFE for Measuring and Engineering Macromolecular Interactions

CFE systems have been used to study and engineer macromolecular interactions involving proteins, nucleic acids, lipids, and small molecules. The open nature and high-throughput potential of CFE systems makes them ideal for probing and engineering these interactions. When working with individual proteins, the features of CFE systems allow for a nearly order of magnitude improvement in speed and throughput when compared to equivalent cell-based processes that are bottlenecked by cloning, transformation, and cell culture.[116,282,556] When working with libraries of proteins, CFE systems offer a variety of ways to couple genotype to phenotype and can achieve library sizes several orders of magnitude greater than their cell-based counterparts.[339,340] Here we summarize the work done to measure and engineer

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molecular interactions, with a focus on protein−protein interactions (PPIs).

3.4.1. High-Throughput Screening of Macromolecular Interactions. Several standard methodologies have been used to quantify PPIs in the complex CFE environment without purification in microplates (Figure 9). Protein complementation assays,[557] where reporter proteins are fragmented and genetically fused to the proteins of interest, have been used with CFE to probe natural PPIs,[558,559] identify inhibitors of PPIs,[560,561] and prototype PPI-based logic gates.[263] Fluorescence correlation spectroscopy, which uses confocal microscopy to observe fluorescently labeled molecules that transit through an observation volume to measure the oligomeric state of the protein(s) (Figure 9),[70,562] can evaluate the interactions and oligomeric state of the expressed proteins or complexes directly in a CFE reaction.[70,556,562−565] Enzyme-linked immunosorbent assays (ELISAs),[566] a gold standard method in PPI screening relying on immobilization of antigens on a surface and detection with an enzyme- or fluorophore-linked antibody, have been utilized to screen antibodies produced in CFE.[188,567,568] The amplified luminescent proximity homogeneous linked immunosorbent assays (AlphaLISA),[569] an in-solution ELISA-like assay,[569] tolerates several crude CFE systems[186,556,570] and is well suited for high-throughput screening because it does not require wash steps. AlphaLISA has been utilized to measure the interaction specificities between several natural proteins,[556,563−565] profile mouse genes for autoantigenicity,[570] as well as evaluate antibodies[116] and computationally designed binding proteins.[189] An *in vitro* two-hybrid (IVT2H) assay, where PPIs are quantified by transcriptional activation of a reporter gene, has also been developed for CFE.[571] Collectively, these measurement techniques enable researchers to probe hundreds to thousands of PPIs using CFE in microplates.

CFE-based self-assembling microarray assays (Section 2.7) can be used to measure PPIs by exposing the microarray to a labeled target protein, where interactions are measured by the intensity of the label measured at each microarray spot. Microarrays have been used to map the interaction network of 841 interactions between 29 human replication proteins,[295] profile antibody target antigens,[572−574] investigate protein small molecule interactions, and more.[575,576]

Microfluidic systems are also commonly employed to measure PPIs. The protein interaction network generator

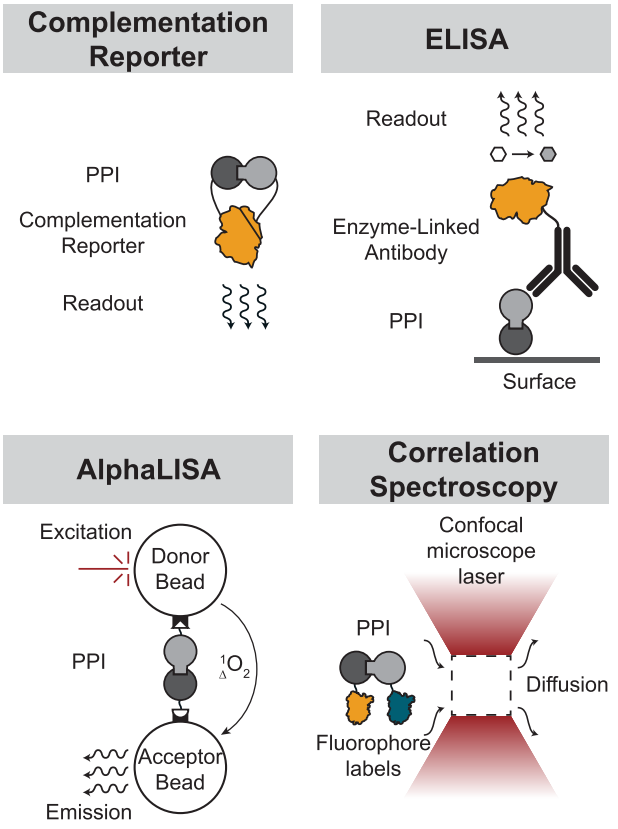

Figure 9. Methods for evaluating PPIs in CFE that do not require purification of the interaction partners.

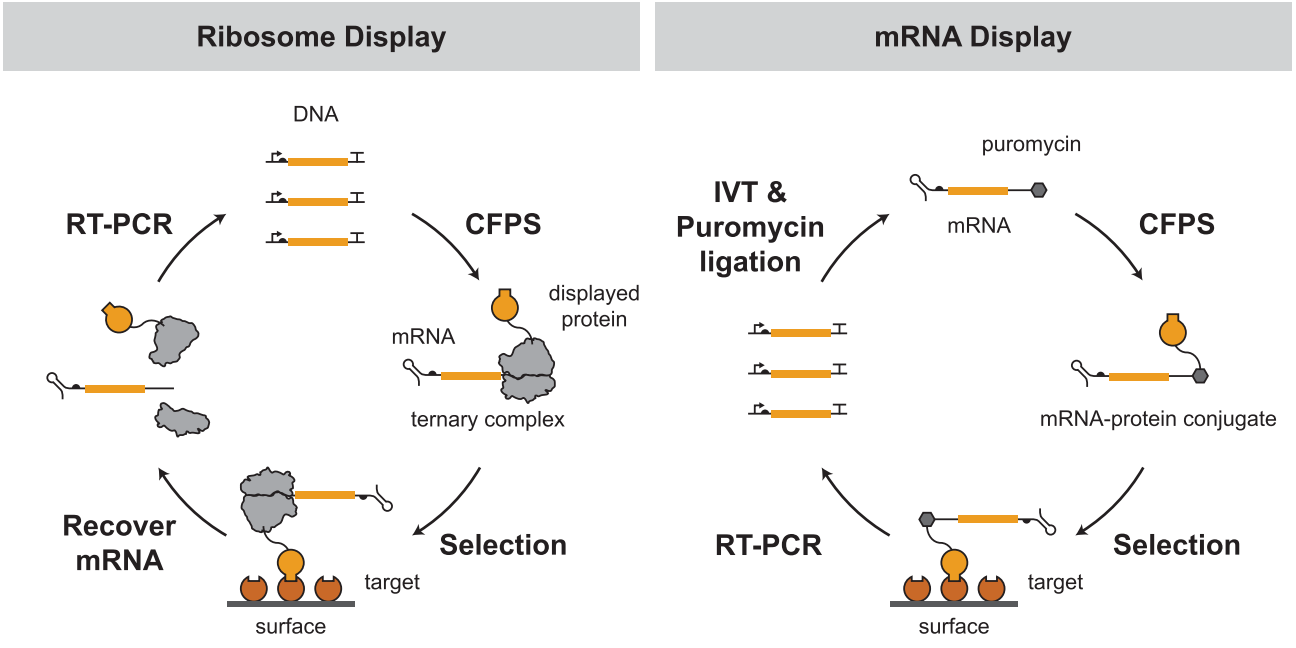

Figure 10. Schemes for ribosome and mRNA display.

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(PING) is a microfluidic system that combines CFE with the mechanical trapping of molecular interactions (MITOMI) to evaluate the interactions of the expressed proteins.[307] The system was used to evaluate 1,849 interactions between 43 *Streptococcus pneumoniae* proteins in quadruplicate, mapping a rich interaction network. PING also enables the evaluation of membrane proteins, with iterations of this technology enabling the simultaneous profiling of 2,100 membrane proteins to characterize host−pathogen interactions.[577] A similar platform has also been used to study and engineer protein−DNA interactions.[303,306] Droplet-based microfluidic systems have further been used to evolve peptide binders using the IVT2H assay.[578]

3.4.2. *In Vitro* Display Technologies. CFE has the unique benefit of enabling researchers to create a direct physical connection between a gene's transcript and its protein product, generating a macromolecular complex linking genotype and phenotype. These approaches, typically referred to as *in vitro* display technologies, have several benefits for both the measuring and engineering the properties of the displayed proteins over cell-based counterparts.[336] Library size in CFE systems is limited by number of ribosomes and DNA or RNA templates present as opposed to the transformation efficiency, enabling library sizes of up to 10^{14},[339,340] appreciably larger than the typical maximum library size of 10^9 to 10^{10} in phage- or cell-based display systems.[338,579] Library assembly and diversification steps can also be performed without transformation and cell growth, leading to faster selection cycles. Furthermore, the nonliving nature of CFE reactions enables methods for creating genotype to phenotype linkages that would not be possible in living systems. Taken together, these features have led to the development of several different *in vitro* display technologies.

Ribosome display maintains the genotype to phenotype linkage by stalling the ribosome on its transcript without releasing the polypeptide chain from its acceptor tRNA[337,580−582] creating a ternary complex that can be used to select for or measure different properties of the displayed protein.[337] The ribosome is stalled on the transcript through the omission of a stop codon or a peptide-based stalling sequence[337,580] (Figure 10). Ribosome display has been used to engineer to a variety of different binding proteins, including single chain variable fragments (scFvs),[583,584] antigen binding fragments (Fabs),[585] designed ankyrin repeat proteins (DARPINs),[586] and single domain antibodies.[587−590] Of note, one of the strongest affinity antibody−antigen interactions reported to date was engineered using ribosome display using extended off rate selections for affinity maturation.[583,591,592] Ribosome display has also been used to select for improved protein stability using chemical denaturants[345] and protease treatment.[346] A limitation of ribosome display is that experiments must be carried out under conditions where the template mRNA is not subject to degradation and the ribosome ternary complex is stable. However, it should be noted that comparisons of display technologies commonly overlook reports that the ternary complex can be stable even under mild denaturing stresses including elevated temperatures.[580,593]

mRNA display establishes a genotype to phenotype linkage by covalently linking the nascent peptide chain to the mRNA using an mRNA-puromycin conjugate[338,340,350,594,595] (Figure 10). Puromycin is an antibiotic that interferes with translation by binding to the ribosomal A site and accepting the nascent peptide chain.[338] By conjugating puromycin to the mRNA transcript, a covalently linked peptide-mRNA fusion can be generated. mRNA display has been used widely for the selection of binding proteins from random linear peptides,[596] antibody mimics,[597] single domain antibodies,[598] and antibody antigen binding fragments.[599,600] Beyond simple binding selections, various versions of mRNA display have also been used for binding selections against whole cell targets,[601] measurements of functional proteomics,[341−343] measurements of binding kinetics,[602] selections for enzyme activity,[349,350,603] selections for stability using chemical denaturants,[347,348] temperature,[349] and proteases,[604] as well as selections to probe the functional areas of random sequence space.[605] mRNA display and derivatives thereof also feature prominently in the RAPID system[230,514] (Section 3.2.1) where they have been used to select linear and cyclic peptides containing noncanonical amino acids with properties akin to natural products.[230,514] Limitations of traditional mRNA display include the stability of mRNA molecule itself as well as the effort required to prepare mRNA-puromycin conjugates. Several modifications have been made to mRNA display methods in order to address these limitations, including the use of cDNA display,[606,607] transcription-translation coupled with association of puromycin linker (TRAP) display,[608] cDNA TRAP display,[609] and click display.[610] These methods enable displayed proteins coupled to cDNA instead of mRNA,[606,607,609,610] rapid and simple puromycin conjugation procedures,[606−610] and one pot transcription and translation reaction formats.[608−610]

Beyond ribosome and mRNA display, many other CFE-based *in vitro* display techniques have been devised. CIS display relies on the ability of the RepA protein to bind the DNA template from which it was expressed, which has been used to select protein binders.[611] Similarly, covalent antibody display (CAD) leverages the P2A enzyme to covalently link the expressed protein to its DNA template, enabling selection of scFvs.[612] The STABLE method utilizes emulsions and streptavidin fused polypeptides to link the biotinylated template DNA to the expressed protein.[613] Similarly, covalent DNA display[614] and SNAP display[615,616] use compartmentalization in emulsions and a fusion of the displayed protein to an enzyme that covalently reacts with a suicide inhibitor conjugated to the DNA template. Several different microbead display systems have been developed,[617−619] all of which utilize *in vitro* compartmentalization in emulsions to immobilize both the CFE-expressed protein and its coding DNA template to a microbead that can be used to perform selections. Liposome display couples genotype and phenotype via compartmentalization using liposomes to enable the engineering of membrane proteins, toxic pore-forming proteins, transporters, and receptors.[620]

CFE display technologies have also been combined with modified next-generation sequencing technologies for high-throughput experimentation. Single molecule interaction sequencing (SMIseq)[344] coupled with ribosome display was used to profile the specificity of a library of 200 scFvs against 55 different human proteins constituting 11,000 possible interactions.[344] Protein display on a massively parallel array (Prot-Map)[319] uses ribosome display coupled with fluorescently labeled target molecules to quantitatively assay binding, which was used to probe the sequence specificity of the M2 anti-FLAG antibody to thousands of possible antigens as well as to study the fitness landscape of more than 100,000 variants

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of the SNAP-tag self-labeling enzyme.[319] In a similar approach dubbed deep screening, ribosome display and a sequencing flow cell were leveraged to quantitatively measure antibody binding affinity for millions of variants simultaneously to generate high quality data for training machine learning models.[320] These techniques highlight the utility of CFE-based display technologies for directed evolution and performing high-throughput functional measurements.

3.5. CFE-Based Biosensors

Biosensors are analytical or diagnostic tools that leverage biological sensing elements (e.g., protein, antibody, nucleic acid, cell) to detect or quantify analytes as a visual or electrical signal output. Historically, whole-cell biosensors have been developed for metabolic pathway engineering, environmental detection,[621−623] and biomedical analysis.[621,624] However, the use of live cells for sensing provides a host of barriers for field-deployable implementation such as membrane permeability limitations, genetic instability, and biocontainment concerns.[625−627] CFE-based biosensors allow for a full use of resources toward production of sensor or reporter machinery making them a compelling option over whole cell biosensors.[1,262,626,628] Reaction conditions, like temperature[629] and pH,[630,631] can also be precisely controlled.

CFE biosensors can detect a range of analytes (e.g., small molecules, metals, nucleic acids, proteins) and have been recently reviewed in detail elsewhere.[623] Cell-free biosensors can be lyophilized and rehydrated from both tube and paper-based formats[142,249,262,632−634] with minimal loss in performance, allowing for long-term storage and application at the point-of-use. Notably, CFE biosensors hold promise to become low-cost, clinically relevant diagnostics as they can also remain active in complex sample matrices (e.g., serum,[551,635] saliva,[636,637] and urine[638]).

Quality biosensor design hinges on selecting a sensor and reporter system that suits the analyte and context it will be sensed in. Most CFE biosensors utilize allosteric transcription factors (aTFs) or aptamers/riboswitches to detect small molecules[638] and ions[143,639] (Figure 11). Nucleic acid detection is typically accomplished with CRISPR-Cas systems and/or toehold switches.[250,640−642] CFE sensors can also be designed for the detection of missing reaction mixture components, such as amino acids.[636,643−647] Recent work has further shown new detection strategies for antibodies[648] and methyltransferases[649] using nucleic acid probes.

Biosensor readouts are typically either optical or electrochemical. Optical reporters have a light-based readout that can be fluorescent (e.g., GFP[650−652]), colorimetric (e.g., β-galactosidase (lacZ)[249,653,654]), or luminescent (e.g., luciferase[637]). Fluorescent and colorimetric readouts are well suited for point-of-use applications due to their simplicity and equipment-less readout. Electrochemical readouts, on the other hand, offer potential in their ability to interface with materials and equipment as portable diagnostics.[655−659] For example, a cell-free biosensing reaction layered over a custom chip with microelectrodes provided a ligand-dependent voltage readout.[656] Other works have shown that commercially sold personal glucose meters can be co-opted for CFE biosensors. To do this, researchers leveraged the electrons generated during the enzymatic oxidation of glucose as a readout for the detection of amino acids in extract-based CFE systems[658] and viruses using purified protein-based systems or PURE.[660] Below we detail the sensing elements used in CFE diagnostics.

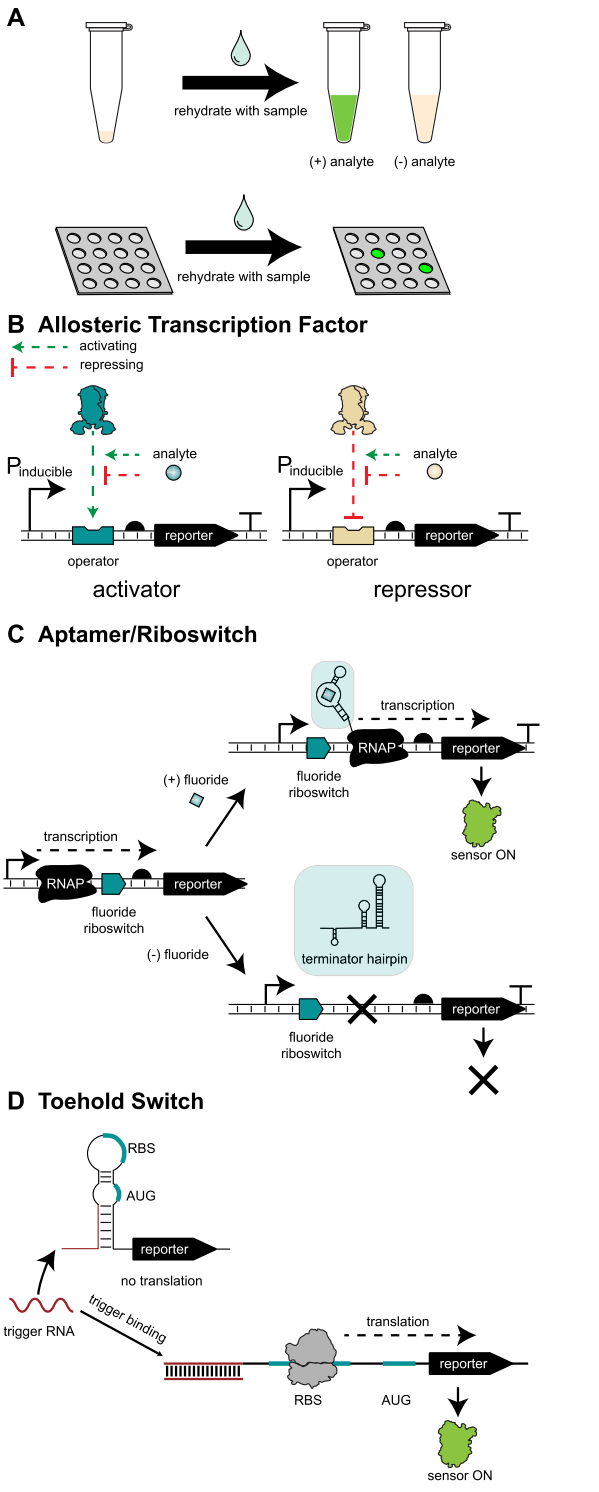

Figure 11. Mechanisms for common sensing elements of cell-free biosensors. (A) Paper and tube-based CFE reactions can be lyophilized for point-of-use application. (B) Common mechanisms of activator and repressor aTFs. Activators activate gene expression upon binding to the operator site and repressors repress gene expression. The aTF-operator binding mechanism is modulated by analyte binding, with analyte-binding either activating or inhibiting aTF-operator binding. (C) Example mechanism of fluoride riboswitch.[143] The riboswitch folds cotranscriptionally into one of two mutually exclusive structures, conditional to fluoride binding. In the presence of fluoride, a pseudoknot enables expression of a reporter. In the absence of fluoride, a terminating hairpin represses expression. (D) Toehold switch mechanism. Toehold switches

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Figure 11. continued

sequester the RBS and start codon RNA into a hairpin loop that is inaccessible to the ribosome. Upon viral trigger RNA binding to the upstream "toehold", the hairpin loop opens, giving the ribosome access to initiate translation.

3.5.1. Allosteric Transcription Factors. Allosteric transcription factors (aTFs) are proteins that bind an analyte (e.g., organic and inorganic small molecules, nucleic acids, proteins) and in turn regulate transcription of a gene downstream of an aTF-specific DNA sequence in the promoter region. aTF-based CFE biosensors have primarily been developed for the purpose of field-deployable small molecule detection, such as for environmental contaminants (e.g., pesticides[142,661] and heavy metals[262,630,639,662]) and human health markers (e.g., drugs[629,638] and metabolites[638]).

aTFs have been used with CFE systems to detect mercury,[629,630,663−666] cyanuric acid,[142] and other analytes.[262,667,668] In these cases, previously identified and optimized aTFs have been tuned in CFE to achieve acceptable sensor performance.[44] The aTF is typically supplemented to the sensing reaction in one of three ways: as a purified component;[669] produced either through a separate CFE reaction that is then mixed with a fresh reaction containing reporter elements or concurrently with the reporter elements;[122,639] or enriched in the extract during cell culture of the extract source strain,[661] which can be mixed with nonenriched extract to achieve the desired sensing results. Further adaptation of an aTF system as a CFE biosensor often involves promoter optimization.[142,661,666] For instance, promoters for the activator AtzR derived from cellular experiments had background expression in CFE in the absence of analyte and needed to be reoptimized in the cell-free environment.[142] Other factors such as sensor/reporter concentration[142,143,638,664,670] and reporter type[143,665] serve as optimization handles for cell-free biosensors. The space of detectable molecules has been broadened beyond analytes that have known aTFs by enzymatically converting an analyte of interest into one with a defined aTF sensor, dubbed metabolic biosensing.[638,661]

3.5.2. Aptamers and Riboswitches. Aptamers and riboswitches are functional nucleic acids that can serve as biological sensing elements seen across all domains of life (Figure 11C).[671−673] Specifically, aptamers are nucleic acids that are capable of binding to specific target molecules[674] and riboswitches are RNAs that use an aptamer to bind to an analyte and regulate gene expression in response to that analyte.[672] A number of riboswitches have been demonstrated in CFE systems and have been reviewed thoroughly elsewhere.[675] Notable applications of CFE riboswitches have been toward artificial cell communication (e.g., theophylline[676−678]) and the detection of molecules relevant to human and environmental health (e.g., thrombin,[679,680] fluoride,[143,681] proteins,[682] tetracycline,[683] histamine,[684] ciprofloxacin,[684] and dopamine[682]).

Aptamers are appealing sensor elements because they can be easily synthesized via PCR, engineered in large libraries, and computationally designed.[685,686] Most *de novo* developed aptamer and analyte binding pairs are engineered to target proteins and small molecules, although metal ions, cells, other nucleic acids, peptides, and carbohydrates have all been targeted as well.[671] The main method for evolving *de novo* aptamers is using Systematic Evolution of Ligands by EXponential enrichment (SELEX), wherein a target molecule is incubated with a library of $10^{14} \sim 10^{16}$ aptamer candidates.[685] In conjunction with library screening, riboswitches can be designed in silico using the thermodynamic and kinetic principles that dictate nucleic acid folding.[687] This was demonstrated for the design of protein-sensing riboswitches in CFE systems.[682] Aptamer engineering methods specific to CFE-biosensors have been developed, such as using water-in-oil droplet sorting in microfluidic chips.[684] In silico methods have also been used to design CFE-riboswitches for detecting proteins[682] and human biomarkers.[682]

A salient demonstration of the potential for riboswitch sensors applied in CFE was the detection of fluoride using a riboswitch controlling expression of an enzymatic colorimetric reporter.[143] This sensor system was lyophilized in microtubes, and reactions were able to sense fluoride at a limit of detection set by the EPA in the lab. To assess the robustness of this diagnostic in the field, diagnostics were deployed to rural Kenya and tested in the hands of nonexperts. The authors found that out of 57 tests, 89.5% of water samples were correctly classified under field conditions.[688] This work was expanded upon by encapsulating the fluoride riboswitch in a lipid vesicle.[681] The sensor's access to analyte and protection from inhibitory enzymes were both controlled by altering the membrane permeability, expanding our repertoire of acceptable sample matrices, highlighting the potential for aptamers applied to cell-free systems.

3.5.3. Nucleic Acid Biosensors. Field-deployable nucleic acid biosensors are a critical need in modern medicine for the rapid and accessible detection of disease.[689] CFE-based nucleic acid biosensors have enabled pathogen detection primarily through leveraging toehold switch sensing elements. RNA-based toehold switches detect specific pathogenic RNA-sequences through a regulating translation. In the absence of pathogen-specific trigger RNA, they sequester the RBS into a hairpin loop that blocks translation by the ribosome. Upon the sequence-specific binding of trigger RNA, the loop unfolds to allow translation and reporter signal.

Extract-based CFE biosensors have been developed to detect a number of global-health related viruses (e.g., SARS-CoV-2,[257,637,652,659,690,691] respiratory syncytial virus,[692] Norovirus,[693] Ebola,[249,257] Zika,[250] and hepatitis[694]), bacterial markers (e.g., typhoid and paratyphoid,[659] antibiotic resistance,[659] gut microbiota[695]), and agriculturally relevant viruses (e.g., Potato Virus Y[653] and cucumber mosaic virus[696]). These sensing reactions were all stably lyophilized and are estimated to cost $0.10-$2 per test.[249,250,693,695]

Toehold switches can be rapidly *de novo* designed to detect pathogen-specific trigger RNA sequences through well-defined and predictable Watson-Crick base pairing.[697,698] Often, toehold switch sensor reactions are preceded by an isothermal amplification step on the sample,[699−702] which enables the amplification of trigger RNA, the conversion of DNA to RNA triggers, or the addition of synthetic sequences to triggers. This has been frequently used to achieve improved detection limits in CFE diagnostics.[250,659,690−693,695] CFE nucleic acid biosensor orthogonality can be further optimized by coupling a toehold switch with CRISPR Cas proteins, which was demonstrated for the discrimination between the African and American Zika strains with single-base resolution.[250] To do this, the authors identified a naturally evolved single nucleotide polymorphism between the two strains that created a strain-

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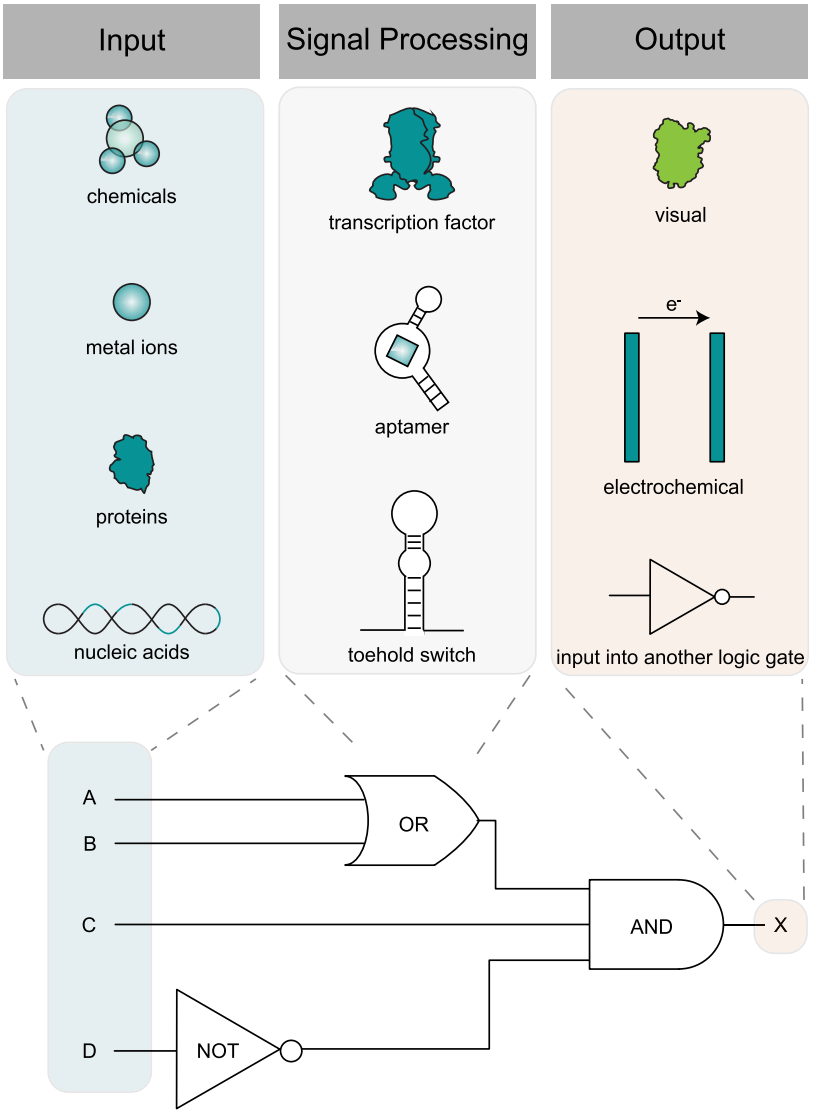

Figure 12. Components of genetic circuits in CFE.

specific PAM site for Cas9 cleavage in only the American ZIKV strain. In a pooled sample of both American and African ZIKV, the authors used isothermal amplification to append a synthetic trigger RNA sequence to the purified viral RNA upstream of the potential Cas9 cleavage site. In the American ZIKV strain, Cas9 cleavage produces a truncated, inactive trigger sequence. Therefore, the sensor only turns on for the African ZIKV strain, which produces full-length trigger RNA in the absence of Cas9 cleavage.

3.6. Genetic Parts and Circuit Prototyping

Characterizing the genetic parts (e.g., promoters, RBSs, terminators) necessary to predictably engineer biology can be laborious with engineering cycles spanning days to months depending on the host organism. Using CFE, large libraries of genetic parts can be quickly generated, assembled with a reporter gene into a transcriptional unit, and assayed at once by running CFE reactions in a variety of formats and volumes.[333,703,704] Genetic parts can be assessed in a 96- or 384-microplate with a fluorescent protein reporter as a readout for relative activity.[112,211,705−707] Higher throughput can be achieved by encapsulating CFE reactions in droplets,[708] where gene variants can be coupled to fluorescent reporters so that fluorescence-activated droplet sorting (FADS) can be used for enrichment, accessing library sizes of $>10^7$ candidates per day.[324,328,709,710] Multiplex characterization of 5′ regulatory sequences has also been demonstrated for pooled CFE reactions using RNA-seq as a readout.[56]

CFE has shown value for prototyping genetic parts from nonmodel organisms. Many nonmodel organisms are emerging as promising commercial chassis strains but are often challenging to engineer due to slow growth rates and limited tools for genetic manipulation. CFE systems utilizing extracts from nonmodel organisms (Section 2.1) sidestep these challenges and make it possible to prototype genetic parts in the context of their native host organism's biological machinery. Using this strategy, genetic parts for *Clostridium autoethanogenum*, *Bacillus megaterium*, *Streptomyces*, *Pseudomonas putida*, and other organisms have been evaluated using their corresponding CFE system.[51,54,56,58,91,112] Ensuring that prototyping results obtained in a CFE system still hold in the context of the living organism is important. To date, clear correlations have been observed between activity in CFE systems and *in vivo*.[56,211,706,707] However, it should be noted that prototyping results can be influenced by the type of DNA

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template (Section 2.3.5)[185,211] and the supplemented reaction components.[711]

Genetic circuits can be constructed from a combination of distinct genetic regulatory elements (e.g., DNA, RNA, proteins) that interact to carry out a given program or function (Figure 12). Although genetic circuits offer exciting possibilities for programming biological behavior,[712] efforts to implement circuits *in vivo* often yield unpredictable results.[261] CFE characterization could be beneficial, as circuit components and inputs can be easily tuned such as by adjusting the amount of DNA added to the open reaction environment. Basic circuit motifs have been implemented in CFE systems by combining regulatory genetic part elements together. AND-gates constructed from small transcriptional activator RNAs (STARs) and toehold switches[713] have been demonstrated along with protein-based cascades, utilizing *E. coli* sigma factors and viral polymerases[42,44,714] and RNA-based cascades.[707,715] Through the combination of these elementary motifs, more complex logic can be achieved such as different types of feed forward loops,[329,715−719] negative autoregulation[720] and oscillators.[721−723] Circuits implemented in CFE and in cells yield largely comparable results, suggesting that CFE can serve as a prototyping platform.[707,713,718] However, like individual genetic parts, results can be convoluted by similar factors mentioned above as well as resource competition[706] and batch to batch variability.[724] Beyond prototyping circuits for cellular applications, CFE can also be used to study genetic circuits that would be challenging or impossible inside of a cell, an excellent example being the recent implementation of a biological circuit operating on a single DNA molecule in a fabricated biochip.[311]

3.7. Enzyme Screening

Enzymes are catalysts that carry out precise chemical transformations. As enzymes have become increasingly important as catalysts for chemical synthesis due to their speed, selectivity for substrates, compatibility with mild physiological conditions (aqueous, pH 7, 37 °C), ability to be assembled into enzymatic cascades, and evolvability, there is also an increasing need for technologies to support enzyme discovery, screening, and evolution.[725,726]

Large libraries of enzymes can be synthesized and screened using linear expression templates in CFE, bypassing traditional time and labor bottlenecks in DNA cloning. These enzymes are often produced in sufficient yield for multiple assays, which enables reactions to be scaled down to μL volumes and used in high-throughput methods (Section 2.7). The open reaction environment of cell-free systems enables enzymes to be tested without the barrier of the cell membrane and, depending on the application, without purification.[727−729] CFE also enables the production of enzymes that may be otherwise toxic to life, due to the *in vitro* nature of the method (Section 3.1.6). Finally, enzymes can be tested within the context of active metabolism, which informs *in vivo* metabolic engineering applications, and provides access to native energy, cofactor, and substrate pools.[730,731]

A common method for studying and engineering enzymes in cell-free systems is to use well-by-well screens. In these methods, enzyme variants are encoded by DNA templates that can be generated by a variety of means, including from commercial gene synthesis, error-prone PCR, and saturation mutagenesis. Enzymes such as [FeFe] hydrogenases and aminoacyl-tRNA synthetases are amenable to CFE-based screening and engineering using these approaches.[532,732] Droplet microfluidics have also been used to increase enzyme screening throughput (Section 2.7). For example, alkaline phosphatases were engineered to have >20-fold improved activity.[318,733] Furthermore, microfluidic chips are capable of synthesizing, immobilizing, washing, and characterizing the kinetic parameters of >1,500 enzyme variants simultaneously.[310] CFE technologies are poised to enable researchers to study and engineer enzymes at depths not previously possible.

Another method for screening and selecting enzymes with new properties using CFE is to compartmentalize reactions to link genotype and phenotype. In a proof-of-concept experiment, water-in-oil emulsions encapsulating *E. coli* S30 extracts that synthesized the methyltransferase from *M. Hae*III could be selected from those synthesizing another protein by using resistance against restriction enzyme digestion.[323] Water-in-oil emulsions are generally compatible with fluorescence-based droplet sorters or microfluidic droplet sorters, enabling large libraries to be screened rapidly.

In theory, any enzyme can be evolved using emulsions provided an appropriate substrate that does not diffuse through the emulsion and the desired chemical reaction can be observed for sorting or selection. A variety of enzymes, including β-galactosidase,[734] penicillin G acylase,[735] horseradish peroxidase,[736] cellulases,[737] proteases,[328] phosphotriesterases,[738] and dehydrogenases[739] have been engineered using this method. Other methods to detect substrate and product, as well as advances to make the technology more broadly accessible, are being actively developed.[740] For example, droplets can be analyzed by using mass spectrometry-based methods, enabling detection of product and substrate without labeling.[741] A secondary way to compartmentalize CFE reactions is to use liposomes.[620] The main advantage of this method is that it can be used to screen membrane proteins, which may not be folded correctly or active in emulsion-based methods.[620] However, this is also compatible with soluble, globular enzymes such as aminoacyl-tRNA synthetases[477] and β-glucuronidase.[742] These droplet-based technologies are significant advances for enzyme engineering.

Looking forward, cell-free enzyme screening holds great potential for high-throughput homologue comparisons from phylogenetic trees,[743] mutagenesis or engineering campaigns,[744] and validating *de novo* design efforts.[745] Key application areas include biological synthesis of therapeutic compounds, carbon capture and storage, as well as degradation of environmental contaminants, such as microplastics.[744,746]

3.8. Enzymatic Cascades and Metabolic Engineering

In addition to screening individual enzymes, cell-free systems provide avenues for multistep biotransformations and biomanufacturing with higher throughput and/or higher efficiency than cellular methods.[747,748]

3.8.1. Purified Enzymes for Pathways. Purified systems provide efficient routes for biochemical conversion outside the constraints of cells and enable predictable, high-titer processes for industrial chemicals with defined reaction parameters.[747−749] These systems can recapitulate pathways from nature,[5] or they may use a subset of enzymes for more direct biosynthetic pathways.[750] Although purified systems typically use cellular expression rather than CFE, many of the same principles apply for cell-free metabolism in purified and crude systems, as discussed below. Purified enzymes enable the

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combination of catabolic and anabolic enzymes from diverse clades without optimizing strains or multigene expression cassettes, enabling up to 99% conversion of sugars into 1,4-butanediol or alpha-ketoglutarate[751] and the production of cannabinoids at titers 2 orders of magnitude greater than *in vivo* platforms.[752] Other significant products synthesized through the combination of known pathways *in vitro* include isobutanol at 5 g/L/h,[753] monoterpenes at 15 g/L/h with yields greater than 95% through 27 enzymes,[754] and myo-inositol produced from starch in 20,000-L reactors.[548]

Purified enzyme systems also provide the ability to move beyond known metabolic pathways and derive non-natural pathways.[755−757] *De novo* pathway design is often bolstered by computational methods that identify enzymes for the desired reactions[758,759] and account for the thermodynamics and cofactor requirements of each step, which can increase the theoretical efficiency of synthetic pathways beyond those observed in nature.[760] Examples of *de novo* pathways implemented with purified enzymes include nonoxidative glycolysis to avoid the carbon loss that occurs in natural glycolysis,[761] reverse β-oxidation for the synthesis of fatty acids,[762,763] the formolase pathway for incorporation of 1-carbon units into metabolism,[764] and the CETCH (crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA) cycle for more efficient carbon fixation than the natural Calvin cycle.[765] In addition to combining disparate natural catalysts into pathways, purified systems can be interfaced with inorganic catalysts to enable a broader diversity of chemical reactions than what is possible in cells.[749] This chemoenzymatic approach is demonstrated by an artificial starch anabolic pathway that employs ZnO-ZrO_2 to hydrogenate CO_2 to methanol and an enzyme cascade to convert the methanol to starch.[764]

The primary limitations in purified systems are the cost of enzyme preparation and the need to recycle cofactors and/or byproducts. Purification costs may be reduced through parallelized workflows[231] or the use of thermotolerant enzymes such that endogenous proteins can be removed through heat denaturation.[548,766] Cofactor recycling in purified systems may simply require additional enzymes,[767,768] but orthogonal cofactor specificities can also be implemented.[769] Significant examples of *in vitro* recycling strategies include salvage pathways to recover nonproductive byproducts in the CETCH carbon fixation cycle[765] and for polyhydroxybutyrate synthesis[770] as well as cofactor pool regulation through a redox purge valve for NAD(P)H[771] or an ATP rheostat.[772] These strategies bring an element of homeostatic regulation to purified systems.[547,747] In addition, one should consider the energy density of substrates and products to enable targeted molecular transformations.

3.8.2. Extract-Based Metabolite Synthesis. While purification offers substantial benefits for enzyme characterization and large-scale biosynthesis, extract-based approaches have utility in prototyping and potential for biomanufacturing. Enzymes in lysate remain active in the soluble fraction of cell extract which was shown to enable conversion of glucose to ethanol,[3] avoiding purification while retaining cofactors and other endogenous components that facilitate enzyme activity.[747] Crude cell extracts thus enable biochemical conversions through interconnected catabolic and anabolic pathways in the absence of cellular barriers, which have been employed for ethanol production with extracts of *C. thermocellum* to assess metabolic bottlenecks[773] and *S. cerevisiae* to maximize ethanol titers *in vitro*.[774] Similarly, extracts from *Streptomyces spp.* have elucidated biochemical mechanisms behind natural products, including fluorinated metabolites,[775] anticancer drugs,[776] and antibiotics.[777−781] Endogenous metabolism in extract can also serve as a precursor to heterologous biochemical synthesis, as seen in the cell-free synthesis of dihydroxyacetone phosphate prior to condensation with butanal to form an unnatural monosaccharide.[782] Alternatively, the metabolism of cell extract can be primed or altered through tuning of growth conditions and media for increased flux toward biochemicals of interest.[84,783,784]

Cell-free metabolism in extracts from engineered strains of *E. coli* or *S. cerevisiae* can also produce heterologous metabolites, such as 2,3-butanediol and itaconic acid, with high volumetric productivities and highlight bottlenecks in metabolic flux[785,786] while displaying robustness to substrates,[787] products,[788] temperatures,[774] and pH ranges[789] that are acutely toxic to living cells.[790] This approach also extends to nonmodel organisms, such as the *in vitro* synthesis of 3-hydroxypropionate in extracts of engineered *Pyrococcus furiosus*.[791] Furthermore, the simplicity of generating extracts from various species enables hybrid cell-free reactions derived from multiple organisms to take advantage of each species' unique biochemical properties in a manner similar to synthetic microbial consortia without the need to balance interspecies growth.[792,793] Although several studies have incorporated mixed extracts for gene expression,[56,111,297,794,795] the primary example of hybrid cell-free metabolism combines cyanobacterial extract for starch catabolism with *E. coli* extract for 2,3-butanediol synthesis.[796]

The combination of rapid biochemical conversions and increased tolerance to harsher conditions has generated interest in cell extracts as potential biomanufacturing platforms.[547,747,797] Although the retention of endogenous metabolism in cell extracts facilitates substrate conversion and cofactor recycling, other endogenous enzymes can drain key intermediates or cofactors from the desired pathway, which limits product yields similarly to cellular systems. Such competing enzymes can be knocked out in the source strain prior to lysis[782,798] or (in the case of enzymes essential for growth) selectively removed from the extract through protease cleavage or purification.[507,799] Enriched extracts may also be heat treated for thermal "purification" of thermotolerant enzymes,[548,766] which represents an intermediary format between fully purified and extract-based cell-free systems. Rather than expressing complete heterologous pathways in cells prior to lysis, many approaches utilize extracts each enriched with one overexpressed enzyme to screen enzyme variants in the context of native metabolism while saving both time and capital costs.[800,801] A similar strategy has been employed for laboratory enzymes, such as polymerases and nucleases, to avoid purification.[802,803]

Mixing extracts individually enriched with a single enzyme has been shown to be a powerful approach for *in vitro* metabolite synthesis. As compared to using extracts from source cells with multiple enzymes overexepressed, this provides greater control over both the identity and relative concentration of catalyst for each step of the pathway. Extract mixing has been employed to study mevalonate,[798] indole-3-acetic acid,[804] butanol,[731,789] limonene,[805] pinene,[806] and chlorogenic acid[807] synthesis and to explore cell-free metabolism.[284] Of note, the number of enzyme variants that can be tested *in vitro* becomes limited by the generation of

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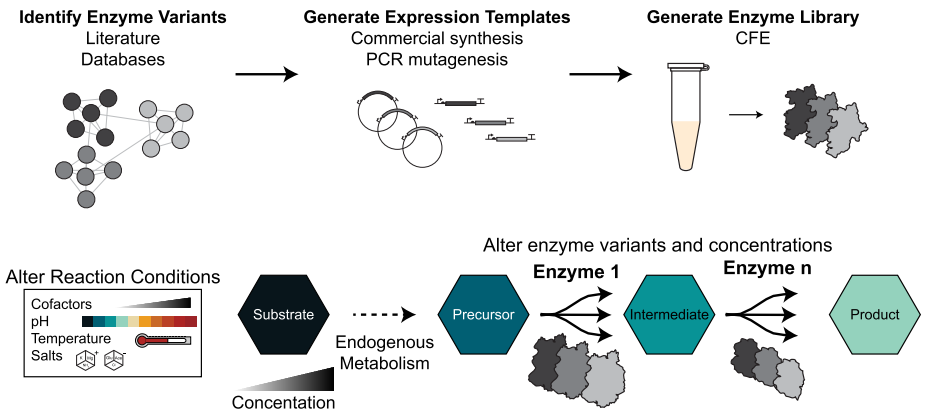

Figure 13. Generalized workflow for cell-free pathway prototyping. Cell-free reactions incorporating CFE with metabolism simplifies high-throughput screening of enzyme libraries and enables control over many more reaction parameters than *in vivo* experiments allow.

many unique cell extracts, the ease of which can vary significantly depending on the scale of available lysis equipment.[20]

Increased flexibility comes from making a blank slate extract capable of CFE, which can generate libraries of enzyme variants without engineering multiple strains (Figure 13). A complete pathway can be expressed in one reaction using multiple plasmids (relying on relative expression levels similar to *in vivo* expression),[731,808] or each enzyme can be expressed separately and quantified prior to combining the pathway (providing greater precision akin to purified systems).[809] Although reagents and metabolic byproducts carried over from protein synthesis can suppress activity of heterologous pathways,[284] the ability to rapidly test large combinatorial libraries of enzymes can outweigh reduced titers by significantly decreasing the length of design-build-test-learn cycles. Cell-free prototyping in *E. coli* extract enabled screening of over 200 pathway variants for 3-hydroxybutryate and butanol biosynthesis to rapidly inform pathway design in *C. autoethanogenum* production strains.[810] Similarly, CFE of 9 heterologous enzymes facilitated the screening of nearly 600 defined pathways for terpene synthesis from glucose to improve limonene titers 25-fold.[811] CFE also enables screening enzymes that compete with a pathway of interest. By combining enzymes from *C. autoethanogenum* suspected to compete with heterologous acetone biosynthesis, an *E. coli* cell-free system identified targets for genomic knockout in the clostridial production strain that substantially increased selectivity for acetone over undesired byproducts.[812] In addition to these cell-free prototyping efforts, the versatility of CFE for metabolite synthesis is highlighted by the wide array of biochemicals synthesized to date. This includes butanol,[810] acetone,[812] terpenes (limonene, pinene, and bisabolene),[811] phenol,[84] 3-hydroxybutyrate (from glucose[810] or whey[813]), styrene,[788] and valinomycin[814] with *E. coli* extracts, unnatural indole alkaloids using PURExpress,[815] and heme using *S. venezuelae* CFE.[62]

The diversity of formats and flexibility of applications for cell-free metabolite synthesis presents benefits for high-throughput prototyping and high-yield biotransformations.[730,748] These cell-free approaches complement traditional *in vivo* metabolic engineering and offer the ability to design biosynthesis platforms with increasing speed and precision to facilitate the sustainable production of chemicals at a range of scales.[816] Addressing cost and scale-up remain the primary hurdles to transition cell-free biosynthesis from a pathway prototyping method to a biomanufacturing platform.[547,747,817]

3.9. Building and Engineering Complex Biological Systems

Cell-free systems derive benefits from the lack of viability constraints, but defining features of cells, such as compartmentalization and self-replication, can also be valuable in nonliving contexts. Research into artificial cells spans many disciplines and applications, generally focusing on top-down reduction of cells into nonliving protocells or bottom-up generation of complex systems.[29,818,819] Although the abiotic context provides immense control over the composition,[820] functionalization,[821] and contents[822] of artificial cells, the goal of self-replication remains difficult to achieve in full.[823] Here we briefly discuss bottom-up efforts to build cell-like structures and complex assemblies, including ribosomes and viruses.

3.9.1. Building Ribosomes. Understanding and engineering ribosomes could repurpose protein translation for synthetic biology applications. The prokaryotic ribosome is a complex molecular machine comprising three ribosomal RNAs (rRNAs) and 54 ribosomal proteins. These assemble into a 70S particle from two parts: the 30S small subunit, comprising the 16S rRNA and 21 r-proteins, and the 50S large subunit, comprising the 23S rRNA, 33 r-proteins, and the 5S rRNA. The total set of r-proteins is referred to as TP70. Ribosome assembly consists of transcription of the rRNA as a single transcript, cleavage of the rRNA transcript to yield the 23S, 16S, and 5S rRNA, maturation of the rRNA involving additional processing and post-transcriptional modifications, and then assembly of individual subunits through well-studied assembly pathways with the r-proteins.[824] Below we describe efforts in ribosome assembly and synthesis, with an emphasis on *E. coli* ribosomes.

An early example of small subunit reconstitution was published in 1968 using 16S rRNA and r-proteins that were isolated from purified small subunits.[825] Recombinant r-proteins were sufficient to reconstitute small subunit assembly when mixed with native 16S rRNA.[826] Similarly, *in vitro* transcribed 16S rRNA could be mixed with native r-proteins to form active small subunits.[827,828] Later experiments showed that the small subunit could be assembled using components purified from nonribosomal sources.[829] Assembly can also be assisted by the addition of ribosome biogenesis factors or chaperones that facilitate folding and assembly at lower temperatures.[827,830,831] In total, this shows that the small

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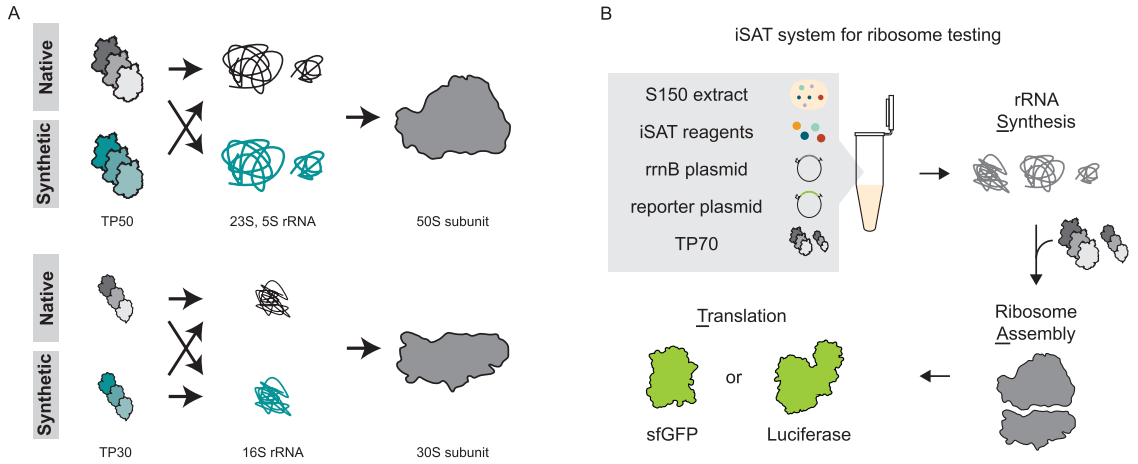

Figure 14. Ribosome synthesis and assembly using the iSAT system.

subunit can be assembled into functional particles within *in vitro* environments.

Reconstitution of the large subunit has proven to be more difficult than that of the small subunit, likely due to the size of the 23S rRNA, essential post-transcriptional modifications of the rRNA, and the complexity of its interactions with the 33 r-proteins. Like the 30S small subunit, reconstitution of the large subunit was first successfully achieved with 23S rRNA, 5S rRNA, and r-proteins that were purified from native 50S subunits.[832,833] R-proteins from recombinant sources could also be used to reconstitute 50S ribosomes with 23S and 5S rRNA isolated from 50S subunits.[834] However, 50S subunits could only be reassembled with minimal activity using *in vitro* transcribed 23S rRNA.[835] This assembly defect is hypothesized to arise from the lack of post-transcriptional modifications on the rRNA. Interestingly, small molecules such as osmolytes and antibiotics were found to improve assembly of 50S subunits from *in vitro* transcribed 23S rRNAs.[836]

Methods to test ribosome assembly and function have also been developed. One-pot integrated rRNA Synthesis, Assembly, and Translation (iSAT; Figure 14)[837] consists of a cellular extract that is depleted of wild-type ribosomes through ultracentrifugation, a plasmid encoding an rRNA operon under the control of a T7 promoter, a plasmid containing an appropriate reporter such as luciferase or sfGFP, and TP70 purified from native ribosomes. All steps of iSAT take place at 37 °C, physiological pH, in buffer and salt conditions that mimic the *E. coli* cytoplasm. Furthermore, the reaction formulation can be changed to enable a wide variety of applications. iSAT enables ribosomes to be synthesized and tested for function, providing a workflow for engineering ribosomes.

A variety of advances have enabled iSAT to become a powerful technology for ribosome synthesis, assembly, and function. For instance, running iSAT reactions in fed-batch mode or in semicontinuous mode increased reaction duration and protein yields by providing necessary phosphoenolpyruvate and NTPs.[838] Additional optimizations included modulating rRNA transcription by adding 3′ modifications to the rRNA transcript and combinatorally optimizing DNA concentrations.[839] Crowding agents and reducing conditions were also found to improve iSAT yields.[840] In total, these optimizations have enabled sfGFP yields at ∼8 μM, or ∼220 μg/mL. iSAT has been modified to run in PURE-like conditions to engineer the 30S subunit, as post-transcriptional modifications of the 16S rRNA are not required for assembly.[828,829] In addition, iSAT enables the study and engineering of ribosomes that are competent for translation but unable to support life in laboratory conditions.[289,841] For example, toward characterizing the mutational flexibility of the active site of the ribosome, the peptidyl transferase center (PTC), ribosomal variants with mutations throughout the PTC were constructed and characterized in iSAT.[841,842] Further, iSAT can be coupled to screening technologies, such as ribosome display and *in vitro* compartmentalization, to evolve the ribosome to enable improved function (e.g., resistance toward antibiotics, improved assembly of active subunits).[289,828,843] iSAT has also been used to advance the concept of "ribosome pool engineering" by identifying sequence-optimized ribosomes with improved protein biosynthesis yields.[844] These advances show how cell-free systems can be used to study and engineer ribosomes for basic science and engineering.

3.9.2. Reconstituting Bacteriophages. Cell-free synthesis of viruses represents a simpler synthetic system than artificial cells due to self-assembly and the lack of membrane chemistry requirements. In addition to enabling basic research of viral assembly without passaging or infecting cells, CFE could serve as a production platform for virus-like particles (VLPs) or phage therapies to target cancer, antibiotic resistance, and other challenges in human health and agriculture.[172] Self-assembling viral capsids have been produced in eukaryotic cell extracts since the 1980s, either through translation of *in vitro* transcribed RNA or through coupled transcription and translation from DNA. For example, hepatitis B and C capsids were expressed in extracts from rabbit reticulocyte,[845] wheat germ,[846] and *P. pastoris*[847] to study assembly and molecular regulation. Similar studies were carried out for cell-free synthesis and assembly of herpes simplex virus capsids in *S. frugiperda* extracts[848,849] and vesicular stomatitis virus in rabbit reticulocyte extracts.[850] In 2020, CFE with extract from *L. tarentolae* provided a platform to study protein interactions important for the assembly of Zika virus.[851] In addition to these eukaryotic examples, *E. coli* extracts have synthesized MS2 and hepatitis B capsids up to 10^{13} particles/mL.[852]

Beyond VLPs and capsids, cell-free systems have demonstrated the capacity to replicate and/or synthesize complete viruses from eukaryotes and prokaryotes. Although the *in vitro* replication of tomato bushy stunt virus in *S. cerevisiae* extract

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was dependent on membrane vesicles,[853] the cell-free synthesis of infectious viruses indicates that living cells with large segments of membrane are not required for viral reproduction.[172] The best explored virus classes in CFE are eukaryote-infecting picornaviruses and *E. coli* bacteriophages, which demonstrate the ability of cell-free systems to carry out large genetic programs and build biological machines. Poliovirus, with a 7.5 kb RNA genome, was the first virus fully reconstituted in an extract-based cell-free system,[854] with expression in HeLa cell extracts achieving titers up to 10^7 infectious particles/mL.[855−857] Similarly, the synthesis of encephalomyocarditis virus, with a 7.8 kb RNA genome, was demonstrated in several eukaryotic cell extracts with titers up to 10^7 particles/mL.[858−860]

Optimized *E. coli* CFE systems have synthesized high titers of increasingly complex phages up to 10^{13} particles/mL.[21] Successfully expressed and assembled phages include MS2 (3.6 kb RNA genome), ΦX174 (5.6 kb ssDNA genome), T7 (40 kb dsDNA genome), and T4 (170 kb dsDNA genome).[44,172,861,862] Cell-free phage synthesis has also been employed as a quality control method to compare the activity of cell extracts prepared with different methods[86] and applied as a screening and engineering platform for potential therapeutics.[863−865] Although this application space has only explored a small fraction of viral diversity to date, cell-free systems offer the potential to synthesize the vast number of sequenced viruses (from both prokaryotes[866] and eukaryotes[867]) for basic and applied research without precise knowledge of host ranges or the ability to culture host organisms. Researchers, however, must remain mindful of ethical and biosafety considerations around viral synthesis, especially for eukaryotic viruses that can infect humans, employing appropriate safeguards and including critical discussion in publications.[868] In addition, a key caution is to ensure that extracts used for phage assembly are free from any living cells.

3.9.3. Building Synthetic Cells. This section focuses on key aspects of synthetic cell research incorporating CFE systems; other aspects of this broad field have been recently reviewed elsewhere.[869] The compartmentalization of CFE systems in droplets or vesicles begins to transition these systems toward cell-like structures capable of replicating genetic information and carrying out the steps of the central dogma[823] while physically linking genotype to phenotype.[323] The functionality of these synthetic cells is determined by the encapsulation mechanism (including polymers, oils, and liposomes; see Section 2.5), the presence and type of protein or nucleic acid functionalization, and the interior composition (Figure 15). Artificial cells based on CFE systems have facilitated a wide range of outputs from continuous reporter protein expression[47] to cytotoxic protein synthesis within mouse models[870] and the parallelized screening of antimicrobial peptides[871] and ncAAs.[872] Furthermore, CFE assembled in compartments on microfluidic chips can provide a platform for synthetic cell studies with movement-restricted reaction arrays. Demonstrations using cell extracts diffusing into compartments coated with DNA have shown cell-like protein expression gradients,[245] temporal propagation of expression,[314] and the ability to tune logic gate speed or precision.[313]

Encapsulated reactions more closely resemble cells when they generate proteins that interact with the surrounding membrane or execute behaviors such as communication and differentiation. Several cytoskeletal proteins have been

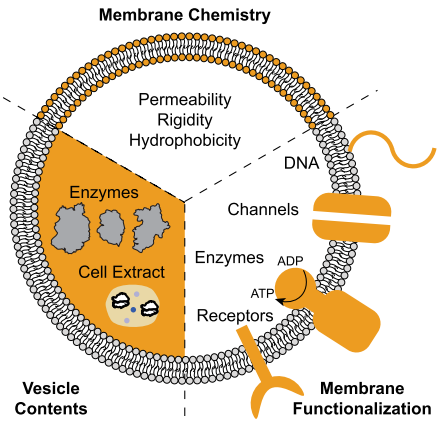

Figure 15. Variables under control for artificial cells. Designing functionalized liposomes enables CFE reactions to more closely resemble cells with complex, context-dependent behaviors. Common variables are highlighted in orange.

synthesized within artificial cells to alter liposome shape or stability, including MreB,[873,874] tubulins,[875] and actin.[876] Additionally, the expression of cell division proteins FtsZ, FtsA, and ZipA within liposomes causes deformation or constriction that could be incorporated along with assembly regulating proteins[877] into a minimal division mechanism.[878−880] In contrast, communication between artificial cells is a more explored research area due to the rich diversity of approaches using functionalized membranes, protein−protein interactions, and signaling pathways to facilitate programs within[881] and between encapsulated cell-free systems.[882,883] Inducible promoters[676] and expression cascades[247] enable context-dependent execution of genetic programs within liposomes, often with the assistance of α-hemolysin for selective permeability or as a component of logic gates.[884,885] The fusion of distinct artificial cell populations can also be mediated by SNARE proteins[886] or complementary DNA strands on the exterior of liposomes to facilitate more complex or interaction-dependent behaviors,[887] such as genetic or metabolic differentiation.[885,886] Furthermore, biochemical communication can extend between living and artificial cells, since the mechanisms implemented in liposomes originally evolved in nature.[888] This typically consists of the exchange of quorum sensing molecules between artificial and living cells in the same solution[889−892] or the implementation of signaling cascades that trigger cellular responses, such as protein synthesis[893] or differentiation.[894] Alternatively, the construction of large liposomes enables the encapsulation of whole cells and other components to form "hybrid cells" capable of layered functionalities.[888,895]

In addition to intercellular communication, the ability to sense and respond to environmental stimuli is a hallmark of life. With this feature in mind, artificial cells have been engineered to respond to changes in pH, osmotic pressure, and light. For example, acid-induced phase separation increased the activity of encapsulated enzymes by raising the local concentration of both enzyme and substrate,[896] and metal−organic frameworks facilitated pH-gated gene expression from encapsulated HeLa cell extract.[897] Pressure-responsive artificial cells have incorporated the membrane protein MscL (large-conductance mechanosensitive channel) coupled with nested vesicles[898] or biosensor cascades[899,900] to link biochemical programs to changes in osmotic pressure, membrane

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asymmetry,[901] and chemical inducers. This channel has also facilitated studies of membrane protein incorporation into vesicles by CFE.[394,902] Additionally, the functionalization of synthetic cell membrane or contents enables light-driven reactions without photoautotrophic cells. These systems typically employ bacteriorhodopsin to generate light-induced proton gradients coupled with ATPase for ATP generation,[903] enabling energy-intensive processes including actin polymerization[876] and combined transcription-translation.[904] Alternatively, artificial cells may incorporate photosynthetic organelles to for light-powered gene expression[905] or enzyme cascades[906] through the combination of natural and synthetic parts.

Despite the ability for compartmentalized CFE reactions to produce a wide range of proteins and execute complex programs, self-replication remains a challenging goal for artificial cell systems.[823] DNA replication in vesicles has been demonstrated successfully alongside protein synthesis,[907,908] but resource limitations in PURE- and extract-based CFE systems limit the extent to which the full complement of gene expression machinery can be regenerated simultaneously.[261,355,909] Optimization of gene expression in artificial cells using highly tuned CFE recipes and/or permeable liposomes in a feeding solution for semicontinuous gene expression show promise,[21] but the dilute environment of cell-free reactions relative to cytoplasm results in lower protein synthesis rates than observed in living cells.[19,179] Serial transfer of PURE reactions enabled the reconstitution of RNA polymerase activity and several key enzymes for translation,[910] and simultaneous generation of RNA polymerase and aminoacyl-tRNA synthetases was enabled by extensively tuning template concentrations.[911] Yet, a major bottleneck remains in regenerating all components with high activity[912] and synthesizing ribosomes fully *in vitro*. Protein synthesis has been demonstrated in liposomes containing ribosomes assembled *in vitro* after harvesting the components from cells,[843] but ribosomal proteins synthesized by PURE show reduced activity,[913] potentially due to the lack of certain assembly cofactors and/or rRNA modifications. However, promising steps have been made toward synthesizing liposomes with both soluble[914] and membrane-bound[412,915] enzymes that could form the basis of replicating artificial cells. Looking ahead, there is a need to integrate artificial cells with functional cell division mechanisms that operate in concert with engineered reactions within the mother and daughter particles. Understanding and engineering smaller self-assembling biological systems, such as viruses and ribosomes, will facilitate further breakthroughs in artificial cell optimization toward fully functional self-replicating systems.

3.10. Biology Education

In addition to numerous opportunities in basic and applied research, cell-free synthetic biology provides a unique platform for education in classrooms ranging from middle school to undergraduate levels. Enzymes provide discrete examples of laboratory-based education for biochemistry, ranging from simple assays to more complex analyses of reaction kinetics.[916,917] However, more complex concepts in molecular and synthetic biology are traditionally taught through lectures alone due to the cost and complexity of cell-based laboratories. This limits hands-on demonstrations and research experiences to well-funded high schools and colleges, typically in extracurricular groups such as the international Genetically Engineered Machines (iGEM) competition[918] and BioBuilder.[919] Cell-free approaches have led to the development of hands-on education modules with the potential for widespread implementation of engaging synthetic biology education due to the minimal equipment and biosafety protocols required.[920] Extract-based approaches with combined transcription-translation enable rapid demonstrations of biology's central dogma through visual reporters, such as fluorescent proteins, that can be seen through inexpensive imagers or the naked eye.[145] More advanced educational kits have explored optogenetics,[921] CRISPR-Cas9 and antibiotic resistance,[922−924] CRISPRi,[925] environmental sampling,[667] or multiple sensory outputs (visual, olfactory, and tactile)[144] to highlight the versatility of biological systems. Furthermore, lyophilization of cell-free reactions increases shelf stability for at least 6 months in a refrigerator, which provides the potential for widespread distribution of low-cost biology education kits around the world from a centralized production facility prior to activation through the simple addition of water and/or DNA. As cell-free biology continues to advance, these systems are poised to transform synthetic biology education by facilitating earlier and more widespread exposure to the field and the rapidly expanding biotechnology industry.[926−928]

4. PERSPECTIVE

CFE systems have significantly advanced over the past 20 years, facilitating numerous applications. As we look to the future, we anticipate several key trends that will shape the evolution of the CFE field.

Increasing CFE reaction yields, reaction longevity, and the diversity of functionally expressible proteins will open completely new use-inspired applications. A CFE batch reaction yield of >10 mg/mL, which is sufficient for self-replication of *E. coli*,[30] will provide new opportunities for large-scale manufacturing and enable applications that require coexpression of multiple genes, such as prototyping molecular assemblies, metabolic networks, and synthetic cells. Achieving low-cost and long-lived reactions will require research into why reactions stop (e.g., via omics methods[81,84−90]), exogenous energy sources (e.g., light[904] and electricity[929]), and alternative organisms. Beyond higher production yields, a key goal should be the manufacture of any proteoform, encompassing all sequence and splice variants of a protein and its numerous possible post-translational modifications.[930] While efforts in glycosylation have advanced, many post-translational modifications remain underrepresented. Indeed, we expect the community to push toward the creation of a universal *in vitro* protein manufacturing system, enabling researchers to choose their preferred translation rates, folding environments, post-translational modifications, and other relevant parameters. The capability to quickly produce any defined proteoform would transform efforts to comprehend and engineer protein function.

Although adoption of CFE systems is increasing, their use in most laboratories is far from routine. To make CFE systems ubiquitous, the community must improve protocol clarity and streamline reagent preparation. Reproducible reagent and cell-extract production across laboratories can be a challenge,[127,210] and most current protocols involve the preparation of cell extract and dozens of reagents, which can be daunting for new researchers. In the past five years, progress has been made in lowering the barrier to entry, with new user-focused primers,[30] video-based protocols,[931] simplified *E. coli* cell-extract prep-

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aration protocols,[87] and efforts to understand the sources of variability in CFE system manufacture.[208,724] However, more work remains to create repeatable, streamlined protocols and CFE systems for routine use outside of expert CFE laboratories. The availability of low cost (i.e., rivaling cost-effective in-house systems), high-yielding commercial CFE systems, would also increase usage and accessibility.

CFE systems are well positioned to generate high-quality data to support the development of biophysical or artificial intelligence models capable of predicting biological function. Through integration with automation, microfabrication, microfluidics, or display techniques, CFE can enable the rapid and regular evaluation of thousands to millions of unique conditions.[319,320,329,344,604]

CFE-based biosensors hold potential as field-deployable and on-demand diagnostic platforms. These sensors will need to demonstrate robust detection of analytes with relevant limits of detection and signal activation outside of the laboratory setting. Identification of new biological sensors, both new analytes that can be measured as well as new sensing modalities, will expand use cases for CFE sensors. Compatibility with complex sample matrices (e.g., wastewater, blood) will be crucial for detecting human health biomarkers and environmental contaminants.

We expect that new distributed manufacturing opportunities will emerge and lead to an entirely new biotechnology industry. Scale-up of protein and biomolecule manufacturing remains relatively underexplored. A key roadblock has been high costs and bioprocess engineering know-how. We anticipate that innovations to further reduce costs as well as purification and downstream quality control processes will increase scale-up applications and distribution of cell-free expression reactions. The development of new materials such as paper, fabric, and hydrogels for distribution is also poised for impact in point-of-use settings.

CFE systems will remain essential tools for studying and building biological systems. Like their early use, CFE systems will continue to contribute to the study and expansion of the genetic code, particularly through the incorporation of noncanonical amino acids into proteins. Efforts to build synthetic cells represent another exciting frontier, with many core functions demonstrated in isolated environments. Future efforts will focus on integrating these functions together and improving their efficiencies to realize fully functional synthetic cells.

The chapters above highlight the enormous growth of CFE systems and their applications. In the coming decade, CFE systems will continue enabling researchers to explore biological systems beyond the cell, transform synthetic biology, and accelerate scientific discovery to address pressing societal challenges.

AUTHOR INFORMATION

Corresponding Author

Michael C. Jewett − *Department of Chemical and Biological Engineering and Chemistry of Life Processes Institute, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States; Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, Illinois 60611, United States; Department of Bioengineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0003-2948-6211; Email: mjewett@stanford.edu

Authors

Andrew C. Hunt − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States;* orcid.org/0000-0001-9620-593X

Blake J. Rasor − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States*

Kosuke Seki − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States;* orcid.org/0000-0002-0413-8184

Holly M. Ekas − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States;* orcid.org/0009-0007-2487-7287

Katherine F. Warfel − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States;* orcid.org/0000-0002-7780-6294

Ashty S. Karim − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States;* orcid.org/0000-0002-5789-7715

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrev.4c00116

Author Contributions

[¶]A.C.H. and B.J.R. contributed equally.

Notes

The authors declare the following competing financial interest(s): M.C.J. is a co-founder and has financial interest in Stemloop, Inc., Pearl Bio, Gauntlet Bio, and Synolo Therapeutics. These interests are reviewed and managed by Northwestern University and Stanford University in accordance with their conflict of interest policies.

Biographies

Andrew Hunt received a B.S. in Chemical Engineering from The New Mexico Institute of Mining and Technology and a Ph.D. in Chemical and Biological Engineering from Northwestern University under the guidance of Dr. Michael Jewett. His Ph.D. research focused on the development of high-throughput screening platforms for protein−protein interactions leveraging cell-free gene expression systems. He is currently a postdoctoral fellow at the University of Washington, where he is using computational protein design to create new enzymes.

Blake Rasor earned a B.S. in Biology and Microbiology from Miami University and a Ph.D. in Chemical and Biological Engineering from Northwestern University, where his research focused on metabolism and pathway prototyping in cell-free systems. He is currently a postdoctoral fellow at the Max Planck Institute for Terrestrial

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Microbiology applying cell-free methodologies to study and engineer photosynthetic systems.

Kosuke Seki is currently a postdoctoral fellow at the University of California, San Francisco under Dr. Tanja Kortemme. He received a Ph.D in Chemical and Biological Engineering with Dr. Michael Jewett using cell-free gene expression systems for genetic code expansion, and a B.S. in Chemical Engineering at the University of California, Irvine. He is currently interested in studying structure−function relationships in proteins.

Holly Ekas received a B.S. in Chemical Engineering from The University of Texas at Austin and a Ph.D. in Chemical and Biological Engineering from Northwestern University under Dr. Michael Jewett. Her Ph.D. work focused on developing high-throughput engineering platforms for cell-free biosensor development.

Katherine Warfel received a B.S. in Chemical Engineering from Bucknell University and a Ph.D in Chemical and Biological Engineering from Northwestern University under the guidance of Dr. Michael Jewett. Her Ph.D. research leveraged cell-free gene expression systems to synthesize glycoconjugate vaccines. Through this work, she engineered membrane-bound glycosylation systems, and optimized the cost and thermostability of cell-free reactions to enhance the production of glycosylated protein vaccines.

Ashty Karim is an assistant professor of Chemical and Biological Engineering at Northwestern University. He earned his B.S. degrees in Chemical Engineering and in Biology from the University of Texas at Austin and received his Ph.D. in Chemical Engineering from Northwestern University where he developed cell-free systems to build and prototype enzymes and enzymatic cascades. Ashty works at the interface of biology and chemistry to develop synthetic biology solutions in sustainability.

Michael Jewett is a Professor of Bioengineering at Stanford University. His research group focuses on advancing synthetic biology research to support planet and societal health, with applications in medicine, manufacturing, sustainability, and education. Dr. Jewett received his PhD in 2005 at Stanford University and completed postdoctoral studies at the Center for Microbial Biotechnology in Denmark and the Harvard Medical School. He began his academic career at Northwestern University in 2009, where he was the Director of the Center for Synthetic Biology.

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