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Cell-free gene expression: an expanded repertoire of applications

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Abstract

Cell-free biology is the activation of biological processes without using intact living cells. It has been used for more than 50 years across the life sciences as a foundational research tool, but a recent technical renaissance has made possible high-yielding (g protein/L) cell-free gene expression systems from model bacteria, the development of cell-free platforms from non-model organisms, and multiplexed strategies for rapidly assessing biological design. These advances provide exciting opportunities to profoundly transform synthetic biology by enabling new approaches to model-driven design of synthetic gene networks, fast and portable sensing of compounds, on-demand biomanufacturing, building cells from the bottom up, and next-generation educational kits.

[H1] Introduction

Cell-free gene expression (CFE) is the activation of transcription and translation using crude cellular extracts instead of intact cells. Because cellular extracts retain the native cellular transcriptional and translational machinery, protein synthesis can be initiated *in vitro* using CFE by supplementing the extracts with exogenous resources, including amino acids, nucleotides, and a high-energy phosphorylated substrate (**Box 1**). By eliminating the constraint of sustaining life, CFE provides unprecedented control over the molecular context for gene expression and metabolism: it enables the experimenter to directly manipulate the system—for example, by adding non-native substrates, purified proteins or RNAs, or recombinant DNA templates. Additionally, cell-free experiments can circumvent mechanisms that have evolved to facilitate species survival, bypass limitations on molecular transport across the cell wall, and provide the unique ability to focus resource utilization on a distinct genetic network or the biosynthesis of a single product.

Owing to these advantages, CFE systems have been harnessed to elucidate key principles of biological systems for more than 50 years. The mechanism of enzymatic DNA replication was first illuminated by directly probing polymerase activity in extracts of *Escherichia coli* and *Xenopus*.[1,2] Similarly, the role of 5'-capping in eukaryotic translation initiation was discovered using an *in vitro* translation assay.[3] Indeed, the Nobel Prize awarded to Marshall Nirenberg for the deduction of the genetic code owed mainly to his seminal experiments in *E. coli* cell-free extracts.[4,5]

While CFE systems have historically been used to answer fundamental research questions, their engineering potential was hampered for decades by a few key constraints: low and variable protein synthesis yields, short reaction durations, reagent costs (particularly for nucleotides and the energy source), small reaction scale, an inability to correctly fold complex proteins and protein assemblies, limited transcription by endogenous cellular RNA polymerases, and popular misconception of the reaction environment as a 'black-box' that could not be controlled.[6-8] Moreover, non-standardized protocols that require specialized, expensive equipment hindered widespread entry to the field. Recently, however, a technical renaissance has begun to systematically address each of these limitations, enabling application of CFE in the burgeoning field of synthetic biology.

Today, protein yields exceed grams of protein produced per liter reaction volume, batch reactions last for more than 10 hours, and reaction scale has reached the 100-liter milestone, a feat deemed impossible just 10 years ago.[9-11] Costs have been reduced by orders of magnitude,[10,12] largely through better understanding of and control over central metabolism and oxidative phosphorylation in extracts.[13] Synthetic approaches for *in vitro* disulfide bond formation and membrane anchoring have made dozens of new classes of

proteins accessible for CFE, including monoclonal antibodies, ion channels, and even the capsids of viable bacteriophages.[14-18] Generalized methods have been developed for preparing transcription- and translation-capable extracts from a wide array of host model organisms, including the activation of endogenous transcription from natural sigma factors.[19-21] Finally, protocols for preparing extract have been simplified from their original formulations[22] and made less expensive and more accessible such that a high-yielding extract from *E. coli* can be prepared in half a day's work with conventional molecular biology equipment.[11,23-26] Taken together, these features have led to an explosion in the scope of CFE systems (**Figure 1**), both for small-scale prototyping of biological processes and also for larger-scale bioengineering efforts.

Here, we review the rapidly growing application space for CFE systems. We first discuss the uses and challenges of cell-free systems for understanding biological principles, with an eye toward forward-engineering cellular function. We then shift to summarize the recent progress and current limitations of scaling up cell-free gene expression reactions for direct practical application, with a focus on point-of-use biosensing and biomanufacturing. We primarily discuss prokaryotic CFE systems, as these have made the most progress and are the most productive, although we do mention eukaryotic extracts that have shown promise for specialty applications. We also predominantly focus on extract-based CFE systems, which are generally less expensive and more versatile than bottom-up reconstituted transcription-translation systems such as the PURE system (protein synthesis using purified recombinant elements).[27] We will not describe in detail the progress made in preparing bacterial extract and reaction mixtures to optimize protein expression yield because this has been recently summarized in several excellent reviews.[6,28,29]

Finally, as a note on terminology: we use the label 'CFE' throughout this review to refer to any methodology for converting DNA into a functional RNA or protein output. This definition encompasses a number of popular terminologies, including cell-free protein synthesis (CFPS), TX-TL (transcription-translation), and *in vitro* transcription-translation (IVTT). We believe that 'cell-free gene expression' is the most accurate label, as it is more inclusive of the steps of gene expression beyond transcription and translation that can also occur *in vitro*, including the synthesis of functional RNAs, eukaryotic transcript splicing, protein folding, and post-translational modification.

[H1] Cell-free probing of cellular functions

Most biochemical studies of cellular function are intrinsically hindered by the cell wall, a highly selective barrier that sequesters reactions of interest from direct user influence. CFE systems are different: the experimenter has barrier-free access to the reaction

environment to directly probe biological function. The ability to prototype individual genetic parts or complex genetic designs (such as genetic response networks or metabolic pathways) *in vitro* before implementing them in cells has thus emerged as an important application area for CFE systems. These experiments can be conducted on miniaturized reaction scales and automated[30] to permit rapid testing of large combinations of genetic parts, serving as a biological analog of combinatorial synthetic chemistry. In this section, we discuss the use of CFE to prototype increasingly complex cellular function, beginning with libraries of simple genetic parts and culminating in the design of artificial minimal cells.

[H2] Genetic part prototyping. Rational and predictable engineering of biological systems is constrained by a limited number of well-characterized genetic elements (such as promoters, ribosome-binding sites, and transcriptional terminators), particularly for non-model or difficult-to-culture hosts. Even well-characterized elements frequently do not work as expected in living cells because of pathway crosstalk, that is, unexpected activity from endogenous regulators of gene expression. CFE systems have begun to address these challenges by enabling rapid, high-throughput *in vitro* prototyping pipelines. Early prototyping studies showed that T7 bacteriophage transcriptional elongation and termination could be measured using cell-free reporter assays.[31,32] Later demonstrations that native bacterial polymerases generated sufficient mRNA *in vitro* to activate cell-free protein synthesis[33] opened the floodgates for cell-free prototyping of more complex regulatory elements, including quorum-sensing promoters,[34,35] light-sensing promoters,[36] allosteric transcription factors,[37-39] synthetic translational riboregulators,[40] and riboswitches.[41,42]

The key methodology in this approach is to rapidly assay the performance of individual genetic parts (for example, promoter initiation rates or transcriptional terminator efficiencies) by designing large libraries of single variants that control expression of an easily measured reporter protein (**Figure 2A**). In one illustrative example, biophysical kinetic rate parameters including the transcription rate, ligand binding affinity, and cooperativity of a xylose repressor were estimated by exhaustively titrating repressor and inducer concentrations in CFE reactions.[30] Importantly, this work was facilitated by an automated liquid-handling robotic system that dispensed single microlitre reactions in which protein and mRNA levels could be monitored in real-time using orthogonal fluorescent reporters, as previously described.[30,43,44] The large reported parameter space, which would have been inaccessible using only manual pipetting, enabled the design of thousands of new promoters and ribosome-binding sites.

Because fluorescent protein expression can be conveniently measured at low reaction volumes using conventional plate readers, screening genetic part libraries with CFE can

be done at small reaction scales (nL to μL) in high throughput (hundreds to thousands of variants tested per experiment) with the aid of robotics. At this level of throughput, the genetic assembly of these libraries of variants can become limiting. That constraint was alleviated by the discovery that cell-free transcription can be initiated from linear expression templates (LETs) generated through PCR. Using LETs as the DNA source in bacterial extracts often requires that the linear DNA is protected from cleavage by the nuclease RecBCD. This can be accomplished by supplementing the reaction with the lambda phage protein GamS, a nuclease inhibitor that binds to and protects the DNA, or by adding oligonucleotide repeats of RecBCD's native substrate χ, a competitive inhibitor.[45-47]

Thus, in the highest-throughput version of this strategy, libraries of genetic parts can be designed rapidly through PCR mutagenesis of a starting sequence, and then directly added to a cell-free reaction, without the need for plasmid construction, transformation, or colony picking (**Figure 2A**). To ensure that single gene variants are maintained, the extracts can be encapsulated in water-in-oil emulsions for droplet-based expression and sorting.[48] CFE reactions compartmentalized into picolitre droplets[49-51] have been used to select[52] and evolve[53,54] enzyme variants through dilution to single variants per droplet, predicted by a Poisson distribution.[55,56] As CFE from a single copy of a gene produces little protein, in-droplet DNA amplification methods, such as digital droplet PCR, are frequently implemented in tandem.[54] An alternative approach to droplets activates transcription and translation on a patterned solid support. Protein arrays have been 'printed' onto fabricated surfaces by chemically conjugating linear DNA templates with surface-reactive handles.[57-61] Because microfluidic control can enable precise spatial and temporal mixing, these arrays are particularly useful for high resolution mapping of multicomponent transcriptional regulation, such as in a quorum sensor.[62]

[H2] Genetic circuit prototyping. In addition to probing individual genetic parts, CFE systems can be used to analyze how genetic parts function together in synthetic genetic control networks or 'circuits' (in analogy to the process of breadboarding **[G]** for electronic components).[43] Whole transcriptional units, each encoded by separate plasmids or LETs, can serve as analogs to logic gates **[G]** ; then, the CFE reaction performs molecular computations or executes defined genetic programs *in vitro* as a prediction for how a circuit would function in cells. A particular benefit of the cell-free experiment is that the relative contribution of each gate to the circuit can easily be manipulated by the experimenter simply by changing the relative level of its DNA template supplied to the reaction (**Figure 2B**). A number of cell-free genetic circuits have been assembled and prototyped using this strategy, including cascades driven by sequential expression of orthogonal polymerases or sigma factors **[G],**[63-65] modulators of retroactivity **[G],**[66] and RNA and/or protein-mediated single-input modules, feedforward loops, and negative autoregulators.[67-72] Even genetic circuits that require dilution over time, such as a ring

oscillator **[G]**, have been implemented in semi-continuous cell-free systems.[73,74] Dialysis setups are frequently necessary to accommodate large and complex cell-free genetic circuits, which can otherwise suffer from byproduct accumulation and substrate depletion.[43,75]

Although miniaturization has enabled large-scale, high-throughput genetic part and circuit testing, a key open question is the extent to which the *in vitro* performance of a part matches its activity in cells (**Figure 2C**). Cell-free predictions of both part 'strength' and circuit 'noise' have been at least qualitatively validated in some studies,[34,35,45,76] and CFE can even be useful to estimate the expression burden a protein might have on a proliferating cell.[77] However, experimentally replicating the reaction conditions found inside cells would likely improve the predictive power of these studies. Even with supplemented molecular crowding agents such as polyethylene glycol or Ficoll, cellular extracts are still tens to hundreds of times more dilute than the cells from which they are made: the intracellular total protein concentration for *E. coli is* ~200 g/L, compared to ~10 g/L total protein in an *E. coli* CFE reaction.[78] This question is particularly germane for the design of genetic parts in non-model organisms, which are limited by slow *in vivo* engineering cycles. CFE preparation protocols have been developed for bacterial genera such as *Vibrio*,[79-81] *Bacillus*,[19,30] *Pseudomonas*,[82] and *Streptomyces*,[83-85] with the hypothesis that CFE predictions for a particular organism are more accurate in the context of a lysate from that same species (**Figure 2C**). Although this hypothesis is not well-tested, it has been elegantly demonstrated that regulatory sequence transcriptional preferences from a 7,000-member sequence library could be rapidly obtained for ten genetically diverse bacterial species by first systematically optimizing the extract preparation protocol of each individual chassis host.[21] The results showed high species-specific correlation between the measured cell-free transcription rate and the rate measured in the corresponding living host. Yet, it remains unclear how effectively a promoter can be forward-engineered to obtain reliable and quantitative protein-level outputs in cells.

Better models of CFE systems would help resolve this uncertainty. Coarse-grained models of CFE estimate that the rate of protein synthesis is at least 1-2 orders of magnitude slower in extract than in cells.[78] Ordinary differential equation models of CFE rely heavily on estimations of transcription and translation rate constants in a continuously monitored reaction.[43,67,86,87] However, performing these estimates under the resource constraints of batch reactions, rather than inside a living cell, can be difficult because pathway crosstalk exists not just for small molecules such as amino acids and nucleotides, but also for proteins such as sigma factors, elongation factors, and RNases.[43,77,88] For instance, it has been shown that translational elongation is a limiting step for *in vitro* protein synthesis, and supplementing with the elongation factor EF-Tu reduces this bottleneck both in extract[89] and purified systems.[27,90] However, shifting to a

faster translation rate may actually reduce protein yields due to the nonlinear dependence of the transcription rate on the concentration of nucleotides and free Mg^{2+} and the depletion of available ribosomes.[75,87] Ideally, metabolic constraints should also be layered atop the predictions for cell-free transcription and translation to explain the rate of energy regeneration, better capturing the dynamics of carbon availability in a batch reaction.[91] However, full-scale models of the transcriptome, metabolome, and proteome of the CFE environment remain elusive, even in reconstituted systems,[92] thereby constraining the predictive ability of cell-free genetic prototyping.

[H2] High-throughput prototyping of protein modifications. Many of the same benefits of using small-scale, high-throughput CFE to rapidly prototype transcription and translation also apply to studying other cellular functions, including metabolism, biosynthesis, and defense against pathogens. However, unlike transcription and translation, the output of these pathways cannot generally be linked to expression of a fluorescent protein, so the reaction scale and the library size are limited by the minimum volume that can be screened using analytical methods. As a result, additional strategies must be implemented to improve assay throughput.

A representative example of prototyping beyond transcription and translation alone is the study of post-translational protein modifications (PTMs) such as phosphorylation and glycosylation. Studying PTMs on proteins in cells is often challenging because the modifications are chemically heterogeneous and compartment-specific. Modifying enzymes are also frequently difficult to express heterologously. As a result, where suitable chemical assays are available, CFE has proven powerful for interrogating PTMs with high sequence specificity. The design for this experiment originates with a decades-old strategy of incorporating isotopically labelled amino acids into proteins to facilitate nuclear magnetic resonance (NMR).[93-95] By supplementing heavy amino acids to the CFE reaction, isotopically labelled proteins can be synthesized at sufficient titer for structural characterization or, in reconstituted systems, for quantitative proteomics.[96,97] Taking this strategy further, small synthetic PTMs can be co-translationally incorporated as noncanonical amino acids in CFE. For example, the effect of serine phosphorylation on MEK1 kinase activity was determined by using extract from a genomically recoded *E. coli* strain that site-specifically incorporated phosphoserine.[98]

More complex PTMs can also be probed using CFE. In a recent study, the sequence preferences of cell-free synthesized glycosyltransferases were determined by detecting the attachment of monosaccharides to a library of sequence-defined peptides immobilized on a gold monolayer using self-assembled monolayer desorption/ionization mass spectrometry (SAMDI-MS) (**Figure 3A**). This CFE assay, called GlycoSCORES, enabled the highest resolution quantification of substrate sequence specificity of glycosylation enzymes to date; the corresponding cellular experiments would have

required thousands of batch cultures.[99] Beyond designing glycosylation sites, cell-free approaches have been extended to study and engineer glycans containing multiple sugars through *in vitro* expression and mixing of the glycosyltransferases. The modularity of this 'GlycoPRIME' strategy enabled construction of 37 putative protein glycosylation pathways, creating 23 unique glycan motifs, 18 of which had not previously been synthesized on proteins.[100] In general, *E. coli* CFE represents an ideal testbed for assaying glycosylation because *E. coli* possesses no native glycosylation machinery.[101,102] Even lipid-bound oligosaccharyltransferases (OSTs), which normally anchor in the cell membrane, can have their activity reconstituted *in vitro* by supplementing extracts with exogenous membrane-mimicking nanodiscs.[103] The ability to accelerate prokaryotic cell-free glycoform screening using cell-free technology could therefore have a transformative impact on the design of glycosylated therapeutics and vaccines.

[H2] Biosynthetic pathway prototyping. Protein synthesis and folding are the most energy-dependent processes for a rapidly growing bacterial cell.[104] To best mimic cellular conditions, cell-free systems must provide the appropriate biochemical environment to meet this ATP demand. Twenty years ago, a series of elegant experiments showed that crude extracts can support highly integrated, multistep metabolic networks that can be understood, modified, and controlled.[105-108] Most importantly, it was then shown that oxidative phosphorylation, mediated by ATP synthases docked in inverted inner membrane vesicles, can regenerate chemical energy *in vitro*.[13] This realization was a crucial factor for achieving cost-effective, high yielding, and long-lasting batch-mode CFE reactions.[13,109] Additionally, though, it hinted at the possibility of using cell-free systems to study multi-enzyme biosynthetic pathways, with the ultimate aim of engineering cellular metabolism.

Such schemes are promising because, for many prototyping workflows, cell-free synthesis of enzymes is often preferable to purifying enzymes from cells. Beyond the obvious elimination of costly and time-consuming protein purification, the physiochemical environment of the extract is more representative of the cellular state than a chemically defined buffer, so the unpurified enzyme may be more likely to retain its native activity. In the original version of this approach (**Figure 3B**), first demonstrated for butanol synthesis,[110] cell-free extracts selectively enriched with an overexpressed enzyme (either pre-enriched in the extract by overexpression in the lysate's source strain or previously expressed *in vitro*) are generated in parallel and then mixed to construct a full biosynthetic pathway. Because design-build-test iterations can be executed just by combining enriched extracts and cofactors at different ratios, aided by robotic liquid handling systems, experimental throughput may be limited by the capacity for analytics.[111] From the biochemist's perspective, this approach offers a high degree of flexibility to model the

kinetics and stability of individual enzymes. For the metabolic engineer, the scheme could accelerate the design of new biosynthetic pathways by optimizing designs (for example, by determining the optimal variants of individual enzymes) before they are expressed in cells in commercial fermenters. Cell-free metabolic engineering has thus emerged as a powerful strategy not just for prototyping synthesis of biofuels, but also of higher-value commodity chemicals, including butanediol, polyhydroxyalkanoates, terpenes, and non-ribosomal peptides.[85,112-116] An early study using CFE of two enzymes for production of 1,4-butanediol was one of the first to show a promising correlation between *in vitro* pathway dynamics and the same genetic constructs implemented in *E. coli* cells.[117] A more recent report demonstrated that CFE coupled with data-driven design could be used to rapidly evaluate hundreds of pathway combinations—selecting enzyme variants and tuning their expression—in *E. coli* extracts to improve productivity of butanol and 3-hydroxybutyrate from a Gram-positive, anaerobic bacterium. This study showed a strong correlation between cell-free and cellular pathway performance, suggesting that the approach could have broad cross-species applicability for engineering non-model microbial metabolism.[118]

[H2] Protein complex and systems-level prototyping. Cell-free systems can also be used to probe other types of pathways. Protein-protein interactions (PPIs), for example, can be discovered at high throughput using a combinatorial mix-and-match approach with protein libraries (**Figure 3C, panel a**). A cell-free strategy is particularly useful for discovering PPIs in organisms such as *Plasmodium vivax* which are otherwise difficult to cultivate in the lab.[119] Moreover, some cellular systems-level characterization of protein function can be performed *in vitro* with CFE (**Figure 3C, panel b)**. By iteratively co-expressing a reporter protein with a library of putative positive and negative effectors of protein synthesis on a chip, a limited determination of gene ontology can be established, using reporter synthesis as a readout of transcriptional inhibition.[120] More recently, strategies have emerged for linking DNA- or RNA-level output to pathway function (**Figure 3C, panel c)**. The target protospacer-adjacent motif (PAM) **[G]** preferences of several cell-free synthesized CRISPR ribonucleoprotein complexes have been determined in high throughput using next-generation sequencing of the target DNA.[121] This study and others[122] highlight the suitability of high-throughput CFE systems for prototyping uncharacterized CRISPR systems.

[H2] Building cells. Moving toward greater complexity, CFE systems are ideally suited for the design and study of synthetic cells integrating multiple genetic and metabolic pathways. This research area is rapidly growing, and several excellent reviews on CFE for synthetic cells have been published.[123,124] One of the first successful proofs-of-concept for this field was the synthesis of self-replicating phages T7 and ΦX174 through *in vitro* transcription and translation of the entire viral genome in 2012.[125] Expression of T4, a phage with a much larger genome, has since been demonstrated in a test tube.[126]

From the inverse perspective, CFE has also largely been the experimental workhorse for building cells from the bottom up. Many laboratories have recapitulated cellular functions by encapsulating CFE systems and synthetic gene circuits inside liposomes **[G]**. An early example of this strategy used hemolysin protein pores in synthetic membranes to enable exchange of nutrients between these artificial cells and an external feeding solution containing NTPs and amino acids (**Figure 4A**).[127] Synthetic membrane-mimicking compartments that encapsulate bacterial extracts have also been made using size-controlled gelation or coacervation. **[G]**.[128-133] Increasingly complex cellular functions have gradually been implemented in minimal cells. For example, photosynthetic energy production has been replicated by incorporating bacteriorhodopsin and ATP synthase, either purified or synthesized *in situ*, into giant unilamellar vesicles **[G]** that encapsulated the protein synthesis machinery.[134] By controlling the loading of specific genetic instructions into particular vesicles, engineered synthetic 'cell-cell' communication networks can be activated by vesicular fusion.[135,136] Organelle-like compartments to spatially separate transcription and translation have been established *in vitro* using agarose microgels functionalized with nucleic acid signal sequences.[128] Synthetic cells made of proteinaceous elastin-like polypeptides (ELPs) that encapsulate DNA encoding the ELP itself can even reproduce and grow.[137] Semiannual Build-a-Cell workshops (http://buildacell.io/) highlight the promise of using encapsulated cell-free systems to uncover the fundamental requirements for replicating, self-sustaining life.

[H2] Cell-free systems for education. Cell-free systems are a powerful tool for understanding biology, starting from the sequence determinants of transcription and translation rate, and scaling to increasingly complex cell-mimicking systems. Many of the benefits of cell-free systems arise from the ease with which they can be manipulated to perform experiments. For these same reasons, and also because cell-free reactions are inexpensive to assemble, the platform has recently been co-opted for use in synthetic biology education (**Figure 4B**). With an eye towards inspiring and training new biologists, chemists, physicists, and engineers, a suite of hands-on educational kits has recently been designed, enabling students to perform biological experiments by adding water and simple reagents to freeze-dried cell-free reactions. Cell-free expression systems can link complex biological concepts to rapid, visual readouts, so students know—after a few hours and with a single glance—the results of their experiments. Cell-free reactions producing fluorescent reporters can be imaged using inexpensive handheld illuminators and incubators, and therefore can be easily implemented for hands-on demonstrations of the central dogma in primary school curricula.[138,139] Students can even be engaged with the experiments on multiple sensory levels by using cell-free reactions encoding olfactory or tactile outputs.[140] More complex supplementary experiments have been designed for teaching about important contemporary biotechnology issues, such as antibiotic

resistance or CRISPR-Cas systems.[141,142] Looking forward, efforts to teach synthetic biology concepts using cell-free systems are poised to have a substantial impact on practical biochemistry and biophysics experiential learning, by expanding educational opportunities to resource-limited schools and culturing an open-source community of educators and young researchers.

[H1] Applications of cell-free systems

The number of direct applications for cell-free systems has exploded over the past decade. In fact, commercialization of cell-free technology has already begun, with multiple companies seeking to redefine the biotechnology industry by working outside of the cell. However, new practical and technical considerations arise when scaling up cell-free systems for commercial applications. In this section, we discuss current progress and future work for two of the most profitable direct applications of CFE: point-of-use biosensing and cell-free biomanufacturing.

[H2] Point-of-use biosensing. In synthetic biology, whole-cell biosensors are created by engineering a host cell to conditionally express a fluorescent or colored reporter protein only in the presence of a target molecule of interest. The sample to test is added to a batch culture of the sensing cells, and reporter synthesis can be monitored visually or electronically to back-estimate the analyte concentration using a calibration curve. Cell-free sensors can work in much the same way: the sample to be tested is added directly to a cell-free reaction that is programmed to conditionally express a reporter protein only when the target analyte is supplied.

Compared to the cellular paradigm, though, cell-free systems offer a number of practical advantages. First, they can detect cell wall-impermeable or cytotoxic analytes. Second, because there is no evolutionary pressure acting on the cell lysate, cell-free sensors are immune to issues such as mutation and plasmid loss that accompany repeated passaging of whole-cell sensors. Finally, *in vitro* systems have a reduced regulatory footprint because there is no need for biocontainment of engineered organisms. Combined, these benefits unlock a novel opportunity for field-deployable biosensing. Rather than growing batch cell cultures in a centralized testing facility and transporting the samples to be tested back to the lab, cell-free sensors can instead be directly brought out to the sampling location—for instance, to detect infection in a patient's blood sample obtained at a remote clinic. A key step toward this goal was the demonstration that CFE reactions can be preserved and distributed at ambient conditions (**Figure 5A**). In 2014, it was shown that cell-free sensors could be lyophilized (freeze-dried) and maintain activity for months, even when dried onto a paper matrix.[143] Because CFE can occur across a range of ambient temperatures, even portable incubators are optional; the reactions can be activated solely using an individual's body heat.[144]

With these technological advances, key progress in cell-free biosensing thus far has been made for the detection of disease-causing viruses and bacteria. In this approach, a sample containing the pathogen is first processed to extract total RNA; then, the RNA is added to a CFE reaction programmed to produce a reporter protein only in the presence of the target nucleic acid sequence. This process differs somewhat from conventional *in vitro* nucleic acid biosensors that do not require gene expression: for instance, strategies where the target nucleic acid stoichiometrically binds to and unquenches a fluorophore in a molecular beacon. **[G]**.[145-147] However, because the reaction occurs isothermally with a single plasmid DNA template, CFE is generally less expensive, easier to multiplex, and more user-friendly outside a laboratory. The strategy has thus been leveraged for the detection of Ebola virus,[143] Zika virus,[148] norovirus,[149] cucumber mosaic virus,[144] and a number of gut-colonizing bacteria, including *Clostridium difficile*.[150]

The molecular design of a nucleic acid biosensor is crucial to prevent off-target activation. To date, most sensors have used a strategy built on genetically encoded toehold-mediated strand displacement (**Figure 5B**).[151] The sensor is designed so that the 5'-end of the toehold switch **[G]** has sequence-specific complementarity with an unstructured RNA 'trigger' sequence unique to the genome of the pathogen being detected.[150] Trigger amplification is often necessary prior to detection of pathogens if the target nucleic acid is in low abundance. For field deployment schemes, this amplification step must be carried out without a thermocycler, so PCR is infeasible. However, nucleic acid sequence based amplification (NASBA)[152] and reverse transcription-recombinase polymerase amplification (RPA)[153] have shown promise as effective isothermal alternatives.[148,149] RNA can be purified from a biological sample (blood, stool, or otherwise) and maintains its stability through sequential nuclease and viral inactivation heating steps.[154] Overall, the entire amplification and sensing reaction requires only three hours when incubated at 37 ºC.[148]

Cell-free detection of small molecules—for example, environmental toxins or cellular metabolites—has made slower progress than the detection of nucleic acids. A key reason for this is that no analog to synthetic riboregulation exists for building sensors for arbitrary small molecules. As a result, most reported successes have comprised natural sensing elements (for example, allosteric transcription factors) that were characterized extensively in cells and then ported over to CFE reactions.[155,156] The transcription factor is added to the reaction in the form of a purified protein[31] through *in situ* expression from an insulated second transcriptional unit,[156,157] through pre-expression in a distinct, earlier CFE reaction,[25,143] or through over-expression in the host strain used to prepare the bacterial extract (**Figure 5C**).[25,158] A cell-free sensor has also been reported for detecting fluoride, a common groundwater contaminant, using a natural riboswitch to control gene expression.[159]

The preparation time required for small molecule detection can be shorter than that for detecting nucleic acids because sample processing steps like nucleic acid amplification and purification are not needed. Epitomizing this strategy, it was recently reported that purified allosteric transcription factors that bind to antibiotics and heavy metals can regulate expression of a fluorescent RNA aptamer in a freeze-dried cell-free transcription-only reaction. Because the timescales for translation and fluorophore maturation are eliminated in this setup, the ROSALIND sensor produces a visible fluorescent output in minutes by using a highly processive T7 RNA polymerase.[160]

The majority of reported cell-free small molecule sensors detect environmental toxins such as mercury,[161] drugs such as gamma-hydroxy-butyrate (GHB),[162] or bacterial quorum sensing signals such as N-butyryl-L-homoserine lactone (C4-HSL).[156] If no known transcriptional biosensor exists to detect the desired molecule, enzymes can also be synthesized in the reaction to first convert the target into a molecule that can be sensed.[163,164] For instance, by co-expressing the transcription factor BenR*,* the hydrolase enzymes HipO and CocE, and a GFP reporter in a single cell-free reaction, a benzoic acid sensor was co-opted for also sensing hippuric acid and cocaine.[164] For other molecules, rational protein engineering has been used to engineer known sensor scaffolds to detect xenobiotic **[G]** compounds, such as endocrine-disrupting compounds found in raw sewage.[161] Taken together, along with advances in computational protein and RNA design for *in silico* sensor engineering, these strategies could expand the spectrum of detectable molecules to enable tailor-made cell-free sensing of any molecule of interest, including new-to-nature compounds.

Despite the great promise and practical advantages of using cell-free sensors in the field, unsolved challenges remain. Chief among these is the difficulty of quantification (**Figure 5D**). In nucleic acid detection, a simple yes or no readout may be sufficient to identify if a pathogen exists in the biological sample. However, quantitative measurement of small molecule concentrations is more difficult because cell-free sensors can be sensitive to variability between batches of extract or to matrix effects, where components of the sample to be assayed actually inhibit transcription or translation. Additional difficulties for quantification arise when enzymatic rather than fluorescent reporters are used. Enzymes that produce visibly colored outputs, such as β-galactosidase,[143] chitinase,[143] or catechol-2,3-dioxygenase[144] are preferable for field-deployment because output can be measured without electronics. However, since the reaction endpoint is set by the amount of reporter substrate supplied to the reaction, the only useful metric to quantify analyte concentration is the speed of response. New strategies and robust standards must therefore be developed to facilitate quantification of target molecules at the point of detection. Toward this end, one promising recent approach for analyte quantification established a time-

dependent, matrix-independent 'ladder' of standards that could also be measured at the point-of-use. The authors applied this strategy to accurately back-estimate physiologically relevant concentrations of zinc in human serum samples.[165]

[H2] *Cell-free biomanufacturing.* Another key prospective application space for CFE is the direct bioproduction of valuable proteins or commodity small molecule products. The rationale for using CFE commercially is that by removing all ancillary growth-related resource utilization, a cell-free reaction could devote all of a cell's transcriptional, translational, and metabolic capacity to making a single product. For an actively growing *E. coli* cell (200 g/L total cytoplasmic protein content, doubling every forty minutes[166]), the total volumetric protein productivity could exceed hundreds of grams cellular protein per L per hr. For small molecules, assuming full substrate conversion and no product toxicity, hundreds of grams per liter per hour could theoretically be made as well.[8] Yet, these productivities are still multiple orders of magnitude higher than actual rates of recombinant cellular production, as cells must also devote resources towards growth and maintenance. This biosynthetic potential could ideally be tapped by cell-free systems.

So far, most attempts to realize that potential have been devoted to the cell-free synthesis of protein biologics. These efforts intensified in the early 2000s with the emergence of new strategies for *in vitro* formation of disulfide bonds, a key structural requirement for antibodies and other therapeutics. Disulfide bond formation is facilitated by chemically inhibiting cytoplasmic reductases found in *E. coli* extracts, creating an oxidized redox environment with glutathione buffer, and supplementing protein chaperones such as DsbC and protein disulfide isomerase. These innovations enabled the synthesis of urokinase, a truncated form of tissue plasminogen activator, and active cytokines such as human granulocyte macrophage colony-stimulating factor (GCSF).[18,167,168] Since then, both antibody fragments and full-length antibodies have been synthesized *in vitro* both in bacterial extracts[169,170] and in Chinese hamster ovary (CHO) cell extracts.[171,172]

Even though most biologics today are made in CHO or other mammalian cells, extracts from mammalian host cells are generally not used for cell-free biomanufacturing. These systems would be prohibitively expensive at an industrial scale because they require continuous filtration (for example, in a microdialysis cassette or in microfluidic channels) to achieve high protein yields.[17,173,174] More recent cell-free approaches have instead aimed to mimic the physiochemical state of the mammalian cytoplasm in productive bacterial extracts. In this way, functional G-protein coupled receptors, such as the β2-adrenergic receptor, can be synthesized in bacterial CFE reactions by docking them to synthetic nanodiscs.[175] Proteins made *in vitro* retain their therapeutic activity. For example, CFE-produced immuno-enhancers against B-cell lymphoma show immunostimulatory effects in culture.[176] In fact, in some cases, a CFE system may be the

most effective way to produce a therapeutic recombinant protein that is toxic when produced by common host organisms. The cytotoxic, chemotherapeutic enzyme onconase can be synthesized at much higher titers *in vitro* than in cells, especially because the open reaction environment enables improvements such as a mid-reaction tRNA dosing regimen.[177]

Individual dose-level quantities of many pharmaceutical proteins can be produced from millilitre-scale CFE reactions, which is a tractable size for on-demand synthesis. As with cell-free biosensors, on-demand biosynthetic reactions can be freeze-dried and hydrated at the point of need to enable cold-chain independent, high-density storage and transport (**Figure 6A**).[178] Pioneering work in this area showed that antimicrobial peptides, vaccine antigens, and antibody analogs synthesized from lyophilized extracts maintained their biological activity,[179] even when sourced from non-*E. coli* hosts.[80] Cryoprotectants can be supplemented to further prolong shelf-stability for more than four months.[180]

In drug manufacturing, the unit operations required for post-fermentation protein purification are often the largest cost contributors. For some proteins made at high yields in *E. coli* extracts, removing or remodeling lipid A endotoxin in the host strain used to produce extract may be sufficient to make a safe therapeutic.[181,182] However, in other cases, on-demand purification, not biosynthesis, of the active pharmaceutical product may be limiting. To this end, in 2016, a fluidic process was developed for rapid end-to-end production of recombinant protein biologics at the point-of-need.[183] More recently, a suitcase-sized platform (termed BioMOD) that performs semi-continuous *in vitro* translation, filtration, affinity tag purification, and polishing was shown to have the capacity for synthesizing GCSF, diphtheria toxoid, and erythropoietin on par with good manufacturing practices (GMP) standards in around eight hours.[184] The approach can be combined with automated microfluidic unit operations to propagate an input DNA sequence through oligonucleotide synthesis, followed by purification, ligation, and *in vitro* transcription and translation, all validated by *in situ* sequencing and electrophoresis.[185] In this way, the entire molecular biology workflow from DNA sequence to functional therapeutic protein could be encoded in a miniature cell-free factory.

CFE systems are especially suitable for the controlled precise production of highly modified proteins for use as therapeutics. A reason for this is that the open cell-free reaction environment permits expression of proteins bearing noncanonical amino acids (ncAAs) using orthogonal translation systems that consist of non-native tRNAs and aminoacyl-tRNA synthetases. The most successful examples of cell-free ncAA incorporation have been done in extracts made from genomically recoded bacterial strains with a knockout of release factor-1, which are supplemented with orthogonal tRNAs to enable amber codon reassignment.[186-189] More recently, multiple codon

reassignments have been performed *in vitro* in a single pot by supplying tRNA-targeting antisense oligonucleotides to the reaction.[190] State-of-the-art CFE systems can achieve yields of greater than 1.7 mg/mL of a reporter protein and incorporate more than 40 identical ncAAs with high fidelity (≥98% efficiency) into a single biopolymer.[188]

Once ncAAs are incorporated at precise positions in a target protein using CFE systems, they act as biorthogonal chemical handles that react with functionalized small molecules to create therapeutic conjugates (**Figure 6B**). One of the first examples of this biorthogonal chemistry was for the cell-free production of antibody-drug conjugates: in 2014, it was shown that para-azido-methyl-phenylalanine, site-specifically incorporated into a cell-free synthesized trastuzumab[191] monoclonal antibody, can be clicked onto the chemotherapeutic drug monomethyl auristatin using the azide-alkyne cycloaddition to obtain high titers.[192] Modified proteins could also potentially be used as protein scaffolds to improve the circulation time of therapeutics in the body. Proteinaceous virus-like particles (VLPs), such as those from the MS2 phage and hepatitis B virus, can be synthesized *in vitro* at very high yields and make particularly effective scaffolds: as they are made up of many repeats of a single coat protein, a single ncAA mutation to the protein allows the particle to be decorated many times over with an antigen, peptide, or small molecule drug.[16]

Importantly, the majority of therapeutic proteins, particularly monoclonal antibodies, are decorated by glycans, not clickable drugs. Synthesizing these glycoproteins *in vitro* has historically been challenging since *E. coli* lacks native glycosylation machinery. One possible solution is to move to eukaryotic CFE reactions, which can be supplemented with purified microsomes **[G]** containing the necessary components for glycosylation. However, these extracts produce much less functional folded protein than bacterial systems.[193,194] An alternative is to prepare CFE systems from specialized cells (often insect, mammalian, or human cells) in which the microsomes remain in the lysate, but these systems remain more expensive than bacterial platforms.[174,195] Bacterial glycoengineering, in which heterologous glycosylation machinery is added to the CFE system, has provided a more convenient approach, (**Figure 6C**). The first demonstration of this approach showed that a purified oligosaccharyltransferase (OST) enzyme and its lipid-linked oligosaccharide (LLO) substrate can be added directly to the CFE reaction for N-linked glycosylation of model proteins in less than a day.[196] More recently, a simpler strategy was described, in which the OSTs and LLOs are overexpressed in the host strain of *E. coli* so that they are pre-enriched in the extract, allowing a protein to be synthesized and glycosylated in the same pot.[197] This technology avoids the need to purify the active glycosylation components and can be used to create a broad range of glycans, including the human trimannose core glycan, in just a few hours at higher yields of glycoprotein that can be achieved in glycoengineered bacterial cells. A culmination of these efforts

641 was the wholly cell-free production of a conjugate vaccine against the pathogen
642 *Francisella tularensis* in single mL-scale CFE reaction that catalyzed the conjugation of a
643 recombinant polysaccharide antigen onto an *in vitro*-synthesized carrier protein. The
644 resulting vaccine showed immunostimulatory effects when injected into mice and is
645 estimated to cost only ~$6 (USD) per dose, which is cost-competitive with conjugate
646 vaccines sold commercially.[181]
647
648 *[H2] Costs and challenges of scaling up cell-free biomanufacturing.* Even with the
649 momentum and recent technical progress for cell-free biomanufacturing, the practical and
650 economic considerations of industrial-scale CFE systems are still mostly untested. By
651 contrast with the abundant academic interest in scaling down CFE reactions for high-
652 throughput biological studies, little work has reported the challenges of scaling them up.
653 Studies from 2011 and 2012 found that many of the process variables and yields scale
654 linearly over a six order of magnitude range in cell-free reaction volume of 100 L.[168,169]
655 However, a systems-level characterization of cell-free bioreactor dynamics would be
656 useful for further optimization efforts and could inform the design of expression systems
657 at an intermediate scale.
658
659 It is instructive here to consider the process and economic feasibility of large-scale
660 biomanufacturing using commonly used CFE systems. To do this, we consider the cell-
661 free synthesis of four representative protein products (T7 RNA polymerase (RNAP), a
662 DTAP vaccine, a drug-conjugated antibody, and subtilisin A), which would be synthesized
663 at scales spanning from a microliter to thousands of liters. T7 RNAP, used for laboratory-
664 scale *in vitro* transcription, can be synthesized in cell-free conditions at sufficient yields to
665 drive synthesis of RNA from a T7 promoter.[64] Diphtheria toxoid (DT) synthesized using
666 continuous-exchange reconstituted cell-free protein synthesis has been made at
667 sufficient concentrations to induce anti-DT antibody production in inoculated mice, with
668 sufficiently low levels of bacterial endotoxin to be within FDA limits for toxoid-based
669 vaccines.[179] Commercial-scale production of glycosylated cytokines has been done at the
670 100 L scale with yields approaching 750 μg/L,[168] which offers promise for high-titer cell-
671 free production of monoclonal antibodies, including those functionalized with
672 chemotherapeutic drugs.[191,192] At 1000 L expression scale, we consider subtilisin, the
673 active protease in commercial laundry detergent and one of the largest-volume
674 commercial commodity proteins.[198] Using reported or estimated cell-free yields for each
675 of these proteins, and 2019 commercial prices, we calculated the maximum possible raw
676 cost of a cell-free reaction that would make cell-free biosynthesis competitive with current
677 production means (**Figure 7A; Supplementary Note**).
678
679 Our simple analyses suggest that cell-free production of laboratory-scale T7 RNAP is
680 economically viable, particularly if little to no purification is necessary. For a DTAP

vaccine, which is already relatively inexpensive on the market, the economics are practical under the stipulation that no purification costs are necessary before injection, noting that some advantages of CFE systems, such as the importance of rapid response to emerging and re-emerging infectious disease threats, are not included in our simple cost model. Although additional costs are incurred by the inducers and glycosylation cofactors supplemented to active cell-free extracts, these may be counterbalanced by the elimination of the cold-chain requirement using freeze-dried reactions. For high-value pharmaceuticals like a drug-conjugated antibody, a cell-free production platform is effective, though with the caveats that purification costs, coupled with limitations on the yield of drug attachment, may dominate the landscape. Because of its low commercial value, subtilisin will likely never be accessible from a cell-free route unless yield per reaction cost decrease by several orders of magnitude.

Our economic analysis is highly contingent on both protein yields and the true cost of CFE. Considering just the raw costs of reagents, neglecting labor and capital, we conservatively estimate that a standard bench-scale CFE reaction formulation costs around $5/mL (USD; **Figure 7B**; **Supplementary Note**). Around 40% of this cost arises from the expensive phosphorylated energy substrate phosphoenolpyruvate (PEP), which is still commonly used. Replacing PEP with non-phosphorylated substrates such as glucose or maltodextrin can nearly halve the overall cost and has been shown to minimally impact protein synthesis yields.[10,199,200] Further cost reductions can be achieved by removing reagents such as dithiotreitol (DTT), coenzyme A (CoA), nicotinamide adenine dinucleotide (NAD), and exogenous tRNAs, replacing nucleoside triphosphates (NTPs) with nucleoside monophosphates (NMPs), and decreasing nucleotide and amino acid concentrations, such that the minimal cost of raw materials could feasibly drop below $1/mL.[10] Given these numerous opportunities to lower costs and increase yields, the application of CFE systems for biomanufacturing remains tantalizing, especially when coupled to the additional flexibility and speed afforded by *in vitro* biosynthesis.

[H1] Conclusions and future directions

Overall, cell-free systems have made enormous technical gains in the past 5-10 years, such that the application space spans disparate fields that include gene expression in non-model organisms, artificial cells, genetic networks, on-demand biosensing and biomanufacturing, and synthetic biology education. However, a number of design goals still need to be met to achieve the true potential of cell-free systems.

With respect to high-throughput, miniaturized CFE systems, an increasing number of non-model chassis strains are becoming available; however, *E. coli* extracts remain the host of choice and nearly the only ones capable of producing protein at cell-comparable yields. For many other organisms, additional work must be done to validate that cell-free

predictions of biological function are well-correlated to cellular studies. Improved kinetic and systems-levels models of CFE systems are key, as most current approaches are still in their infancy, even for well-studied prokaryotes. Single-molecule experiments (for instance, optical tweezers or single-molecule Förster resonance energy transfer) could be useful for measuring some of the necessary biophysical rate constants in a cell-like physiochemical environment, absent the requirement of sustaining life. Similarly, except for the few successful approaches for optimizing cell-free glycolytic metabolism,[117,201,202] metabolic modelling has been constrained by the difficulty in performing functional metabolomic studies in extracts.[203,204] Promising advances in real-time NMR spectroscopy, initially applied to CFE expression from rabbit reticulocyte lysate, could be of some use here,[205] especially to complement recent studies of the bacterial cell-free proteome.[206,207]

With regard to direct applications for CFE, much progress has been made both for biosensing and biomanufacturing. However, additional studies must demonstrate batch production at scale for high-volume production of sensors or therapeutics. Moreover, studies that explore the potential to harness ensembles of catalytic proteins prepared inexpensively from crude lysates of cells for the production of chemicals and hybrid molecules are warranted to unlock the potential of cell-free metabolic engineering at a commercial scale.[8] Regardless of the final product, batch-to-batch consistency of protein yield between extracts and reagent lots, which has proven difficult at small production volumes across academic labs,[208] must be addressed for many long-term goals, including universal calibration of cell-free sensors and robust production of GMP-quality batches of therapeutic proteins. Robust cell-free biomanufacturing may require longer reaction durations than current practice (weeks rather than hours), and therefore an improved understanding of how to prolong cell-free metabolism. For such long timescales to be reached, it may be important to consider the genetic stability of circular DNA templates over extended periods of time, a question that has not previously been explored. Due to the importance of glycosylation for many therapeutics, further advances in the biosynthesis of diverse sugar structures are needed for intentional engineering of natural and novel glycosylated products. Finally, simple, portable purification systems to reliably produce FDA-compliant vaccines and therapeutics from CFE would significantly advance current biomanufacturing paradigms.

In the early years, CFE systems were used only when working in cells was inconvenient or intractable, yet they still uncovered some of the most important insights into our understanding of how genetic information is transferred. As methods for CFE continue to become easier, cheaper, and more widespread, we anticipate that the application space will continue to dramatically increase, because, for many biological applications, cells can be an unnecessary and unsought complexity. CFE systems have already brought design

rules to synthetic biology and introduced practical solutions for challenges in education, diagnostics, and manufacturing. The next phase for cell-free technologies will build upon and expand beyond these efforts toward new and unexpected application spaces to address society's most pressing global challenges.

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Author contributions

The authors contributed to all aspects of the article.

Competing interests

M.C.J. has a financial interest in SwiftScale Biologics and Design Pharmaceuticals Inc. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors declare no conflicts of interest.

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Figure legends

Figure 1. Timeline of cell-free gene expression systems. Practical improvements in cell-free gene expression systems, particularly in the early 2000s, have improved yields of model protein synthesis and have made protocols more accessible and generalizable. These improvements have enabled a host of applications including rapidly prototyping cellular systems, biosensing, and production of therapeutic recombinant proteins.

Figure 2. Cell-free gene expression for prototyping cellular mechanisms of transcription and translation. (A) An example is given for rapidly characterizing the effect of promoter sequence on the transcription rate. Large sequence spaces can be efficiently explored in cell-free systems using linear expression templates that are supplied to individual wells of a high-throughput assay or encapsulated into droplets. Thus, a library of promoter variants can be efficiently screened for transcriptional activity in the absence of cellular expression constraints using a simple RNA or protein-based fluorescent readout. (B) Beyond prototyping individual parts such as promoters, larger genetic networks can be constructed *in vitro* by combining parts with known transfer functions. In the case shown here, a bi-stable toggle switch is constructed by choosing well-characterized inducible promoters with transfer functions that match the desired output model. I1 and I2 are inducers for transcriptional regulators R1 and R2 with designed input-output performance. Optimization of parts at high-throughput in cell-free systems can thus enable rational design before the final network is introduced into cells for higher-order logic functions. (C) Cell-free prototyping can be much more rapid than cellular experiments because genetic constructs do not need to be assembled into a plasmid or genomically integrated for testing. This advantage is particularly helpful for studying non-model, slow-growing host organisms, or for studying the effects of perturbations to the physiochemical environment, though the extent to which cell-free experiments replicate cellular results remains poorly understood.

Figure 3. Cell-free systems for prototyping proteins and pathways. (A) Post-translational modifications can be rapidly assayed by expressing modifying enzymes and target

proteins in vitro and reading out activity using a high-throughput platform such as mass spectrometry. For example, modifying enzymes that add sugars or oligosaccharides can create a product that is heavier due to the mass of the substrate (top). Incorporation of noncanonical amino acids can also create a mass difference in the synthesized proteins (bottom). (B) Biosynthetic pathways can be reconstructed using cell-free expression of individual pathway enzymes and then mixing enriched extracts and cofactors with a high degree of modularity. (Ca) Protein-protein interactions can be screened in high-throughput using combinatorial mixing. In this case, combinatorial mixing demonstrates that the test protein can interact with Protein A, but not Protein H. (Cb) Sequential CFE can identify proteins that positively or negatively affect cellular processes reconstituted in lysates. Putative effectors can be expressed in vitro in separate wells of a plate, and their positive or negative impact on protein synthesis can be read out by expressing a reporter protein in the same well. (Cc) CFE of CRISPR-Cas complexes can identify protospacer adjacent motif (PAM) requirements and rapidly screen potential small molecule inhibitors in extracts by linking Cas-mediated cleavage to a measurable expression output through sequencing or reporter protein synthesis. In the figure, the Cas nuclease, guide RNA, and target reporter protein are co-expressed in a single reaction, and on-target Cas-mediated cleavage results in decreased fluorescent protein synthesis.

Figure 4. Building cells and synthetic biology education. (A) Synthetic cells typically consist of encapsulated cellular extract in a vesicle or other microcompartment which can exchange spent reaction products and fresh monomers for protein synthesis with a feed solution. Expression of proton pumps, SNAREs, or phase-separating polymers can be used to mimic cellular behaviors for energy generation, vesicular communication, and organelle formation. (B) Freeze-dried CFE reactions can be used as an inexpensive hands-on experimental platform for teaching students about the central dogma and connected phenomena, using a range of possible sensory outputs to enable student-led experimental inquiry.

Figure 5. Design of cell-free biosensors. (A) Schematic for point-of-use cell-free biosensing. Freeze-dried sensor reactions can be assembled centrally and directly shipped to the point-of-testing, hydrated with the sample of interest, and will produce a fluorescent or colorimetric output that can be measured by eye in the absence of sophisticated electronics, even when immobilized on a paper substrate. (B) Operation of a cell-free nucleic acid sensor. RNAs or DNAs to be sensed are identified computationally by finding organism-specific sequences that are compatible with toehold strand displacement reactions, and toehold sensors are designed to produce the reporter protein only in the presence of the target sequence. Sample preparation requires nucleic acid extraction, often through heat inactivation or affinity purification, and isothermal amplification of target sequences. The resulting RNA pool is then supplied directly to the freeze-dried cell-free gene expression (CFE) reaction. (C) Operation of a cell-free small molecule sensor. An allosteric transcription factor is activated by a molecular cue to turn on transcription of a downstream reporter. For transcriptional activity and regulation from the bacterial promoter in a cell-free environment, a consensus promoter sequence is often required alongside a natural or engineered operator sequence. (D) Challenges in cell-

free biosensing include nonlinearities observed in both target amplification and sensor transfer functions, as well as the difficulty in interpolating the speed of an enzymatic response to a quantitative concentration metric.

Figure 6. Cell-free biomanufacturing platforms. (A) Cold-chain-independent freeze-dried lysate, DNA, and cell-free gene expression (CFE) reagents can be transported. Upon rehydration and mixing, CFE can be carried out to produce a wide variety of proteins and chemicals. (B) By incorporating noncanonical amino acids (ncAAs) into virus-like particle (VLP)-forming heterodimers and antibodies and/or antibody fragments, CFE can produce antibody-drug conjugates and VLPs for drug delivery using bioconjugate chemistry. (C) Making lysate out of E. coli enriched with glycan biosynthesis enzymes and an oligosaccharyltransferase (OST) allows for in vitro glycosylation during CFE of a target protein.

Figure 7. The economics of cell-free gene expression. (A) Approximate costs and revenues from production of laboratory-grade enzyme, point-of-use vaccine doses, bio-manufactured medicines, and commodity chemicals. All listed costs are US dollars. For high-value commodity products (such as enzymes and medicines), cell-free biosynthesis is a viable route, particularly for products that are difficult to make inside cells. For point-of-use vaccine production, the cost comparison may be on par but freeze-dried cell-free reactions offer benefits to offset cold chain costs. However, for most low-value proteins produced in cells, the economics of cell-free systems are not currently feasible. For cost comparisons, we used the following market prices: T7 RNA polymerase ($13,250/mg); DTAP vaccine ($200/mg); antibody-drug conjugate (Mylotarg; $5,467/mg); Subtilisin A ($0.022/mg). Further assumptions and costing are detailed in the Supplementary Note, noting that our economics are highly dependent on estimates of protein expression yields and reaction costs. (B) The cost of a cell-free protein synthesis reaction. Neglecting any necessary cofactors, the formulation of a cell-free reaction from published methods,[209] based solely on the cost of raw materials from traditional chemical manufacturers is approximately five cents for a typical ten microliter reaction. Removal of expensive phosphorylated energy substrates and non-mandatory reaction cofactors, as well as reduction in the supply of nucleotide triphosphates, amino acids, and supplemented DNA may bring this cost down by a factor of 5-10. Additional economies of scale could also reduce the cost per reaction.

Box 1: Overview of cell-free gene expression

The CFE reaction is prepared from three components: cell extract, a reaction mixture, and a mixture of DNA and inducers encoding the genetic instructions for the reaction.

- *Extract*. The extract contains ribosomes, RNA polymerase, and other transcription and translation accessory proteins (such as sigma factors, initiation factors, and elongation factors) derived from a source strain. Often, the source strain has been genome-optimized to reduce protease and nuclease activity. The source strain is grown in enriched media and lysed by homogenization, sonication, bead-beating, or freeze-thawing to obtain functional extract. Additional post-lysis purification

steps, including ribosomal runoff reaction and dialysis, may also be performed depending on the application.[210,211]

- *Reaction Mixture*. This mixture of supplemented cofactors for protein synthesis includes amino acids, nucleotides, salts (ammonium, magnesium and potassium cations), an energy source (sugars or polysaccharides, glycolytic intermediates, or creatine phosphate), molecular crowding agents (often polyethylene glycol, putrescine, spermidine, or Ficoll), metabolic cofactors (nicotinamide adenine dinucleotide and coenzyme A), buffer, tRNAs, and other metabolic additives (oxalate to inhibit gluconeogenesis, glutathione for disulfide bond formation, and folinic acid for formylmethionine synthesis).[13,109,212]
- *DNA template*: The template supplied to the reaction can be plasmid DNA or a linear expression template obtained from PCR, usually at 1-20 nM concentration.

The three components are incubated together at temperatures from 16 °C to 37 °C, and protein synthesis reactions typically occur over 1-24 hours in batch operation.[9] The typical reaction volume is on the order of 10 µL, but the reaction may be carried out in 1 mL tubes or on the bottom of well-plates to maximize the oxygenated headspace, facilitating oxidative phosphorylation.[9,64] The reaction volume can vary across orders of magnitude (10^{-15} to 10^{3} L) depending on the application. Typical protein yields in a reaction are in the hundreds of µg/mL range, with protein production reported above 2 mg/mL for model proteins in conventional batch reactions.[199] Higher yields can be achieved by prolonging the reactions through continuous exchange of reaction byproducts and a nutrient feed stream, either in an ultrafiltration cell,[213] dialysis cassette,[214] or through microfluidic pumping.[49] For conventional reporter proteins such as green fluorescent protein (GFP)[215] or its variants,[216] these yields are sufficiently high that gene expression can be measured across several orders of magnitude.

Glossary

breadboarding: The modular assembly of electronic circuits by combining well-characterized components (e.g., resistors, voltage sources) onto a plastic board; the term has been co-opted for synthetic biology to describe the assembly of genetic regulatory networks

logic gates: An electronic component that can compute a basic Boolean binary operation (e.g., AND, OR, NOT)

sigma factors: The accessory protein components that form the *holo* bacterial RNA polymerase capable of transcription initiation

retroactivity: An effect where downstream effectors cause a genetic load on upstream components of a multicomponent metabolic or genetic network

ring oscillator: A genetic circuit in which three species are interchanged (A → B → C → A) and their concentrations vary with fixed period

protospacer-adjacent motif (PAM): A 3'-recognition sequence for CRISPR-Cas proteins that determines the sites of RNA-mediated DNA cleavage

liposomes: Spherical compartment composed of phospholipid bilayers that spontaneously forms when fatty acids aggregate in water

coacervation: Liquid-liquid phase separation that occurs in polymer solutions

giant unilamellar vesicles: A liposome containing a single bilayer with size on the order of a whole cell or larger (radius ~1-100 μm)

molecular beacon: A nucleic acid duplex in which one strand is covalently linked to a fluorophore and the other is linked to a fluorescence quencher, so that fluorescence is only observed upon disruption of the duplex

toehold switch: A genetic regulator in which mRNA translation is inhibited by sterically blocking a ribosome-binding site with a complementary strand of mRNA in the 5' untranslated region, which can only be translationally competent if the duplex is disrupted by a complementary (trigger) strand

xenobiotic: Molecules that are not produced in any known natural biological systems

microsomes: Small vesicles originating from the endoplasmic reticulum when cells are sheared and centrifuged

ToC blurb

Cell-free gene expression (CFE) systems have long been used to address fundamental research questions. Now, owing to technological advances, CFE is finding wider applications in the field of synthetic biology, including in biosensing, biomanufacturing and the design of gene networks.