Cell-free gene expression: an expanded repertoire of applications

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Abstract | Cell-free biology is the activation of biological processes without the use of 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 facilitated high-yielding (grams of protein per litre), 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 the model-driven design of synthetic gene networks, the fast and portable sensing of compounds, on-demand biomanufacturing, building cells from the bottom up, and next-generation educational kits.

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 secondary energy 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 was due 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 a 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 the widespread use of CFE. Recently, however, a technical renaissance has begun to systematically address each of these limitations, enabling the application of CFE in the burgeoning field of synthetic biology.

Today, protein yields exceed grams of protein produced per litre of reaction volume, batch reactions last for more than 10 h and the reaction scale has reached the 100-litre milestone, a feat deemed impossible just 10 years ago[9–11]. Costs have been reduced by orders of magnitude[10,12], largely through the 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-capable 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 extracts 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 (FIG. 1), both for the small-scale

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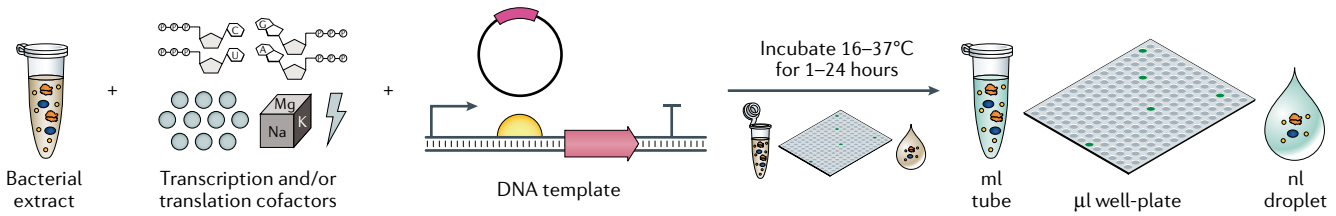
Box 1 | **Overview of cell-free gene expression**

The cell-free gene expression reaction is prepared from three components: the cell extract, a reaction mixture, and a mixture of DNA and inducers encoding the genetic instructions for the reaction (see the figure).

- *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. Metabolic enzymes for energy and cofactor regeneration are also present. 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 a functional extract. Additional post-lysis purification steps, including ribosomal runoff reaction and dialysis, may also be performed, depending on the application[209,210].
- *Reaction mixture*. This mixture of supplemented cofactors for protein synthesis includes amino acids, nucleotides, salts (for example, ammonium, magnesium and potassium cations), polyamines (for example, putrescine, spermidine), an energy source (sugars or polysaccharides, glycolytic intermediates or creatine phosphate), molecular crowding agents (for example, polyethylene glycol or Ficoll), metabolic cofactors (for example, nicotinamide adenine dinucleotide and coenzyme A), buffer, transfer RNAs, and other metabolic additives (for example, oxalate to inhibit gluconeogenesis, glutathione for disulfide bond formation and folinic acid for formylmethionine synthesis)[13,109,211].
- *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 h in batch operation[9]. The standard 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 range of hundreds of micrograms per millilitre, 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 by-products and a nutrient feed stream, either in an ultrafiltration cell[212], dialysis cassette[213] or through microfluidic pumping[49]. For conventional reporter proteins, such as green fluorescent protein (GFP)[214] or its variants[215], these yields are sufficiently high that gene expression can be measured across several orders of magnitude.



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 towards forward-engineering cellular function. We then summarize the recent progress in, and current limitations of, scaling up CFE 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 extracts and reaction mixtures to optimize protein expression yield as 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 several popular terminologies, including cell-free protein synthesis, transcription–translation and in vitro transcription–translation. 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.

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

Fig. 1 | **Timeline of cell-free gene expression systems.** Practical improvements in cell-free gene expression systems, particularly over the past 20 years, 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. RNAP, RNA polymerase.

Methodology | Discovery | Application

1961
1964
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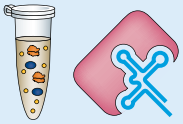
Use of *E. coli* extracts to determine the genetic code[4,5]

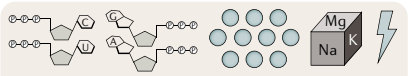


Geoffrey Zubay publishes S30 extract protocol[22]

Prolonged transcription and translation using continuous-exchange cell-free protein synthesis[213]

Isotopically labelled amino acid supplementation to facilitate protein NMR[93]

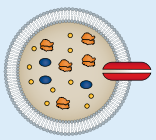
In vitro compartmentalization for rapid protein evolution[54]



Cytoplasmic mimicry, strain engineering and new energy substrates improve protein titres and reduce costs[10–13,105–109,210–212]

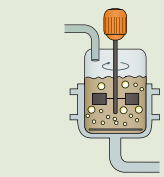
In vitro transcription and translation first demonstrated in purified, reconstituted system (PURE)[27]

Extract encapsulated in vesicles for long-lived cell-free bioreactor[127]

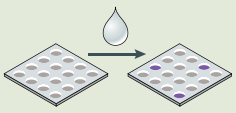


Extract centrifugation at 12,000×*g* (S12 prep)[26]

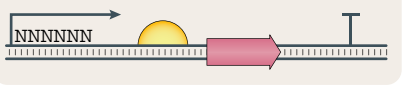
Linear scale-up of cell-free expression to the 100-litre scale[168]



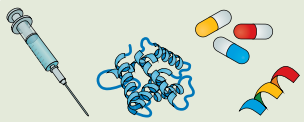
Nucleic acid detection using freeze-dried cell-free expression platform[143,148]



Bacterial RNAP used for cell-free transcription, enabling new circuit and sensor function[33]

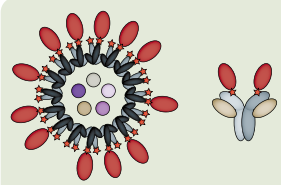


Therapeutic proteins produced for on-demand biomanufacturing[179]



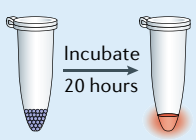
Cell-free synthesis of functional bacteriophage particles[64,125,126]

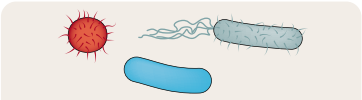
New protocols for cheaper, faster and more accessible lysis methods (sonication, bead-beating, flash-freezing)[23–25]



Cell-free non-standard amino acid incorporation into proteins at g/l quantities with >95% fidelity[188]

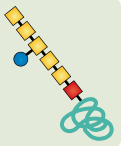
Synthetic biology education kits developed from freeze-dried cell-free reactions[138–142]





Extract protocols created for additional prokaryotic chassis hosts, including Streptomyces, Pseudomonas, Vibrio, and Bacillus[30,79–85]

Synthesis of glycoproteins in cell-free *E. coli* extracts[103,197]



High-throughput prototyping of glycosyltransferase and CRISPR sequence preferences using cell-free expression[100,121]

a biological analogue 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.

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 the 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 (FIG. 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.

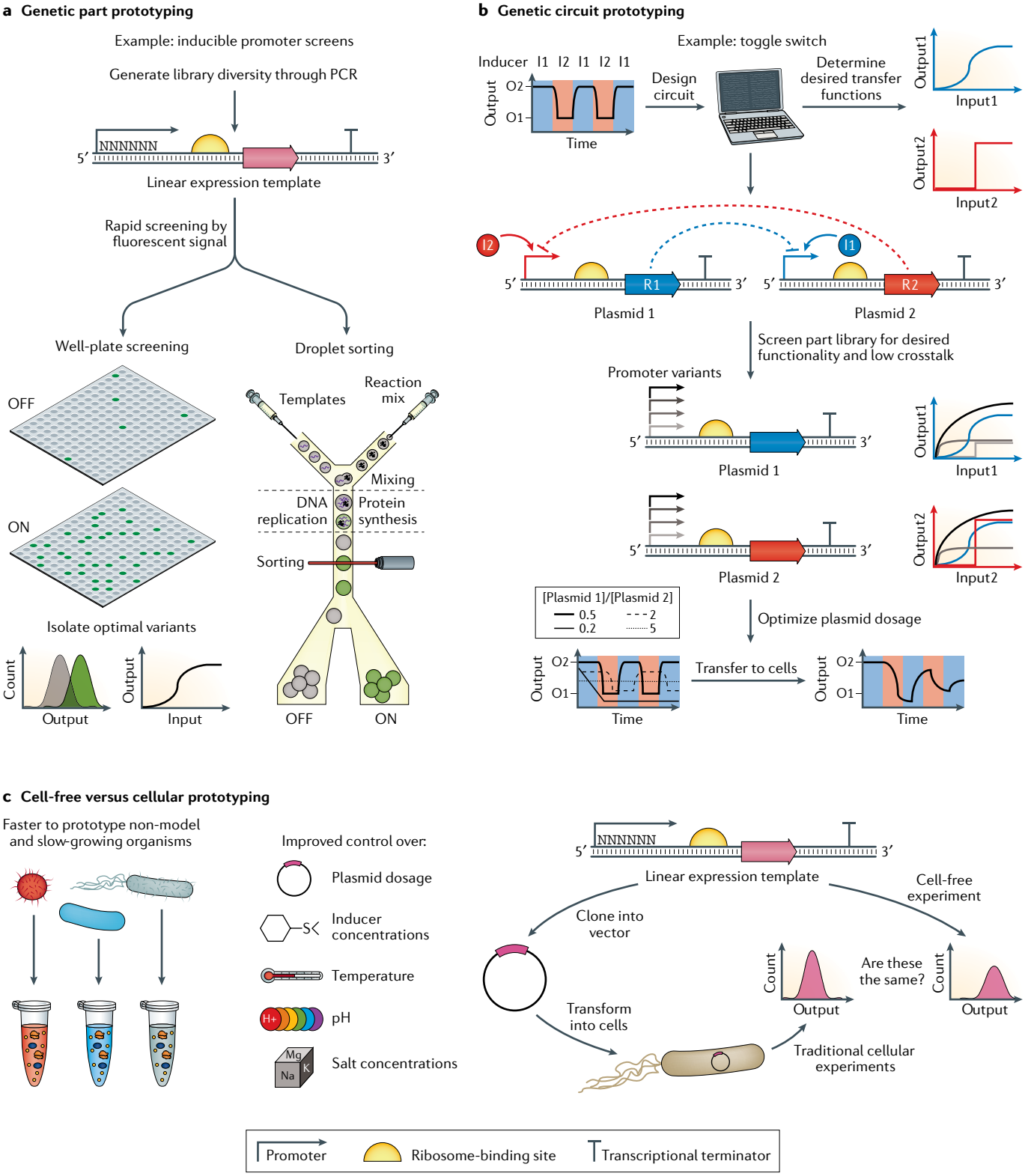
Because fluorescent protein expression can be conveniently measured at low reaction volumes using conventional plate readers, screening genetic part libraries with CFE can be done at small reaction scales (nanolitre to microlitre) in high throughput (hundreds to thousands of variants tested per experiment) with the aid of robotics. At this level of throughput, the genetic assembly of these libraries of variants can become limiting; this 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 (FIG. 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, as predicted by a Poisson distribution[55,56]; reactions have been carried out in volumes as small as femtolitres[56]. As CFE from a single copy of a gene produces minimal amounts of protein, in-droplet DNA amplification methods, such as digital droplet PCR, are frequently implemented in tandem[54]. Alternatively, transcription and translation can be activated directly on a patterned solid support. Protein arrays have been programmably '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 the high resolution mapping of multicomponent transcriptional regulation such as in a quorum sensor[62].

Genetic circuit prototyping. In addition to probing individual genetic parts, CFE systems can be used to analyse how genetic parts function together in synthetic genetic control networks or 'circuits' (in analogy to the process of breadboarding for electronic components)[43].

Fig. 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 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-based 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 the effects of perturbations to the physiochemical environment, though the extent to which cell-free experiments replicate cellular results remains not well defined.

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



Whole transcriptional units, each encoded by separate plasmids or LETs, can serve as analogues to a logic gate; then, the CFE reaction performs molecular computations or executes defined genetic programmes 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 (FIG. 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[63–65], modulators of retroactivity[66], and RNA and/or protein-mediated

Logic gate
An electronic component that can compute a basic Boolean binary operation (for example, 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 a fixed period.

single-input modules, feedforward loops and negative autoregulators[67–72]. Even genetic circuits that require dilution over time, such as a ring oscillator, 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 by-product 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 (FIG. 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 when supplemented with 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 of 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 (FIG. 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 10 genetically diverse bacterial species by first systematically optimizing the extract preparation protocol of each individual chassis host[21]. The results showed a 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 order of magnitude slower in extracts 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 supplementation with 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 non-linear dependence of the transcription rate on the concentration of nucleotides and free Mg^{2+} as well as 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.

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 defence 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; furthermore, 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 NMR[93–95]. By supplementing heavy amino acids to the CFE reaction, isotopically labelled proteins can be synthesized at sufficient titres 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 non-canonical amino acids in CFE. For example, the effect of serine phosphorylation on MEK1 kinase activity was determined by using extracts 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 (FIG. 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.

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, it hinted at the possibility of using cell-free systems to study multi-enzyme biosynthetic pathways, with the 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 (FIG. 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 these pathways are expressed in cells in commercial fermenters. Cell-free metabolic engineering has thus emerged as a powerful strategy, not just for prototyping the 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 the 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 a 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 the 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 the engineering of non-model microbial metabolism[118].

Protein complex and systems-level prototyping. Cell-free systems can also be used to probe other types of pathways. Protein–protein interactions, for example, can be discovered at high throughput using a combinatorial mix-and-match approach with protein libraries (FIG. 3Ca). A cell-free strategy is particularly useful for discovering protein–protein interactions 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 (FIG. 3Cb). 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-level or RNA-level output to pathway function (FIG. 3Cc). The target protospacer-adjacent motif (PAM) 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.

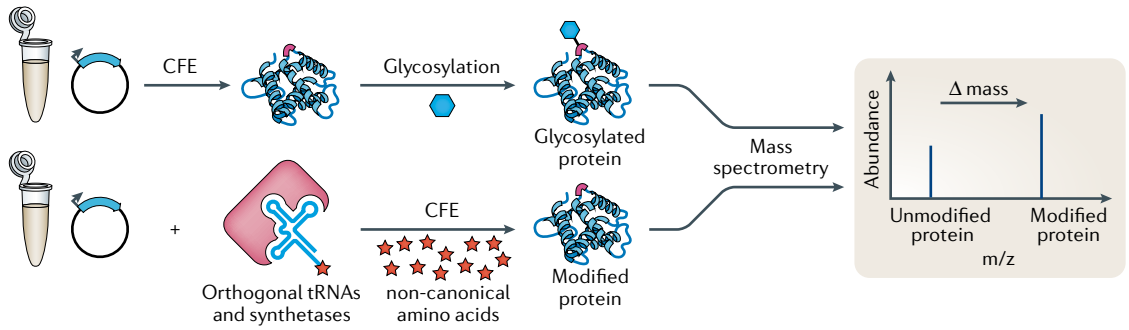
Building cells. Moving towards greater complexity, CFE systems are ideally suited for the design and study of synthetic cells integrating multiple genetic and metabolic pathways. This research area is rapidly growing, and several excellent reviews on CFE for synthetic cells have been published[123,124]. 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 (REF.[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. An early

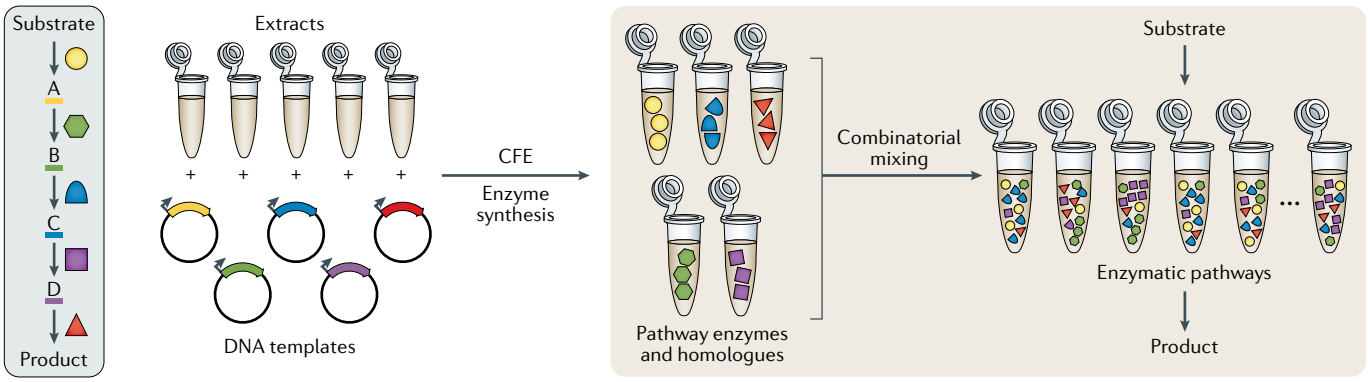
Protospacer-adjacent motif (PAM). A 3′-recognition sequence for CRISPR–Cas proteins that determines the sites of RNA-mediated DNA cleavage.

Liposomes
Spherical compartment composed of phospholipid bilayers that spontaneously forms when fatty acids aggregate in water.

A Protein modification prototyping

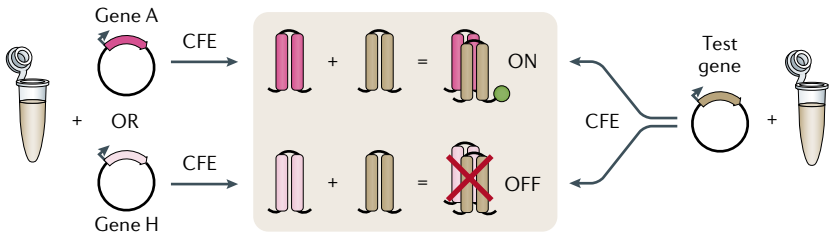


B Enzymatic pathway prototyping

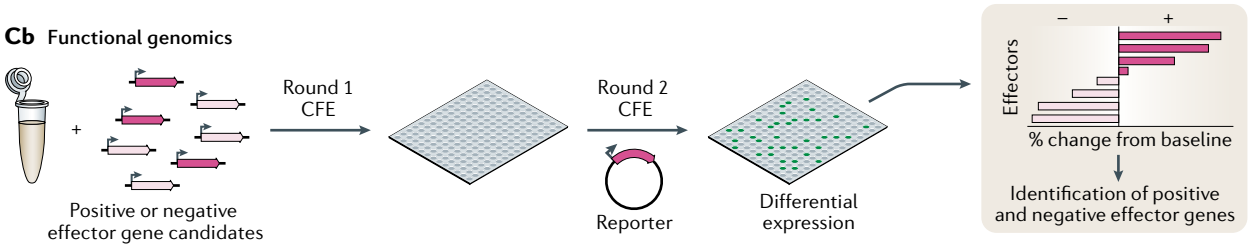


C Protein complex prototyping

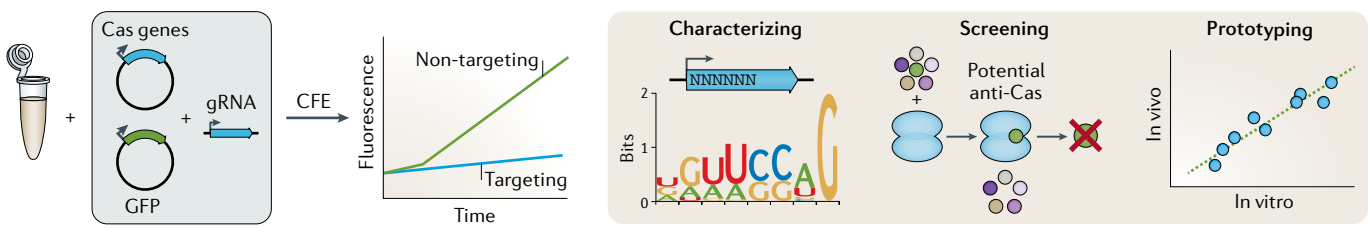
Ca Protein–protein interactions



Cb Functional genomics



Cc CRISPR–Cas complexes



◀ Fig. 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 non-canonical 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 cell-free gene expression (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 effects 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 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 (gRNA) and a target reporter protein are co-expressed in a single reaction, and on-target Cas-mediated cleavage results in decreased green fluorescent protein (GFP) synthesis. tRNA, transfer RNA.

Coacervation
Liquid–liquid phase separation that occurs in polymer solutions.

Giant unilamellar vesicles
Liposomes containing a single bilayer with a size in the order of a whole cell or larger (radius ~1–100 μm).

example of this strategy used haemolysin protein pores in synthetic membranes to enable the exchange of nutrients between these artificial cells and an external feeding solution containing nucleoside triphosphates and amino acids[127] (FIG. 4a). Synthetic membrane-mimicking compartments that encapsulate bacterial extracts have also been made using size-controlled gelation or coacervation[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 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 that encapsulate DNA encoding the polypeptide itself can even reproduce and grow[137]. Semi-annual Build-a-Cell workshops highlight the promise of using encapsulated cell-free systems to uncover the fundamental requirements for replicating, self-sustaining life.

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 (FIG. 4b). With an eye towards inspiring and training new biologists, chemists, physicists, computer scientists 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 hand-held 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.

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 the current progress and future work for two applications of CFE: point-of-use biosensing and cell-free biomanufacturing.

Point-of-use biosensing. In synthetic biology, whole-cell biosensors are created by engineering a host cell to conditionally express a fluorescent or coloured reporter protein only in the presence of a target molecule of interest. The test sample 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 test sample is added directly to a cell-free reaction that is programmed to conditionally express a reporter protein only when the target analyte is supplied.

However, compared to the cellular paradigm, cell-free systems offer several 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 test samples back to the lab, cell-free sensors can instead be directly brought out to the

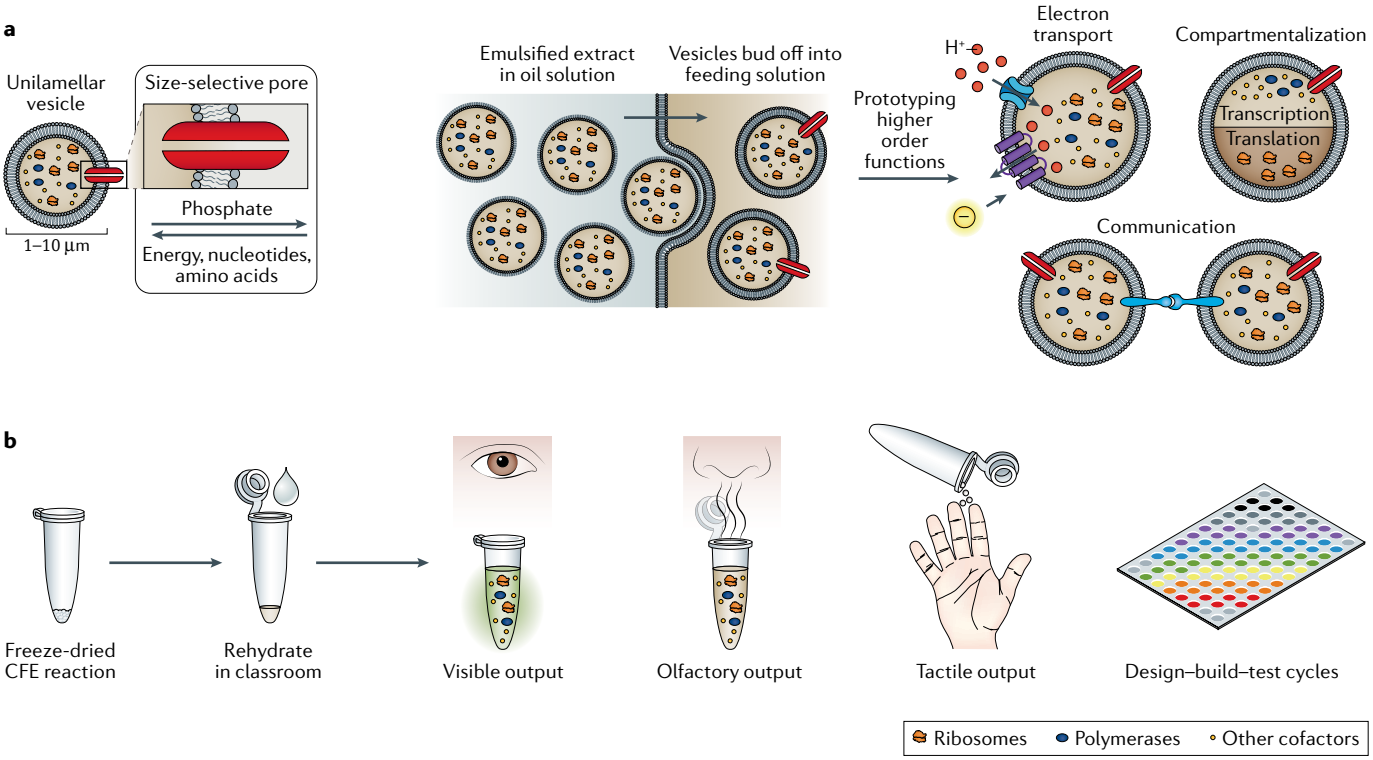


Fig. 4 | **Building cells and synthetic biology education. a** | Synthetic cells typically consist of encapsulated cellular extract in a vesicle or other microcompartment, which can exchange spent reaction products and fresh monomers for protein synthesis with a feed solution. Expression of proton pumps, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) or phase-separating polymers can be used to mimic cellular behaviours for energy generation, vesicular communication and organelle formation. **b** | Freeze-dried cell-free gene expression (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.

sampling location, for instance, to detect infection in a patient's blood sample obtained at a remote clinic. A key step towards this goal was the demonstration that CFE reactions can be preserved and distributed at ambient conditions (FIG. 5a). In 2014, it was shown that cell-free sensors could be lyophilized (freeze-dried) and that they maintain their 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 by simply using an individual's body heat[144].

With these technological advances, key progress in cell-free biosensing has thus far been made in the detection of disease-causing viruses and bacteria. In this approach, a sample containing the pathogen is first processed to extract total RNA; the RNA is then 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 such as, for instance, strategies where the target nucleic acid stoichiometrically binds to and unquenches a fluorophore in a molecular beacon[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[151] (FIG. 5b). The sensor is designed such that the 5′-end of the toehold switch 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[152] and reverse transcription–recombinase polymerase amplification[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 3 h when incubated at 37 °C (REF.[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 analogue to synthetic riboregulation exists for building sensors for arbitrary small

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.

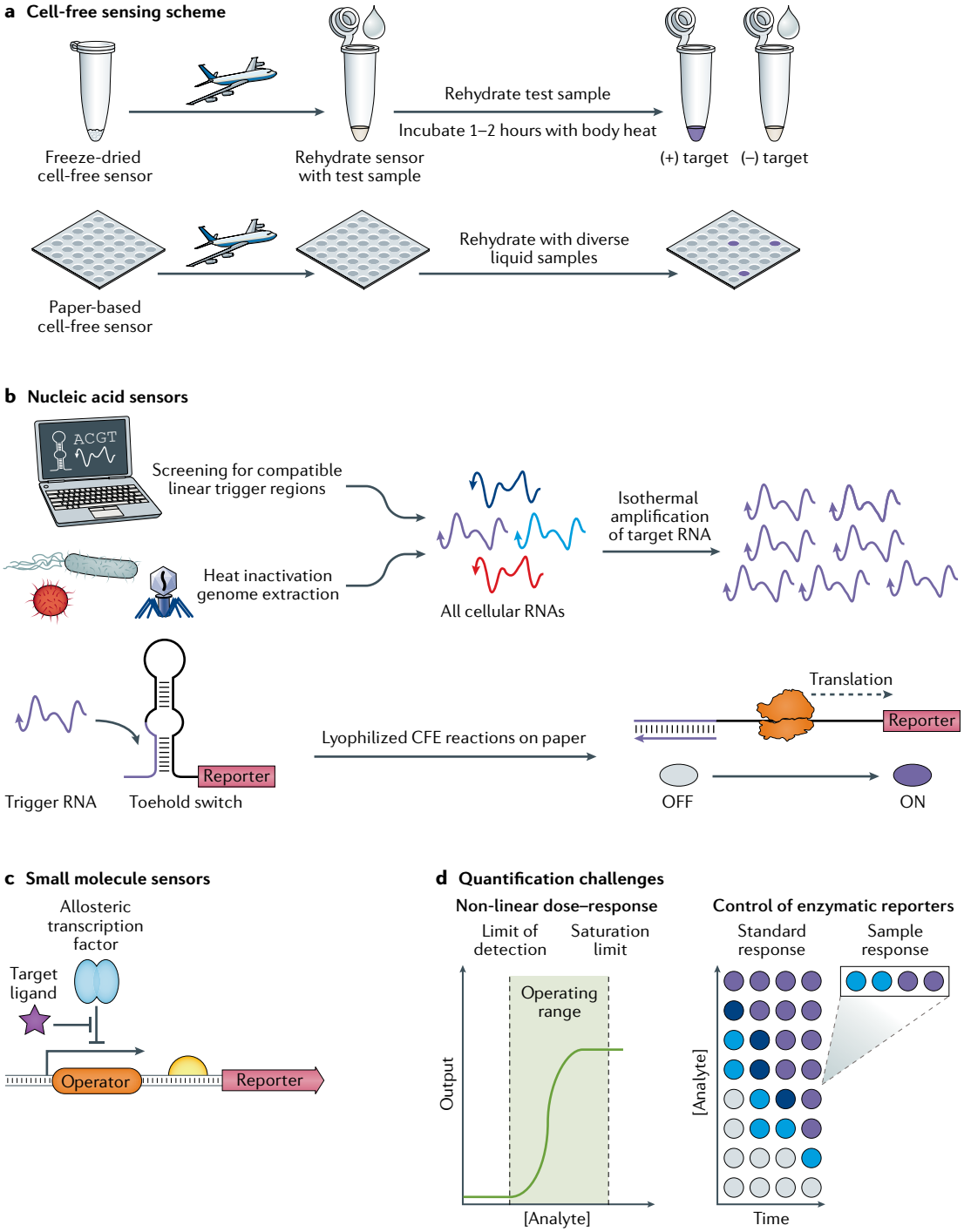


Fig. 5 | **Design of cell-free biosensors. a** | Schematic for point-of-use cell-free biosensing. Freeze-dried sensor reactions can be assembled centrally and directly shipped to the point of testing and hydrated with the sample of interest. The reactions will produce a fluorescent or colorimetric output that can be measured by the naked 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 non-linearities 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.

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[25,158] (FIG. 5c). 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, such as 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[162], or bacterial quorum sensing signals such as N-butyryl-L-homoserine lactone[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 to also sense hippuric acid and cocaine[164]. For other molecules, rational protein engineering has been used to engineer known sensor scaffolds to detect xenobiotic 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 promise and practical advantages of using cell-free sensors in the field, unsolved challenges remain; chief among these is the difficulty of quantification (FIG. 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 extract batches 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 coloured 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 end point is set by the amount of reporter substrate supplied to the reaction, the only useful metric to quantify analyte concentrations is the speed of response. New strategies and robust standards must therefore be developed to facilitate the quantification of target molecules at the point of detection. Towards 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].

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 40 min (REF.[166])), the total volumetric protein productivity could exceed hundreds of grams of cellular protein per litre per hour. For small molecules, assuming full substrate conversion and no product toxicity, hundreds of grams per litre per hour could theoretically be made as well[8]. Yet, these productivities are still 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.

Thus far, most attempts to realize the above biosynthetic 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 (GM-CSF)[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 cell extracts[171,172].

Most biologics today are made in Chinese hamster ovary or other mammalian cells. However, cell-free systems based on extracts from mammalian host cells are generally not used for biomanufacturing — mammalian CFE systems would be prohibitively expensive at an industrial scale because they require semi-continuous operation to achieve high protein yields. In these systems, microdialysis cassettes are typically used to enable continuous feeding of energy substrates and removal of small molecule byproducts[17,173,174]. More

Xenobiotic
Molecules that are not produced in any known natural biological systems.

recent cell-free approaches have instead aimed to mimic the physiochemical state of the mammalian cytoplasm in productive bacterial extracts. In this way, even 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 titres in vitro than in cells, especially because the open reaction environment enables improvements such as a mid-reaction transfer RNA (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[178] (FIG. 6a). Pioneering work in this area showed that antimicrobial peptides, vaccine antigens and antibody analogues 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 4 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 remodelling

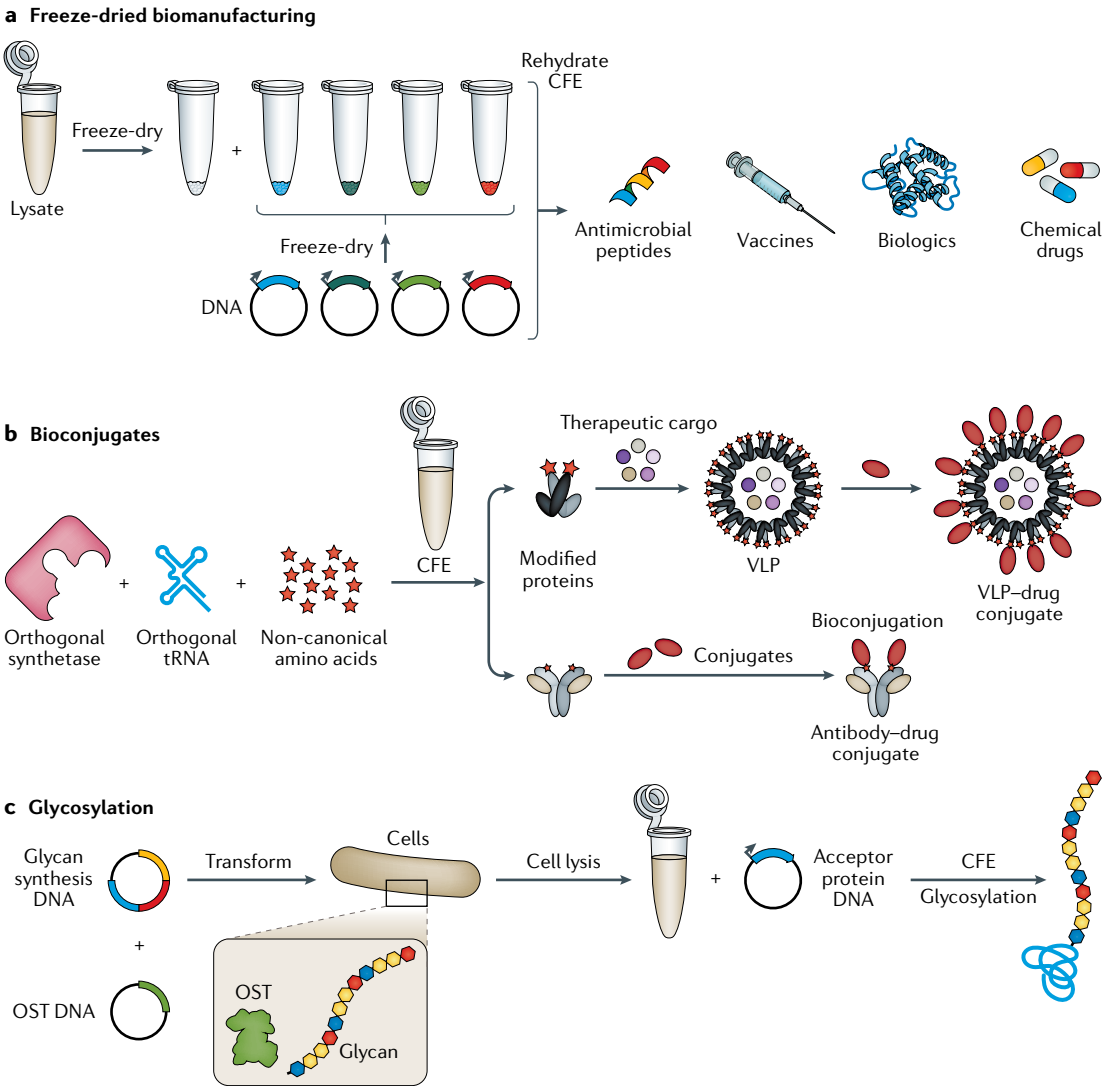


Fig. 6 | **Cell-free biomanufacturing platforms. a** | Cold chain-independent freeze-dried lysate, DNA and cell-free gene expression (CFE) reagents can be transported. Upon rehydration and mixing, CFE can be carried out to produce a wide variety of proteins and chemicals. **b** | By incorporating non-canonical amino acids 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. tRNA, transfer RNA.

lipid A endotoxin in the host strain used to produce the 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 practice standards in around 8 h (REF.[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 well suited 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 non-canonical 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 achieved in extracts made from genomically recoded bacterial strains with a knockout of release factor 1, 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 (FIG. 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-azidomethyl-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, 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. Indeed, 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].

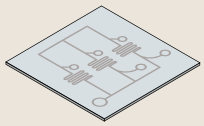
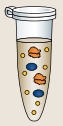
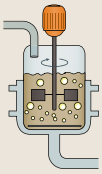
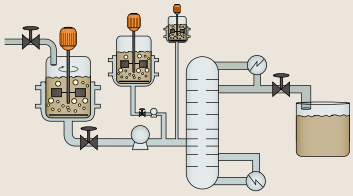
Importantly, most therapeutic proteins, particularly monoclonal antibodies, are decorated by glycans, not clickable drugs. Synthesizing these glycoproteins in vitro has historically been challenging since *E. coli* lacks native glycosylation machinery. One possible solution is to move to eukaryotic CFE reactions, which can be supplemented with purified microsomes 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 (FIG. 6c). The first demonstration of this approach showed that a purified OST enzyme and its lipid-linked oligosaccharide 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 lipid-linked oligosaccharides 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 was the cell-free production of a conjugate vaccine against the pathogen *Francisella tularensis* in a single millilitre-scale CFE reaction that catalysed the conjugation of a recombinant polysaccharide antigen onto an in vitro-synthesized carrier protein. The resulting vaccine showed immunostimulatory effects when injected into mice and is estimated to cost ~US$6 per dose, which is cost-competitive with conjugate vaccines sold commercially[181].

Costs and challenges of scaling up cell-free biomanufacturing. Even with the momentum and recent technical progress for cell-free biomanufacturing, the practical and economic considerations of industrial-scale CFE systems remain mostly untested. By contrast with the abundant academic interest in scaling down CFE reactions for high-throughput biological studies, little work has reported the challenges of scaling them up. Studies from 2011 and 2012 found that many of the process variables and yields scale linearly over a range of six orders of magnitude in a cell-free reaction volume of 1001 (REFS[168,169]). However, a systems-level characterization of cell-free bioreactor dynamics would be useful for further optimization efforts and could inform the design of expression systems at an intermediate scale.

It is instructive here to consider the process and economic feasibility of large-scale biomanufacturing with commonly used CFE systems. To do this, we consider the cell-free synthesis of four representative protein products (T7 RNA polymerase (RNAP), a DTaP vaccine, a drug-conjugated antibody and subtilisin A), which would be synthesized at scales spanning from a microlitre to thousands of litres. T7 RNAP, used for laboratory-scale in vitro transcription, can be synthesized in cell-free

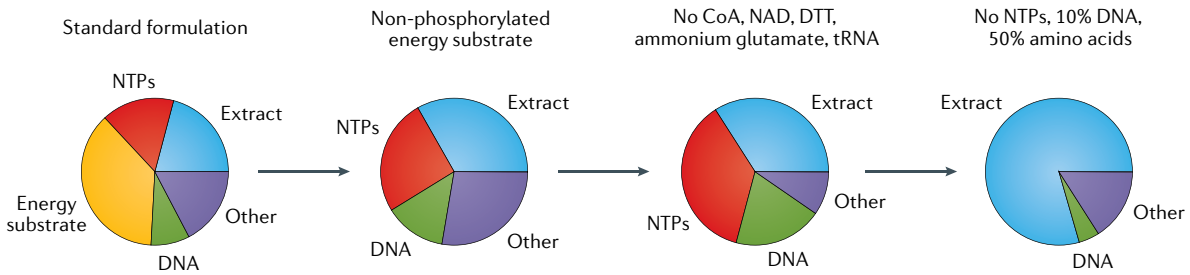
Microsomes
Small vesicles originating from the endoplasmic reticulum when cells are sheared and centrifuged.

a

Approximate batch size	V = 1 µl	V = 1 ml	V = 100 l	V = 1,000 l (continuous operation)
Example product	Laboratory use T7 RNA polymerase	Point-of-use vaccine dose	Pharmaceutical antibody–drug conjugate	Commodity grade subtilisin
Commercial price ($/mg)	$13,250	$200	$5,500	$0.022
Estimated cell-free yield (mg/ml reaction)	1.0	0.02	0.250	2.0
Estimated purification cost	0×	0×	10×	1×
Estimated breakeven cost ($/ml reaction)	$13,250	$4	$120	$0.022

b



$/ml reaction	$4.67	$2.93	$2.03	$0.87

Fig. 7 | **The economics of cell-free gene expression. a** | Approximate costs and revenues from the production of laboratory-grade enzyme, point-of-use vaccine doses, biomanufactured 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[216], based solely on the cost of raw materials from traditional chemical manufacturers, is approximately five cents for a typical 10 µl reaction. Removal of expensive phosphorylated energy substrates and non-mandatory reaction cofactors as well as reduction in the supply of nucleoside triphosphate (NTP), 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. CoA, coenzyme A; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; tRNA, transfer RNA.

conditions at sufficient yields to drive the synthesis of RNA from a T7 promoter[64]. Diphtheria toxoid synthesized using continuous-exchange reconstituted cell-free protein synthesis has been made at sufficient concentrations to induce anti-diphtheria toxoid antibody production in inoculated mice, with sufficiently low levels of bacterial endotoxin to be within FDA limits for toxoid-based vaccines[179]. Commercial-scale production of glycosylated cytokines has been achieved at the 100-litre scale with yields approaching 750 µg/l (REF.[168]), which offers promise for high-titre, cell-free production of monoclonal antibodies, including those functionalized with chemotherapeutic drugs[191,192]. At the 1,000-litre expression scale, we consider subtilisin, the active protease in commercial laundry detergent and one of the largest-volume commercial commodity proteins[198]. Using reported or estimated cell-free yields for each of these proteins, and 2019 commercial prices, we calculated the maximum possible raw cost of a cell-free reaction that would make cell-free biosynthesis competitive with current production means (FIG. 7a; Supplementary Note).

Our simple analyses suggest that cell-free production of laboratory-scale T7 RNAP is 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 little to 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, such as 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 yields 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 labour and capital, we conservatively estimate that a standard bench-scale CFE reaction formulation costs around US$5/ml (FIG. 7b; Supplementary Note). Approximately 40% of this cost arises from the expensive phosphorylated energy substrate phosphoenolpyruvate, which is still commonly used. Replacing this 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 dithiothreitol, coenzyme A, nicotinamide adenine dinucleotide and exogenous tRNAs, replacing nucleoside triphosphates with nucleoside monophosphates, and decreasing nucleotide and amino acid concentrations, such that the minimal cost of raw materials could feasibly drop below US$1/ml (REF.[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.

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, several 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 practically 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, without 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 regards to direct applications for CFE, much progress has been made in both 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 good manufacturing practice-quality batches of therapeutic proteins. Robust cell-free biomanufacturing would benefit from longer reaction durations than current practice (for example, days or 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 the 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 given that, 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 these efforts towards 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.

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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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