

REVIEW

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# Synthetic Biology Goes Cell-Free

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## Abstract

Cell-free systems (CFS) have recently evolved into key platforms for synthetic biology applications. Many synthetic biology tools have traditionally relied on cell-based systems, and while their adoption has shown great progress, the constraints inherent to the use of cellular hosts have limited their reach and scope. Cell-free systems, which can be thought of as programmable liquids, have removed many of these complexities and have brought about exciting opportunities for rational design and manipulation of biological systems. Here we review how these simple and accessible enzymatic systems are poised to accelerate the rate of advancement in synthetic biology and, more broadly, biotechnology.

## Moving towards a new bioengineering platform

Since its emergence, the field of synthetic biology has given rise to the development of many technologies that are implemented using the whole cell [1]. These have included biosensors capable of detecting broad ranges of analytes [2–5], systems that can count [6] or perform complex logic [7–10], engines for the bioproduction of valuable commodities [11–14], gene-circuit-driven chassis for regenerative medicine [15, 16], and engineered CAR-T cells [17]. Such technologies are on track to transform many aspects of modern life, yet their requirement for a cellular host has limited their reach and scope. For example, concerns over biosafety have restricted the use of engineered cells, and the systems they host, largely to laboratory settings. The self-replicability of cell-based systems carries the risk of “escape” or contamination that could impact human health, food security, and the environment. While the development of safeguards to prevent these types of events is an active area of research [18, 19], failure-free implementation of such systems is not a trivial task.

Another substantial limitation of cell-based synthetic biology is the requirement for laborious genetic encoding of its design features into a living cell, which can limit its functionality and significantly slow down design–build–test cycles. In cell-based systems, genetic instructions often need to be assembled into a vector, imported into the cell, and maintained by using a selectable marker or by genomic integration. Only then can the instructions be evaluated. Furthermore, designs must be iteratively tested to minimize cross-talk with endogenous molecular programs while balancing between the metabolic burden on the cellular host and the desired outcome.

Cell-free systems offer a means to circumvent many of these limitations. They were originally conceived as tools to facilitate *in vitro* protein synthesis and consist of molecular machinery extracted from cells. They typically contain enzymes necessary for transcription and translation, and accordingly are able to perform the fundamental processes of the central dogma (DNA→RNA→protein) independent of a cell. These systems can be derived from eukaryotes (e.g., vertebrates, plants, insects, fungi) [20–27] or prokaryotes (e.g., *Escherichia coli*, *Vibrio natriegens*, *Bacillus subtilis*) [28–43] and may be prepared as either purified components [36, 44] or semi-processed cellular extracts [38]. CFS can be made sterile via simple filtration, which provides for a biosafe format for use outside of the lab.

The open nature of CFS means that there is no physical barrier (e.g., a cell wall) to programming and modification. CFS can be augmented with proteins or small molecules that improve the performance of synthetic gene networks [45, 46] or the productivity of reactions [39, 47]. More importantly, genetically encoded instructions can be added directly to CFS at desired concentrations and stoichiometries using linear or circular formats. This means that conceptual designs can go from computational instructions to chemical synthesis and amplification (e.g., through PCR) to CFS without the need for selective markers or cell-based cloning steps. Such simplicity allows for rapid prototyping of molecular tools.

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Importantly, CFS can be freeze-dried, enabling room temperature storage and distribution [46, 48]. Freeze-dried cell-free (FD-CF) systems can then be activated at the time of need simply by adding water [46]. This feature has been used to deploy biosafe, genetically encoded tools outside of the laboratory as diagnostics and as platforms for biomanufacturing [49, 50], as well as their deployment in altogether new contexts, such as global health and education.

Below we will discuss how CFS are enabling new technologies and accelerating the coming revolution in bio-engineering, highlighting some of the most active areas of research in the cell-free community (Fig. 1).

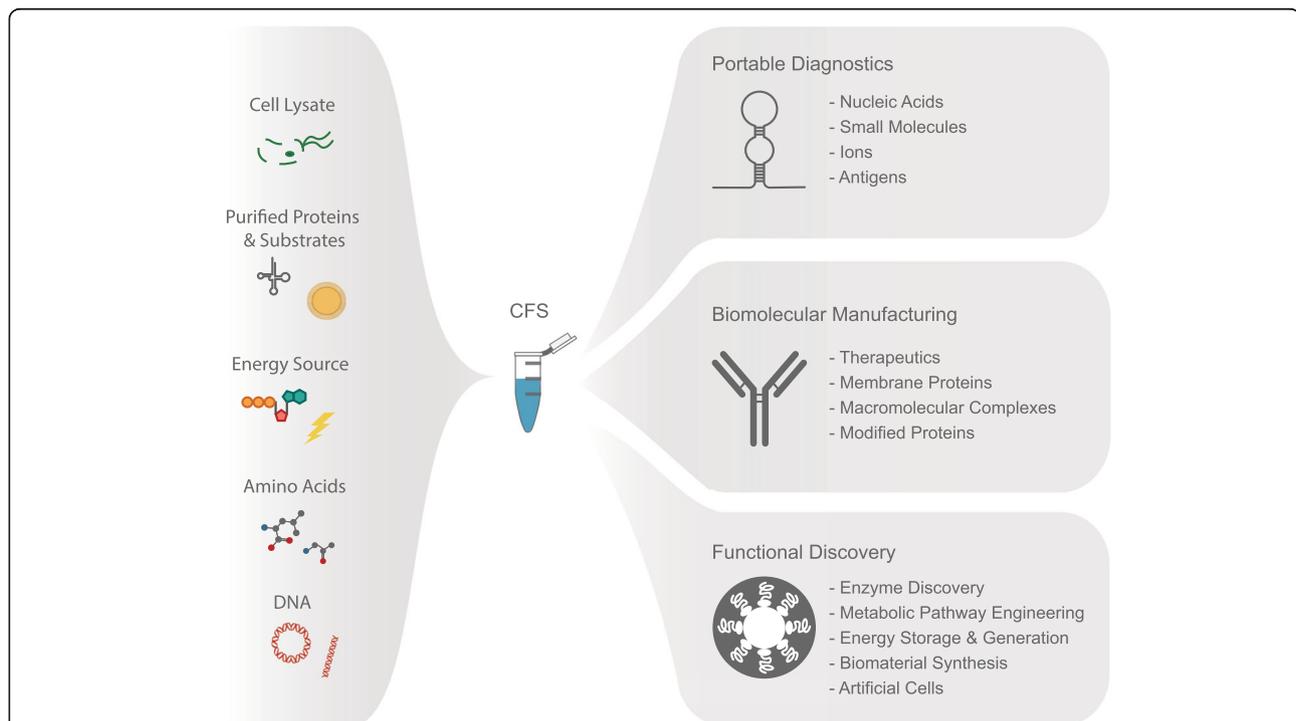
### Development of sensors

Molecular recognition underlies almost every biological process, including the nucleic acid base pairing that imparts specific syntax to the central dogma. Scientists and engineers have long worked to usher these processes into cell-free *in vitro* environments to understand and exploit their underlying molecular mechanisms for purposes such as diagnostics and detection of molecules. One of the fruits from such efforts is the polymerase chain reaction (PCR), which is now an indispensable tool utilized in most molecular biology laboratories, including those for clinical diagnostics. There is currently a

growing need for de-centralized, portable diagnostics that can be rapidly deployed in the field, for instance during infectious disease outbreaks or for agricultural purposes. However, sensing technologies such as PCR and others have largely remained confined to laboratories in large urban centers due to their requirement for specialized equipment and personnel.

The biosafe and stable nature of FD-CF systems offers an alternative molecular venue to address the unmet need for distributed and low-cost sensing. Here, the transcription and translation properties of CFS can be used to host gene circuit-based sensors that can detect small molecules and nucleic acids with exquisite sensitivity and specificity. Many of the biosensors and circuits that have been developed for cell-based applications can be operated in the cell-free environment. These include, among others, many classic switches (e.g., TetO- and LacI-based systems), logic gates, negative feedback loops, transcriptional cascades [37, 41, 53–56] and ring oscillators [57]. This cross-compatibility between CFS and cell-based systems has also been exploited for rapid prototyping of regulatory elements that can be brought back to the cell-based environment.

FD-CF systems do not require a temperature-controlled environment and cold-chain logistics intrinsic to many other diagnostic approaches, as they remain



**Fig. 1** Cell-free protein expression systems and their applications. Capitalizing on their open nature, CFS can be rationally assembled to include cell lysates, purified proteins, energy sources (e.g., ATP), amino acids, other substrates (such as modified tRNAs and membrane mimics) and RNA or DNA (circular or linear). CFS can be applied in portable diagnostic devices [46, 50] and also hold great potential for biomolecular manufacturing [49, 51]. Additionally, CFS can enable discovery of novel enzymes (e.g., through directed evolution) [52]

active for at least a year without refrigeration, enabling room temperature storage and distribution [46]. This, however, does not circumvent the challenges arising from handling these molecular tools in liquid phase—for instance upon their resuspension outside of the laboratory environment. Inspired by systems like pH paper and lateral-flow diagnostics, we embedded FD-CF reactions into porous materials (e.g., paper), demonstrating that low-volume reactions (1–2  $\mu\text{L}$ ) could readily be achieved within this medium. Such paper-based cell-free systems enabled the deployment of poised synthetic gene networks outside of the laboratory in a contained and biosafe format for the first time [46].

With this new ruggedized paper-based format, simple sensing such as anhydrotetracycline (ATc)-inducible expression of GFP and mCherry was established [46]. However, to demonstrate the real-world potential for this system, a sensing platform that could be rationally designed to detect a wide range of practical analytes was needed. This was realized with the introduction of toehold switches [58], a new class of riboregulators, into FD-CF reactions. The use of toehold switches, which can be designed to recognize virtually any sequence of interest, was first demonstrated in paper-based FD-CF reactions for the detection of genes responsible for antibiotic resistance and strain-specific detection of the Ebola virus [46]. While the demonstration of this sensing capacity in a portable format was exciting, the system lacked the sensitivity necessary to detect RNA levels generally present in patient samples.

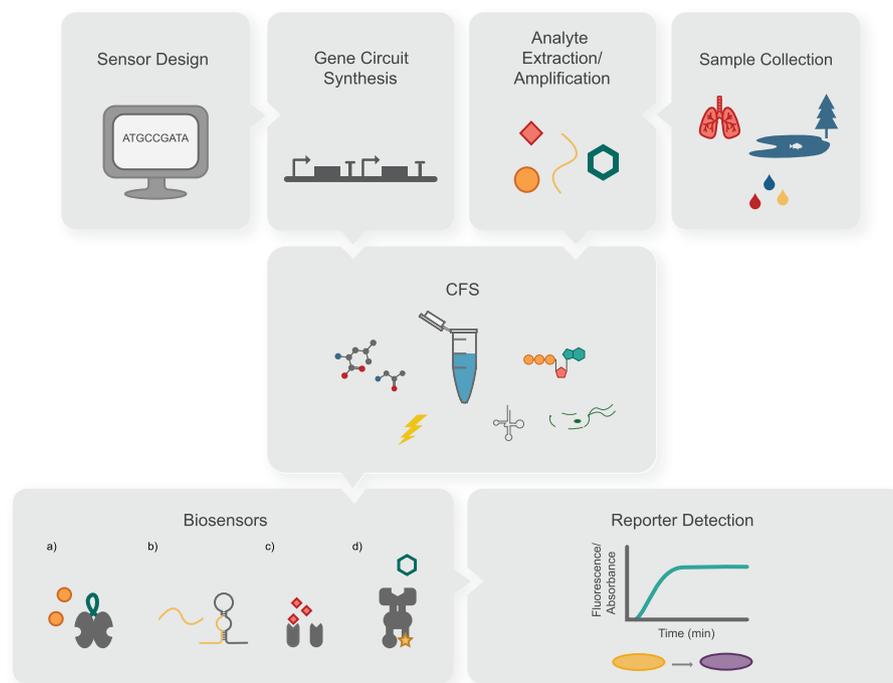
This sensitivity challenge was addressed by placing an isothermal amplification step (e.g., NASBA) in the workflow upstream of the cell-free reaction. This improved the threshold of detection by orders of magnitude ( $10^6$ ). Since isothermal amplification is a primer-directed process, combination with toehold-based sensing results in two sequence-specific checkpoints. An opportunity to test out the improved system presented itself in early 2016 when the outbreak of the mosquito-borne Zika virus was reported in Brazil. With the improved embodiment, FD-CF toehold sensors could detect all global strains of the Zika virus at clinically relevant concentrations (down to 2.8 femtomolar) from viremic plasma [50]. Moreover, powered by the first CRISPR-based system in an in vitro diagnostic system, viral genotypes could be distinguished with single base pair resolution (e.g., American vs African Zika strains). Most recently the Collins group extended these concepts in a *tour de force* effort that demonstrated quantitative detection of ten gut bacterial species from patient samples [59]. This work demonstrated detection at clinically relevant concentrations with sensing performance that mapped well with parallel measurements done with RT-qPCR.

It also showcased the ability to detect a toxin-related sequence for the diagnosis of *Clostridium difficile* infections.

Following the initial work outlining the potential for the FD-CF format, a body of work ensued demonstrating many biosensing applications and improvements on FD-CF preparations. In one of the earliest examples, Duyen et al. developed a sensor for the detection of antibiotic contamination based on protein synthesis inhibition caused by some antibiotics [60]. The Freemont group applied their expertise in CFS to develop sensors for the detection of *Pseudomonas aeruginosa* in cystic fibrosis patient samples [61], demonstrating that the quorum-sensing molecule from *P. aeruginosa* (3-oxo-C12-HSL) could be detected down to low nanomolar concentrations. Another novel approach used CFS to express engineered protein fusions containing nuclear receptor ligand binding domains for the detection of endocrine-disrupting compounds [62, 63]. This work showcased sensitivity in the nanomolar range, and, interestingly, demonstrated that CFS could operate in the presence of contaminants in environmental and clinical samples. In another example, detection of mercury contamination using the mercury(II)-responsive transcriptional repressor MerR was accomplished [45] (Fig. 2).

### Manufacturing of therapeutics

Another active area in CFS research is the biomanufacturing of therapeutics and other protein-based reagents. Natural biological systems have evolved a remarkable capacity to synthesize a variety of molecules ranging from metabolites to biopolymers. Cell-free protein expression systems allow the incorporation of such reactions into a highly controlled process that allows production of molecules as needed and in the field. Our primary focus here will be on a subset of biopolymers, namely therapeutic proteins. The ongoing work in this field rests on decades of research that have led to the productive and practical systems currently available [28, 29, 36–38, 40]. Recent advances in high-throughput preparation techniques [40, 45] and in the development of systems that can use more economical energy sources [64, 65] have made CFS highly accessible. Meanwhile, significant strides are being made towards resolving various protein folding issues and shortcomings in post-translational modifications [66] associated with traditional CFS. Recent advances have showcased the potential for scaling up cell-free reactions, with some having demonstrated reaction volumes reaching 100 liters [67, 68] to 1000 liters [69]. Cell-free expression has been used as a platform for the production of a wide range of potential therapeutics, some of which have been summarized in Table 1. A number of these products have been validated in animal models [49, 76].



**Fig. 2** Overview of the use of biosensors in CFS. The general workflow usually involves in silico design of gene circuits encoding biosensors and reporter proteins, followed by chemical synthesis of such circuits. Meanwhile, patient or environmental samples are collected, target analytes are extracted, and, in some cases, amplified. The gene circuits and target analytes are then added to CFS. Examples of biosensors in CFS have included **a**) mercury (II) detection using the MerR repressor [45], **b**) viral and bacterial nucleic acid sensing using toehold switch-based sensors [46, 50, 59], **c**) identification of *P. aeruginosa* infection by its quorum sensing molecule, 3-oxo-C12-HSL, using the LasRV sensor [61] and **d**) recognition of an endocrine-disrupting compound by utilizing an allosterically activated fusion protein containing the ligand binding domain of a human estrogen receptor [62, 63]. Reporters (e.g., colorimetric or fluorescent) can then be produced, contingent upon analyte detection, enabling clinical diagnosis (e.g., using standard spectrophotometers)

Two primary modes of CFS have been pursued. The first, used by commercial efforts such as Sutro [94], focuses on large, centralized production. This approach leverages the advantages of synthesis outside of the cell for biomanufacturing. For these applications, CFS not only allow for rapid production, but also significantly speed up the drug development process [95]. Remarkably, Sutro has reportedly increased their cell-free production to an incredible 1000 liters [69], showcasing the scalability of centralized cell-free production. The second mode uses FD-CF systems to de-centralize biomanufacturing capacity for small-batch production of therapeutics, with applications in global health and emergency response [49, 73, 96, 97]. Using this mode of production, we have recently demonstrated the proof-of-concept capacity to manufacture over 50 therapeutics and lab reagents, including proteins (e.g., vaccines, antibodies, and antimicrobial peptides) and small molecules [49], with applications outside of the laboratory setting.

Cell-free biomanufacturing is particularly well-suited for vaccine production due to its potential for rapid scale-up in response to public health emergencies. Successful cell-free expression of a number of recombinant

vaccines (e.g., botulinum, diphtheria, anthrax) has been demonstrated [49, 86–90, 98], with some having been validated in animal models, such as mice [49, 90]. Considering the low dose requirements (microgram range) for many of these therapeutics, commercialization of CFS-derived vaccines will likely see rapid growth in the coming years. Production of antibodies has also been an area of focus for the cell-free community [20, 49, 51, 74–80, 99, 100]. Due to their compact size and relatively high expression levels in CFS, single-domain antibodies have garnered particular attention and seem strategically well-placed to serve the emerging needs in personalized medicine, i.e., for therapeutics and diagnostics.

Antibiotic resistance has been recognized as a major threat to global health, resulting in approximately two million illnesses and 23,000 deaths in the US alone every year [101]. Accordingly, cell-free production of antimicrobial compounds, including antimicrobial peptides and small molecule drugs, has become the focus of some groups [49, 93]. A number of labs have also demonstrated the power of CFS to express phages [56, 102–104]. The upward trend in the reported antibiotic resistance cases has led to a resurgence in viewing phage

**Table 1** Examples of potential therapeutics expressed in CFS to date

Therapeutic proteins	Granulocyte macrophage colony-stimulating factor (GM-CSF) [68, 70]
	Erythropoietin [70–72]
	Cytotoxic protein onconase [73]
	Antibodies [51, 74, 75] and antibody fragments [49, 76–79]
	Bispecific antibodies [80]
	Antibody-drug conjugates [49, 81]
	Tissue-type plasminogen activator [82–85]
Vaccine antigens	Picornaviral capsid intermediate structures [86]
	Trimeric influenza hemagglutinin head [87] and stem [88] proteins
	Trivalent vaccine based on Hc fragments of botulinum toxins A, B, and E [89]
	Anthrax protective antigen and diphtheria toxoid [49]
Virus-like particles	A B-cell lymphoma vaccine [90]
	Anti-hepatitis B VLPs [91]
	A virus-like nanoparticle scaffold for vaccines and drug delivery [92]
Antimicrobials	Antimicrobial peptides [49, 93]
	Small molecules such as violacein [49, 56]

therapy as a potentially viable alternative to current antibiotic regimens [101, 105]. The use of phages has also been evaluated as an effective treatment strategy for a number of plant diseases, with some phages now being commercially available for mass consumption [106]. CFS-based production of these non-traditional antimicrobials could play a significant role in battling the antibiotic resistance crisis and could also help improve food security around the globe.

Below, we will highlight some of the areas in which CFS have shown great potential for enhancing current methods of therapeutics development and manufacturing. These advances are rapidly transforming CFS into an integral part of the manufacturing ecosystem.

### Membrane proteins

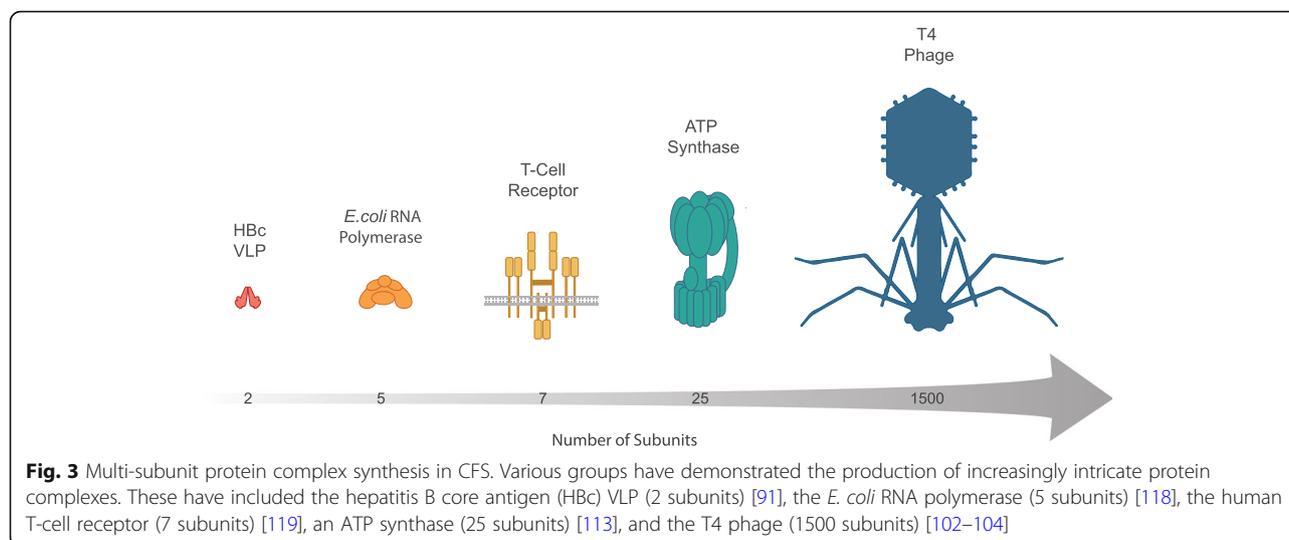
While approximately 70% of all drugs act on membrane proteins [107], working with these proteins is notoriously difficult due to their enrichment in hydrophobic surfaces. Cell-based expression of membrane proteins is often fraught with challenges, such as toxicity caused by their membrane incorporation or their incompatibility with the host's physiology [108]. Recently, cell-free approaches have been used to tackle this challenging category of proteins, the coding sequences of which comprise 20–30% of all known genes [107]. When compared to current cell-based methods, CFS can be a powerful tool in the production of soluble active membrane proteins [109]. The ability to

integrate steps that can tackle the challenging aspects of membrane protein synthesis is particularly valuable. For instance, previous efforts in cell-based systems have demonstrated that membrane mimics can be successfully used to synthesize and stabilize a wide range of membrane proteins such as G-protein-coupled receptors [110, 111], the epidermal growth factor receptor [71], hepatitis C virus membrane proteins [112], and an ATP synthase [109, 113]. These mimics include surfactants, liposomes, and nanodiscs [114–116] and can be added directly to CFS co-translationally or post-translationally. There is also evidence suggesting that functioning single-span membrane proteins can be synthesized simply in the presence of an oil–water interface (e.g., through the use of emulsions) [117].

### Macromolecular production

Molecular research has highlighted the importance of protein–protein interactions and the resulting complexes that these interactions can generate. Whether it is for the biophysical study of these complexes or as vehicles for new therapeutic delivery (e.g., virus-like scaffolds for vaccines), there is a growing need for developing robust tools aimed at synthesis of such complexes. As in the case of membrane proteins, CFS have also demonstrated higher yields, compared to in vivo strategies, in the production of macromolecular assemblies such as virus-like particles (VLPs) [109]. Groundbreaking work by the Swartz group, demonstrating the cell-free expression of hepatitis B core antigen VLP (2 subunits) [91] in an *E. coli*-based cell-free system, opened the door to other researchers expressing a variety of macromolecular assemblies including the *E. coli* RNA polymerase (5 subunits) [118] and an ATP synthase (25 subunits) [113]. Earlier work with reticulocyte lysate had also demonstrated cell-free expression of the human T-cell receptor (7 subunits) [119]. Remarkably, a number of bacteriophages have now also been successfully expressed in CFS, including the T4 phage, which structurally contains 1500 proteins from 50 genes [56, 102–104] (Fig. 3).

Non-identical subunits of a protein complex are often referred to as hetero subunits. In some instances, such hetero subunits require co-translation to yield active complexes [120]. Thus, the ability of CFS to concurrently translate multiple mRNAs facilitates the production of active complexes composed of a number of different subunits [121]. Some CFS such as *E. coli*-based preparations are generally not capable of producing proteins that contain disulfide bonds, which are critical to numerous pharmaceutically relevant proteins (e.g., antibodies and many cytokines) [121]. However, recent efforts have augmented these systems to enable the production of complex proteins requiring multiple disulfide bonds [85, 99, 122], expanding the range of therapies that can be made in CFS.



### Modification of proteins and codon tables

Effectiveness of many protein-based therapeutics hinges upon precise control over natural or non-natural modification of their peptide sequences. One of the most compelling uses of such modifications is in the development of antibody–drug conjugates (ADCs), which are quickly gaining favor as a new class of therapeutics against cancer. Classic conjugation techniques result in a heterogeneous mixture of labeled antibodies due to their reliance on arbitrary conjugation to multiple amino acid side chains. Recent studies, however, suggest that pharmacologic properties of ADCs could be improved through site-specific conjugation. Non-natural amino acids provide an efficient avenue for such site-specific conjugation [123]. To date, co-translational incorporation of over 100 different non-natural amino acids has been demonstrated *in vivo* [124], allowing for a wide range of modifications [125–129]. Many of these modifications have been demonstrated in the cell-free context for a variety of applications, including orientation-controlled immobilization [92, 98] and site-specific functionalization (e.g., phosphorylation [130], PEGylation [131], or drug conjugation [81]) [132–134].

CFS platforms circumvent some of the cell-based toxicity and permeability limitations and offer greater control and versatility in making protein modifications [109, 135]. Incorporation of non-natural amino acids in cell-based approaches has typically relied on repurposing stop codons to minimize the negative impacts of recoding on cell-viability [109]. In a cell-free system, however, the entire codon table can in theory be reprogrammed, allowing not only for the incorporation of non-natural amino acids, but also for the creation of entirely novel codon tables.

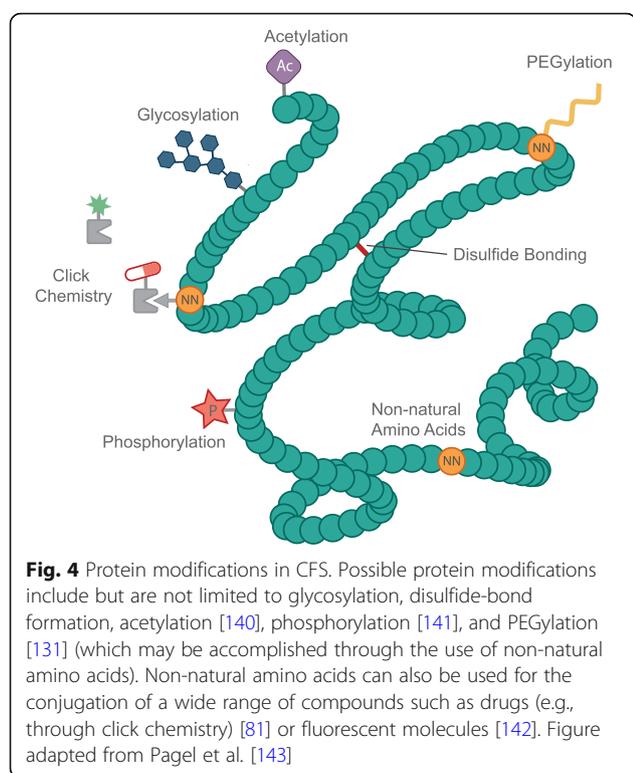
Taken to its extreme, the latter could help with the protection of intellectual property. DNA sequences could be obfuscated such that they are rendered non-functional outside of their specialized cell-free context.

This obfuscated code would make proprietary designs difficult to copy. Codon obfuscation could also pose serious challenges for the detection of DNA sequences that may be employed by malevolent entities. For example, DNA synthesis companies would have a much more difficult time screening against DNA sequences that could be used for nefarious activities (e.g., bioterrorism). Recent work has shown that the size of the codon table can also be expanded by augmenting the four-letter genetic alphabet with unnatural base pairs [136, 137]. Thus, proteins made in CFS could—at least in theory—hold an unlimited number of non-natural amino acids.

CFS can also be employed for making naturally occurring modifications to proteins. An example of these is the grafting of sugars (i.e., glycans) referred to as glycosylation. Successful production of many therapeutics is often contingent upon highly efficient glycosylation, as lack of proper glycosylation can reduce the efficacy and circulation half-life of many therapeutic proteins [138]. Some CFS (e.g., insect, Chinese hamster ovary, and human K562 extract-based systems) are inherently capable of glycosylation. However, their repertoire of glycan structures tends to be limited to those naturally synthesized by their lysates' source cell type. Additionally, glycosylation in these systems often requires recapitulation of the source cell's protein trafficking mechanisms [109]. Thus, creation of synthetic glycosylation pathways in CFS has become an area of focus in recent years [135, 139]. Success in this domain will likely serve as a key catalyst in bringing cell-free-produced vaccines and other therapeutics to the masses. Figure 4 outlines some of the possible protein modifications in CFS.

### Directed evolution

Directed evolution is a powerful tool for aptamer and protein engineering that uses iterative rounds of mutagenesis



and selection to modify or tune specific bimolecular properties (e.g., an enzyme's substrate activity). Utility of aptamers or proteins, in a given context, with respect to their corresponding nucleotide sequences is often described as a fitness landscape. Directed evolution provides a massively parallel method for searching through a fitness landscape to find optimal variants and their corresponding genotypes [144]. This generally requires one-to-one mapping of phenotype to genotype. Although cells have a built-in capacity for such mapping due to their compartmentalized nature, using cells to conduct directed evolution can impose limits on the size of candidate libraries screened, and restricts the type of solvents, buffers, and temperatures that can be sampled [145]. As a result, cell-free directed evolution platforms have gained favor [145], starting with the first truly cell-free systems published in the late 90s [146, 147]. More recently, connecting phenotype to genotype has been accomplished through artificial compartmentalization (e.g., using emulsion, microbeads, and liposomes) [145, 148–151]. Applications have included the design and optimization of Fab antibody fragments [77, 152], membrane proteins [151], and, as we will discuss below, enzyme discovery [52].

### Platform for discovery

Engineered transcription and translation systems can also greatly catalyze research in the laboratory. As previously mentioned, the absence of a cell wall means that

candidate genes can be readily screened for function. It also means that substrates, including those difficult to use in the cellular context, can easily be brought into contact with enzyme libraries to screen for novel reactions. Below we look at some of the recent work using CFS as a platform for discovery.

### Biosynthetic pathways

From the early days of synthetic biology, it was clear that there was great potential for synergy with the field of chemical synthesis. Metabolic pathways responsible for the synthesis of valuable compounds (e.g., drugs, scents, and flavors) were thus moved out of organisms that did not easily lend themselves to production and into heterologous hosts, such as yeast. This microorganism-based approach has been incredibly successful and has led to the assembly of genes from disparate sources to create engineered pathways. Enzyme-based catalysis has the advantage of allowing for stereo-selectivity in aqueous, low-energy reactions (e.g., green chemistry) [153]. By leveraging naturally occurring pathways, it has been possible to generate tremendous chemical diversity, as seen in isoprenoids, from simple precursors [154]. An exemplar of this approach is the synthesis of amorpha-4,11-diene and artemisinic acid, which are precursors to the anti-malarial compound artemisinin [154–157]. This process has been repeated for other pharmaceutical pathways, enabling the production of opioids [158, 159] and taxol [160], as well as for the generation of molecules for the energy industry and the agriculture sector [13, 161].

While microorganisms are currently a mainstay for biomanufacturing of commodities, their use for these purposes is nontrivial. For example, assembly, fine-tuning, and host strain integration of the industrialized pathway for the bioproduction of artemisinic acid is estimated to have taken over 150 person-years [162]. Another challenge to microbial bioproduction is that a significant portion of inputs are lost to general cellular metabolism and growth, reducing efficiency of the overall process [67, 134]. Cell-free synthetic biology alleviates some of these challenges. For instance, enzyme discovery—the identification of enzymes that can be used for biosynthetic purposes—via CFS has proven to be effective. Enzymes and their homologs can be rapidly screened for performance without the cumbersome steps required for cell-based screening (e.g., plasmid assembly and transformation). This approach can be extended to simple prototyping of pathways or the automated multiplexed shuffling of complex pathway components. Unlike with cell-based prototyping, the cell-free environment allows for the use of enzymes encoded as linear constructs (DNA or RNA). Substrate preference can also be evaluated without the need for enzyme purification.

In many cases, enzymes and pathways discovered in CFS will be brought back into cells for scale-up [163]. However, there is a growing case for using CFS directly as the production medium. Commercial ventures (e.g., Sutro, Greenlight) have already demonstrated that CFS can provide economic advantages for the production of protein and RNA products [69]. Thus, it would be reasonable to think that a similar approach could provide a viable source of high-value small molecules. Such systems have the advantage of enabling bioproduction without metabolic inefficiencies, toxicity limitations, complex extraction steps, or the need for integration into a host strain [67, 134, 164]. Combined with the capacity for efficient prototyping, these systems are generating significant enthusiasm. The field is now beginning to focus on more complex pathways (more than eight enzymes) and larger reaction volumes (> 100 L) [67].

Single enzyme reactions are highly simplified cell-free systems that have been used for decades at scale for washing (e.g., dish and laundry detergents) and for processing food, wood pulp, and fuel [165]. Once fully operationalized, more complex cell-free enzymatic pathways could revolutionize the chemical industry and enable greater accessibility to bioproduction. Earlier attempts at engineering such pathways outside of a cell were generally made by using purified components. These pathways have included those designed for the production of amorpho-4,11-diene [166], isoprene [167], fatty acids [168], and nucleotides [169]. Recent work has showcased the use of 27 purified enzymes that can work together to convert glucose into terpenes such as limonene, pinene, and sabinene [170]. Here, production can operate continuously for 5 days with a single addition of glucose, with glucose conversion of greater than 95%, to generate high product concentrations (> 15 g/L) that are well above levels toxic to microbes. While exciting, expression and purification of each individual component for such an approach is quite laborious.

Transitioning these metabolic pathways into CFS, where expression of enzyme-encoding sequences could lead to the self-assembly of pathways, would be incredibly enabling. To date, a number of reports have validated this approach. Three- and six-enzyme pathways have recently been generated *de novo* from DNA inputs in CFS to produce N-acetylglucosamine and a peptidoglycan precursor, respectively [171, 172]. A five-enzyme pathway that transforms tryptophan into a bioactive pigment called violacein has also been demonstrated [49, 56]. Additionally, a combinatorial strategy has recently been used to build a 17-step enzyme pathway for *n*-butanol [173]. It is intriguing to envision how this approach could influence the synthesis of high-value commodities (e.g., small-molecule drugs, cosmetic ingredients, food additives, and scented compounds), and move

production towards more sustainable enzyme-catalyzed processes.

The cell-free assembly of engineered metabolic pathways has led to parallel approaches in the areas of energy production, biomaterials, and even the development of artificial cells. Below we introduce some of the related efforts in these fields.

### Energy storage and generation

Cell-free enzymatic pathways have recently been used to create biobatteries with small environmental footprints and energy-storage densities superior to that of current lithium-ion devices [174]. Moreover, previous studies have demonstrated ATP generation on electrode surfaces [175, 176]. Since both the assembly of ATP synthase [113] and the synthesis of membrane proteins into tethered lipid bilayers [177] have been shown in CFS, one potential application of CFS could be rapid prototyping and construction of novel energy-generating biodevices that would be capable of producing electricity from low-value commodities (i.e., biomass or waste) [109]. One could readily imagine CFS simply powered by light [178] or electricity, which could help lower the cost of manufacturing industrially relevant biomolecules as discussed above.

### Biomaterials

As noted earlier, CFS have not only been used to screen the natural diversity of enzymes, but also to sculpt enzymatic activity. In an example of this, Bawazer et al. used CFS to synthesize solid-state materials [52]. A cell-free system was used to exert evolutionary selection on biomineralizing enzymes called silicateins that are capable of synthesizing silicon dioxide or titanium dioxide. DNA fragments coding for two isoforms of silicatein were digested and reassembled by DNA shuffling to create a library of chimeric enzymes. Through a clever scheme of selection, variants were then chosen for their ability to deposit silica or titanium dioxide onto microbeads in an oil-water emulsion. The success of this methodology through the use of CFS raises the exciting prospect of using green chemistry for the deposition of semi-conductor materials. This type of green deposition could also be modified such that it is guided by a CFS-compatible photolithography technique similar to that demonstrated by the Bar-Ziv group [55, 179, 180].

### Artificial cells

Artificial cells have traditionally been defined as encapsulated bioactive materials (e.g., RNA, DNA, and enzymes) within a membrane compiled to perform a designated function [134]. Incorporation of CFS into liposomes pre-dates much of the cell-free synthetic biology discussed above [181, 182] and provides a powerful

platform for engineering artificial cells [37, 151, 183–185]. Artificial cells have many important applications; they can be used to link phenotype to genotype in vitro for directed evolution applications, and to spatially separate synthesis of different proteins [185]. There is also evidence indicating that confinement, a feature common to many types of artificial cells, can be used to boost protein expression yields of CFS [186]. Furthermore, artificial cells may allow for prolonged expression without relying on traditional dialysis methods that are often used to provide a continuous supply of reaction precursors. For example, early work by the Noireaux group showed that membrane-based artificial cells can be augmented with  $\alpha$ -hemolysin pore proteins from *Staphylococcus aureus* in order to achieve selective permeability for nutrients [182, 187].

Artificial cells may also be constructed in the form of solid-state two-dimensional compartments. Silicon has been used to fabricate two-dimensional artificial cells capable of carrying out many of the features possible in cell-based systems. These features include simple metabolism, operation of gene circuits (e.g., oscillators), and even communication between compartments. Control over fabrication geometry allows for precise evaluation of the effects of diffusion gradients and can help tune protein turnover [55, 179].

Looking forward, perhaps one of the most exciting and promising applications of artificial cells is the ability to express membrane proteins efficiently. This could allow for cell-free engineering of signaling pathways [188], such as those involving G-protein-coupled receptors (GPCRs) [189, 190]. Approximately 34% of all FDA-approved drugs act on GPCR targets [191]. As such, artificial cells could become an invaluable tool in the drug discovery process. Artificial cells also have the potential to be used for in vivo therapeutics. For example, they could be designed to perform sensing, logic, or therapeutic functions. Artificial cells may be designed to accumulate at a tumor site through the enhanced permeability and retention (EPR) effect [192] or by using targeting molecules on their surface. They can also be constructed to protect therapeutic enzymes while being permeable to specific substrates and products, thus increasing active circulation time and expanding their therapeutic potential [193, 194].

## Education

Given their potential for biosafety and portability, cell-free systems offer a great platform for teaching key concepts in synthetic biology. The Cold Spring Harbor Laboratory course in synthetic biology, for example, includes modules that utilize cell-free systems [195]. In recent work led by Jim Collins and Michael Jewett, the ability of CFS to support on-demand and

on-site sensing and manufacturing was further extended to bring synthetic biology capabilities to the classroom [196, 197]. Here FD-CF components were used to create kits that enable students to experience rational design of reactions, such as creating their own unique colors by mixing DNA coding for different fluorescent proteins. Other applications included the on-demand creation of fluorescent hydrogels, scents, and even sensors that could distinguish between DNA from banana, kiwi, and strawberry. Reflecting an important trend in the field of synthetic biology, this work included the testing of tools under field conditions with the help of high school students. This work sets the important groundwork for inspiring curiosity and passion in students who will drive the next generation of synthetic biology.

## The future of biotechnology with cell-free systems

The merger of cell-free systems with the vast array of genetically programmable tools is transforming the synthetic biology landscape, creating powerful in vitro platforms. These platforms have already begun to bring about de-centralization of health care through portable diagnostics and drug manufacturing. They also have great potential for the efficient, centralized production of high-value commodities. Cell-free synthetic biology approaches will take biology and biotechnology to new horizons and will surely produce many creative and unexpected outcomes. We expect the field to continue to expand and to merge with other engineered systems. One could envision programmed interactions with materials on the nano-scale and interplay with a variety of engineered enzymes. We are excited to see how CFS will bring synthetic biology closer to electronics, computation, and machine learning.

## Acknowledgments

Not applicable.

## Authors' contributions

AT and KP co-authored and edited the manuscript. KJ edited the manuscript and created the figures as well as their legends. All authors read and approved the final manuscript.

## Funding

This work was supported by the CIHR Foundation Grant Program (201610FDN-375469), CIHR/IDRC team grant (149783), and the Canada Research Chair Program (CIHR, 950-231075) to K.P.

## Availability of data and materials

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

Published online: 08 August 2019

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