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On-demand biomanufacturing through synthetic biology approach

Chenwang Tang [a,1], Lin Wang [b,1], Lei Zang [b], Qing Wang [b], Dianpeng Qi [a,**], Zhuojun Dai [b,*]

[a] MIIT Key Laboratory of Critical Materials Technology for New Energy Conversion and Storage; National and Local Joint Engineering Laboratory for Synthesis, Transformation and Separation of Extreme Environmental Nutrients, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin, 150001, China

[b] Materials Synthetic Biology Center, CAS Key Laboratory of Quantitative Engineering Biology, Guangdong Provincial Key Laboratory of Synthetic Genomics, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518055, China

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ABSTRACT

Biopharmaceuticals including protein therapeutics, engineered protein-based vaccines and monoclonal antibodies, are currently the mainstay products of the biotechnology industry. However, the need for specialized equipment and refrigeration during production and distribution poses challenges for the delivery of these technologies to the field and low-resource area. With the development of synthetic biology, multiple studies rewire the cell-free system or living cells to impact the portable, on-site and on-demand manufacturing of biomolecules. Here, we review these efforts and suggest future directions.

1. Introduction

Biologic medicines, such as recombinantly expressed protein hormones, cytokines, replacement enzymes, blood factors, engineered protein-based vaccines and antibodies, are currently the mainstay products of the biotechnology industry [1–3]. They are routinely utilized to treat diseases such as cancer and autoimmune disorders, and can precisely improve a patient's physiology with fewer side effects than traditional small molecules drugs. The current standard manufacturing scheme is generally optimized for fed-batch bioreactors, followed by a combination of different filtration and chromatography unit operations to achieve the required purity and yield [4]. This centralized scheme heavily relies on the infrastructure, such as large fermenters (up to 25, 000 L) and chromatography columns (up to 2 m in diameter with 10 cm–20 cm bed height) [5]. These pose several issues. First, the expenses in building the necessary equipment, as well as associated piping and hardware costs (including preparing, holding and cleaning) limit the essential drugs production in developing countries [6–8]. Besides, the conventional method cannot address the need in emergency situations, such as the outbreaks of infectious disease. Large-scale preparation in advance is a tangible solution, but usually suffers from huge waste and cold-chain limitation [9–11]. Since it is difficult to predict the type and amount of drugs in need, multiple facilities have to be built (requiring high capital investment and maintenance cost) with a large number of strains cultivated to cover the possible pharmaceuticals. Lastly, the emergence of personalized medicine, battlefield medicine as well as smaller product campaigns associated with orphan drugs and smaller disease paradigms put more pressure on oversized bioprocess plants [12–14]. In all, there is a growing need for rapid, flexible, low-cost and portable biomanufacturing systems that can produce the biomolecules on-demand and on-site.

With more than twenty years of development in fundamental research and technology translation, synthetic biology is progressively impacting a variety of spaces including biomanufacturing, food, agriculture, materials as well as healthcare [15–18]. In particular, synthetic biology uses the engineering principles and biological disciplines to re-design the naturally existing biological systems, or design and construct new biological parts, genetic networks and systems to achieve the logical form of cellular control for desired applications [19–21]. In the last decade, efforts have been made in assembling cell-free system and rewiring the living cellular hosts to address the demands in the versatile and small-scale biomanufacturing (Fig. 1). In this review, we detail these strategies, as well as flaws and potential future solutions.

* Corresponding author.
** Corresponding author.
E-mail addresses: dpqi@hit.edu.cn (D. Qi), zj.dai@siat.ac.cn (Z. Dai).
[1] These authors contribute equally.

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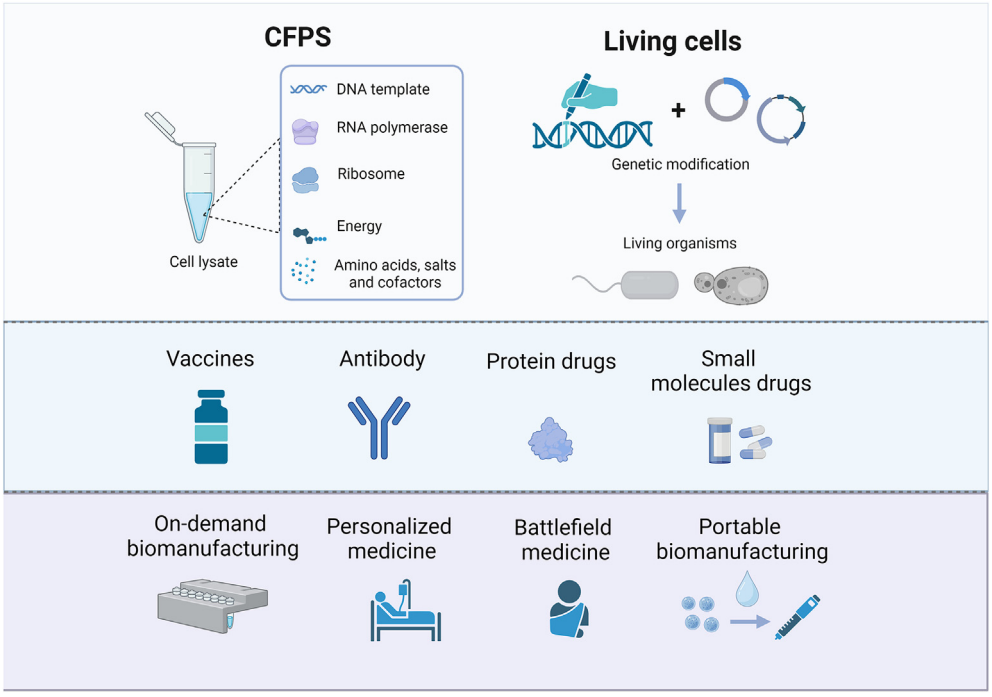

Fig. 1. On-demand biomanufacturing. In this review, we summarize approaches of engineering cell-free protein synthesis (CFPS) and living cellular hosts to accomplish the on-demand biomanufacturing. Compared with conventional large-scale fermentation, on-demand biomanufacturing can be flexible and portable to meet requirements under different situations.

1.1. Engineering cell-free system for portable biomolecules manufacturing

Cell-free protein synthesis (CFPS) applies transcriptional and translational machinery to synthesize protein in vitro without the use of living cells [14,22]. Consequently, the protein synthesis environment of CFPS is not restricted by cell walls or homeostasis conditions necessary to maintain cell viability (Table 1). It has been developed and used for more than 60 years to understand fundamental molecular biology and biochemistry. Recently, it has been applied to monitor and screen the molecular switch dynamics and complex gene circuits [23,24]. Most of these efforts by far have focused on the solution-phase reactions, which are not stable and impractical for handling outside the lab. In 2014, Pardee et al. reported a method of embedding cell-free synthetic gene networks onto paper and other materials for rapid sensing and diagnoses (Fig. 2a) [25,26]. In particular, they freeze-dried cell-free system onto the paper or other porous substrates such that the materials possessed the fundamental transcription and translation properties. These materials were stable at the room temperature and could be activated by simply supplying water. By this strategy, the authors demonstrated the storage and operation of multiple synthetic networks, including the rapid screening of gene constructs and diagnostics of strain-specific Ebola virus. By these examples, they have shown that the paper-based cell-free system could be produced, stored, operated at a low cost, and highly portable for distribution.

In 2016, Pardee et al. have further extended this portable cell-free strategy to produce various therapeutics including antimicrobial peptides (AMPs), vaccines, combinatorial antibodies and small molecules on-site and on-demand (Fig. 2b) [27]. They compressed the buffers, cellular machinery, and molecular instructions into a single freeze-dried reaction pellet, activated the reaction by adding water and yielded the desired product within 1–2 h. The biosynthesis was conducted without the need for specialized equipment and skills. The system could be applied to global health and personalized medicine, making the scalable molecular synthesis decentralized. These two studies have rewired the format of cell-free system by immobilizing the essential elements onto the paper or freeze-drying the machinery into pellets. Simply by doing so greatly enhances the stability of the multi-enzyme system and facilitates the distribution and operation.

Cell-free strategy is able to synthesize small molecules and non-ribosomal peptides. Pardee et al. used freeze-dried cell-free (FD-CF) system to reconstitute the pathway of violacein, which was known with its diverse biological activities including anticancer and antibacterial properties [27]. In *Chromobacterium violaceum*, violacein is synthesized by five-enzyme pathway (VioA, VioB, VioC, VioD and VioE) through the transformation of two L-tryptophan molecules [38]. The authors have confirmed the expression of each enzyme by western blot in FD-CF reaction. Then, L-tryptophan was used as the reaction substrate and they rehydrated FD-CF reaction pellets with the combinations of VioA to VioE template to enable the synthesis of the desired product.

Zhuang et al. developed cell-free platform for rapid biosynthesis of nonribosomal peptide valinomycin [39]. Valinomycin can dissipate essential transmembrane electrochemical gradients and cause tremendous metabolic disorders. Therefore, it is widely recognized as an ionophore and used as drug with antifungal, antimicrobial and anticancer efficacy. In nature, valinomycin is synthesized by several *Streptomyces* strains via valinomycin synthetase, which contains two distinct proteins named VIm1 and VIm2 [40,41]. The authors developed a cell-free

Table 1
Comparison of cell-free protein synthesis systems and living cells.

Properties	CFPS systems	Living cells
Portable and on-demand [27]	Fast	Slow
Time for each run [28]	Fast	Slow
Protein separation and purification [28]	Simple	Complex
Cost [27,29]	High	Low
Expression of transmembrane protein [30]	Simple	Complex
Incorporation of unnatural amino acids [31]	Simple	Complex
Tolerance of toxic compounds [32]	High	Low
Concentration of chemicals or proteins [33]	Simple	Complex
Protein folding [34]	Simple	Complex
Self-replication [35]	Complex	Simple
Raw materials [36]	Complex	Simple
Industrialization [29]	Complex	Simple

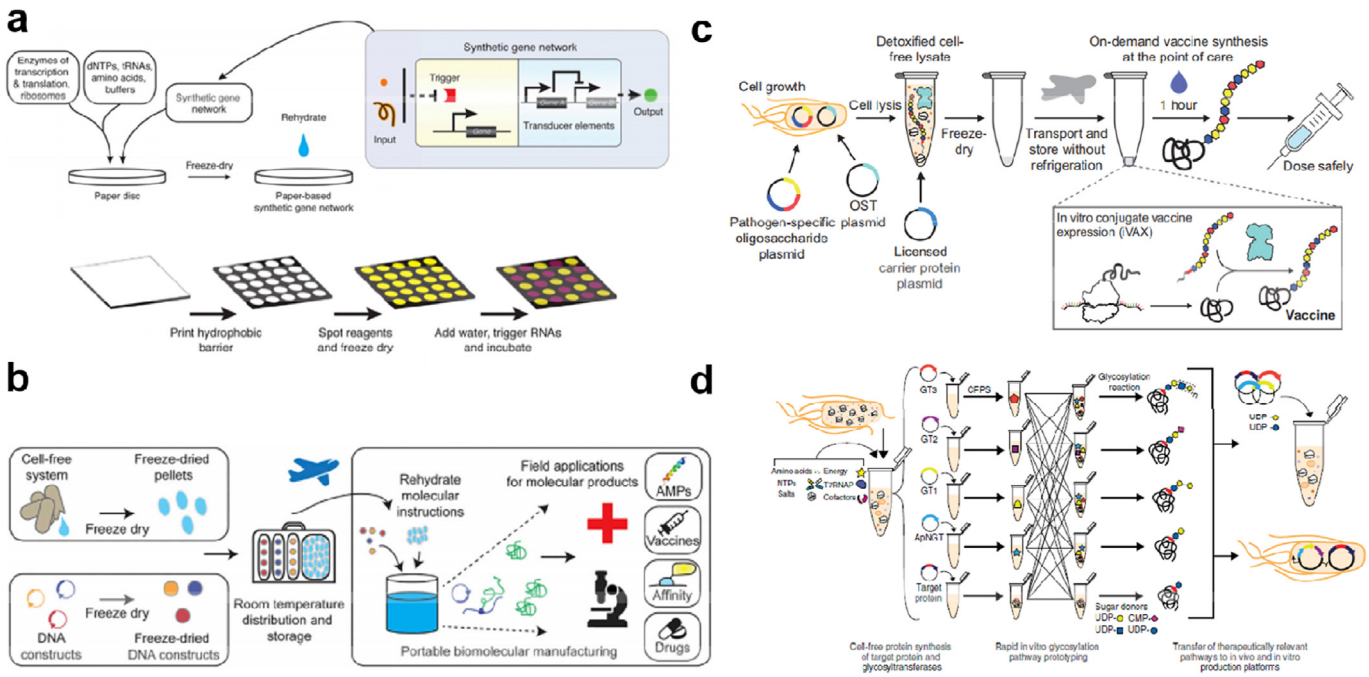

Fig. 2. Engineering cell-free protein synthesis platform for on-demand biomanufacturing.

a. Embedding cell-free synthetic gene networks onto papers and other materials for rapid sensing and diagnoses [25].
b. Engineering the freeze-dried cell-free system to produce various therapeutics including antimicrobial peptides (AMPs), vaccines, combinatorial antibodies and small molecules on-site and on-demand [27].
c. A modular technology for in vitro conjugate vaccine expression (iVAX) in a portable and on-demand fashion [28].
d. Cell-free workflow for modular synthesis, assembly and discovery of multi-enzyme glycosylation pathways in vitro [37].

metabolic engineering (CFME) method to direct the synthesis of valinomycin. They introduced genes encoding Vlm1 and Vlm2 individually into the source strain *Escherichia coli* BAP1. These cells were then induced for heterologous expression of each enzyme and then lysed to generate Vlm1 and Vlm2 enriched cell lysates, respectively. The *sfp* gene was chromosomally integrated into the engineered strain, making the Vlm1 and Vlm2 in their active *holo* form modified by the encoded Sfp. The biosynthesis of valinomycin was initiated by directly mixing two cell lysates, and the cofactors from the cell lysate were sufficient to drive the synthesis of the desired product.

Besides the optimization in the format of cell-free system, cell-free machinery can be modified for more sophisticated therapeutic production. For example, glycosylation is present in over 70% of protein therapeutics (e.g., conjugate vaccines) and profoundly affects the immunogenicity and activity of the therapeutics. However, most of the lab-used prokaryotic species such as *Escherichia coli* lack endogenous glycosylation enzymes. To overcome the limitations, Jaroentomeechai et al. used a glyco-optimized *E. coli* strain to source the cell extracts containing the oligosaccharyltransferases (OSTs) that can transfer the prebuilt sugars from lipid-linked oligosaccharides (LLOs) onto the target proteins [42]. The engineered *E. coli* modified with (i) genomic mutations that benefit glycosylation reactions and (ii) plasmid DNA for producing essential glycosylation components (i.e., oligosaccharyltransferases (OSTs) and lipid-linked oligosaccharides (LLOs)) served as the source strain for producing crude S30 extracts. Biosynthesis of *N*-glycoproteins was initiated by priming the extract of the source strain with DNA encoding the acceptor protein of interest. The platform enabled a one-pot reaction scheme for efficient and site-specific glycosylation of target proteins. Stark et al. further used the system to produce *anti-Francisella. Tularensis* conjugate vaccine by attaching the FtO-PS (*F. tularensis* Schu S4 O-antigen polysaccharide, 826-Da repeating tetrasaccharide unit) structure to diverse carrier proteins in vitro (Fig. 2c) [28]. The authors sourced the cell extracts from *E. coli* cells expressing the FtO-PS biosynthetic pathway [43,44], and the oligosaccharyltransferase (OST) enzyme CjPglB. Plasmid encoding the carrier protein was incubated in the lysate containing the lipid-linked FtO-PS and active CjPglB to generate glycosylated protein. They have further engineered the detoxified strain and freeze-dried the lysate. Upon rehydration, the reaction synthesized clinically relevant doses of conjugate vaccines in 1 h.

Most methods use OST to implement glycosylation. However, OSTs are difficult to express because they are integral membrane proteins that often contain multiple subunits. The LLO substrate specificities of OSTs limit the modularity and the diversity of glycan structures that can be transferred onto proteins. In comparison, *N*-glycosyltransferases (NGTs) show remarkable advantages since NGTs are cytoplasmic bacterial enzymes that transfer a glucose residue from a uracil-diphosphate-glucose (UDP-Glc) sugar donor onto asparagine side-chains. This primer can then be sequentially elaborated by co-expressed glycosyltransferases (GTs). By this strategy, Kightlinger et al. developed a cell-free workflow, named as GlycoPRIME for modular synthesis, assembly and discovery of multienzyme glycosylation pathways in vitro (Fig. 2d) [37,45]. They incorporated a recently found *N*-glycosyltransferase from *Actinobacillus pleuropneumoniae* (ApNGT) into the system so that the ApNGT could recognize the classic *N*-X-S/T amino acid motif, and site-specifically install a single *N*-linked glucose primer onto proteins. They have further selected and expressed 24 bacterial and eukaryotic GTs and combined them to create 37 putative biosynthetic pathways to elaborate the glucose installed by ApNGT on a model glycoprotein substrate. These pathways yielded 18 glycan structures that had not yet been reported on proteins and provided new biosynthetic routes to therapeutically modifications (e.g. a protein vaccine candidate with α-galactose adjuvant motif) in a one-pot cell-free system. By enabling the rapid synthesis and assembly of glycosylation enzymes, Glyco-PRIME demonstrates the potential to further expand the glycoengineering toolkit towards the generation of glycoproteins on-demand and by design.

1.2. Rewired cells for on-demand biologics production

Living cells have long been used as workhorse for conventional manufacturing of protein biopharmaceuticals in centralized, large-scale and single-product facilities [2,46–48]. With the fast development in synthetic biology, new methods using rewired cells are emerging for portable and on-demand production of drugs for small patient populations or individuals. *E. coli* are commonly used microorganisms to produce heterologous proteins for therapeutic use, accounting for ~30% of biopharmaceuticals (particularly non-glycosylated proteins), due to their rapid growth, high yield of the product, cost effectiveness and simple scale-up process. The availability of various expression vectors and strains, relatively easy protein folding mechanisms and bioprocess technologies, make them very attractive for industrial applications. However, synthesis of recombinant proteins using bacterial hosts entails multiple steps including culturing, disruption of bacteria by physical or chemical means, and subsequent isolation and purification of the desired product. For industrial operations, these steps are usually carried out on a large scale; consequently, each step requires a sophisticated and delicate infrastructure to ensure efficiency and product quality. While critical for producing molecules in large amounts, this format is not flexible or economically suited for producing or characterizing diverse biologics when only a small amount is needed for each. In 2019, Dai et al. took advantage of recent developments in synthetic biology and stimulus-responsive biomaterials to integrate multiple steps of production, disruption and separation into a concise format to build a microbial swarmbot (MSB) platform (Fig. 3a) [49]. They first programmed the density-dependent autolysis of engineered bacteria by a genetic circuit, and encapsulated these bacteria into a micro-sized hydrogel capsules. When the local cell density inside the capsule was sufficiently high, autonomous partial lysis would occur and allow the cells to release their contents, including the protein product of interest. The bacterial growth changed the local environmental conditions (pH and ionic strength), driving the volume phase transition of the encapsulating material. Consequently, the released protein was transported from the interior to exterior with the shrinking of growth-sensitive capsules, while cells and large debris were trapped inside the capsules. They used nutrient replenishment as a cue to swell the capsule again, while it reset the capsule environment and allowed the cell density to resume. Over 25 different proteins including some therapeutics have been produced by this portable and flexible way.

Yeasts are unicellular organisms and are among the simplest eukaryotes. They have a sub-cellular organization similar to that of higher eukaryotes and contain a nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, secretory vesicles, vacuoles and microbodies [53,54]. *Saccharomyces cerevisiae* (Baker's yeast) have been accepted as "generally recognized as safe'' since they have been used as a component of human diet for centuries. Johnston et al. used a temperature-responsive, shear-thinning hydrogel to compartmentalize *S. cerevisiae* into hydrogel (Fig. 3b) [50]. The F127-bisurethane methacrylate (F127-BUM) exhibiting a temperature-dependent sol-gel transition (~17 °C), was employed to immobilize yeast cells through the

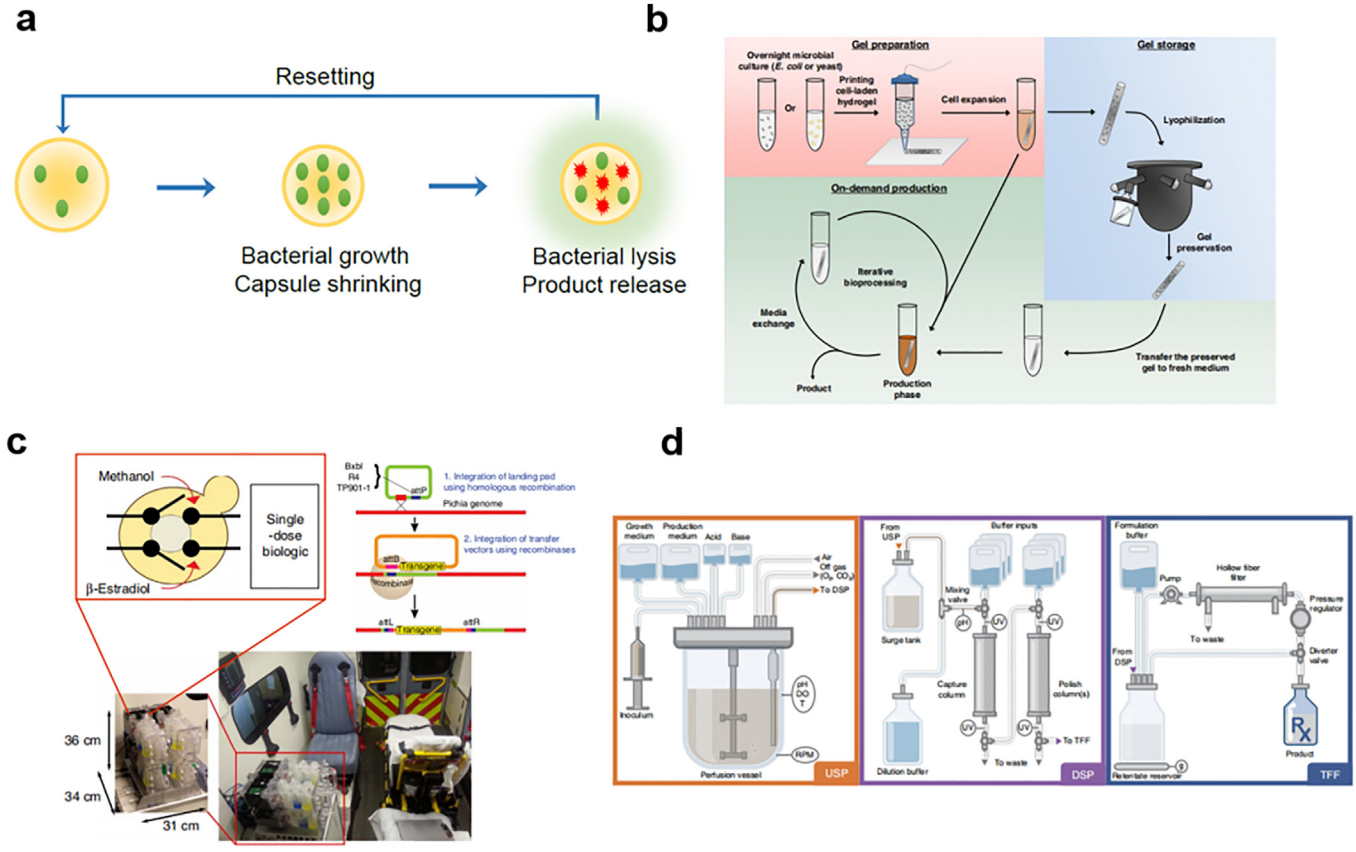

Fig. 3. Engineering living cells for portable and on-demand biomanufacturing.

a. Developing microbial swarmbot (MSB) platform that integrates the multiple steps of production, disruption and separation in a concise format [49].
b. Engineering a temperature-responsive, shear-thinning hydrogel system to harness the bioactivity of embedded microbes for on-demand production of small molecules and peptides [50].
c. An integrated, benchtop and portable microfluidic device containing genetically engineered *P. pastoris* to generate multiple therapeutic proteins [51].
d. An automated desktop multi-product manufacturing system named InSCyT (Integrated Scalable Cyto-Technology) to integrate fermentation, sensing, input and output, purification and analysis automatically [52].

extrusion of a 3D printer [55]. The formed cell-embedded bioreactors could produce small molecules and antimicrobial peptides. More importantly, these hydrogels provided protection from preservation techniques such as lyophilization and sustained the metabolic function of the encapsulated cells for over 1 year of repeated use. The preserved gels could be rehydrated and incubated in fresh medium continuously to produce the desired product.

Pichia pastoris is another regularly used chassis for recombinant protein expression host due to its clear genetic background, simple gene programming operations, high-efficiency in secretion and post-translational modification [56,57]. To date, more than 500 different proteins, including simple peptides, enzymes, hormones, monoclonal antibodies and FDA-approved treatments, have been expressed in *P. pastoris*. In addition to traditional industrial fermentation process, emerging studies are reprogramming *P. pastoris* for on-demand biomolecules production. Perez-Pinera et al. used genetically engineered *P. pastoris* strains to secrete multiple proteins on programmable cues in an integrated, benchtop, and portable microfluidic device (Fig. 3c) [51]. First, they introduced an *attB* homology site into the *P. pastoris* genome, allowing it to integrate single-copy plasmids by recombinases, and used this method to construct an inducible transcription system activated by β-estradiol. The system was orthogonal to the methanol-inducible transcription system (AOX1 promoter), and therefore could control the secretion of two proteins by different chemical input. The engineered dual-biologics-producing (rHGH and IFNα2b) *P. pastoris* were cultured in an integrated, milliliter-scale microfluidic device to form a benchtop microbial reactor. The system enabled fast and flexible protein expression and secretion, and produced near-single-dose levels of rHGH and IFNα2b within 24 h.

In conventional biomanufacturing, it is regular to produce only one drug in one pipeline. However, it is economically difficult to produce multiple drugs using multiple parallel manufacturing platforms. To tackle the problem, Cao et al. used the above-mentioned double-inducible *P. pastoris* to further construct a variety of strains that could co-express two biologics with dynamical control over the ratio, and separated the biologics through the downstream processing [58]. For example, they produced a cocktail of two therapeutic monoclonal antibodies, anti-programmed cell death 1 (*anti*-PD1) and anti-cytotoxic T-lymphocyte-associated antigen 4 (*anti*-CTLA4) in a single batch fermentation. To simplify the purification process, they eluted multiple proteins sequentially using the buffer with different salt concentration from the same columns. To further increase the throughput, an IPTG (isopropyl β-D-1-thiohomolactopyranoside)-induced expression system was further introduced to achieve the orthogonal control of three biologics production (methanol-induced expression of rHGH, β-estradiol-induced expression of granulocyte-colony stimulating factor (G-CSF), and IPTG-induced expression of IFNα-2b) in a single strain.

Using the *P. pastoris* as the chassis cell, Crowell et al. developed an automated desktop multi-product manufacturing system named InSCyT (Integrated Scalable Cyto-Technology) to integrate fermentation, sensing, input and output, purification and analysis automatically (Fig. 3d) [52]. InSCyT used fluidically connected modules for the upstream fermentation and downstream processing. They performed continuous fermentation through in-tank perfusion in a sub-liter benchtop bioreactor to reduce the volume of the bioreactor and enable high space-time yield. The bioreactor was equipped with sensors to control fermentation parameters such as pH, temperature and dissolved oxygen. The purification module was designed to enable either two or three stages of chromatographic separation by straight-through processing with no intermediate holding tanks or adjustments between purification steps. The final module in the system was a tangential flow-filtration system for buffer exchange and formulation to a final liquid dosage form of the product. A custom integrated software architecture unified operation of all three modules with appropriate controls as a fully automated single system. They have successfully implemented multiple therapeutic protein products using the platform, including rHGH, IFNα2b and G-CSF. The system can produce hundreds to thousands of doses of clinical-quality protein biologics end-to-end in about three days.

2. Discussion

By far, most of the studies in portable and on-demand biomanufacturing focus on developing and optimizing CFPS system or rewiring the regular chassis including *E. coli*, *S. cerevisiae* and *P. pastoris*. With the vast progress, problems remain in multiple aspects such as downstream processing and limitation in the regular chassis. In addition, the purification scheme is still tedious even for small-scale manufacturing, and there lacks portable device to examine the quality of the biologics, such as rapid sterility testing of cell products and portable biotoxicity analysis. A possible solution is to use recombinant cell-free system PURE (protein synthesis using recombinant elements). The system contains 32–36 known proteins to enable both the transcription and translation machinery [59–63]. If all the recombinant are elements decorated with the same affinity tag, the expressed protein of interest can be purified by the reverse affinity chromatography [64]. The clear background of the PURE system may also permit the direct use of biologics based on the actual requirement.

Besides *E. coli*, *S. cerevisiae* and *P. pastoris*, other chassis microbes may also be engineered due to their unique advantages in resilience to the harsh environment and metabolic diversity. For example, *Bacillus. subtilis* are aerobic soil bacteria and identified as an attractive host for bioproductions. They have been widely used to produce recombinant proteins, vitamins, inositol, acetoin, hyaluronan and some other chemicals [65,66]. Compared with other cells and some of its close relatives, *B. subtilis* have excellent protein secretion ability and can be edited with developed toolbox, making them an attractive host for protein production [67,68]. Importantly, *B. subtilis* have the ability to generate spores when the environment is not conducive to its own growth and reproduction [69–72]. Utilizing this property, Yang et al. programmed the *B. subtilis*, which could form spores and be resistant to desiccation and radiation, for long-term "gene" storage with excellent stability [73]. They have further utilized the protein expression and secretory systems of vegetative *B. subtilis* cells to design a pipeline to produce nanobodies, making the technology stable with applications in extreme environments.

Most of the chassis cells are heterotrophic. Therefore, the biomanufacturing by these cells depends on the supply of nutrients. The introduction of phototrophic members may address this problem since the phototrophs such as cyanobacteria can convert carbon dioxide into organic carbon to maintain the growth and metabolism functions of themselves. Among the photoautotrophs, cyanobacteria are known to be the first oxygenic photosynthetic microorganisms which have contributed to Earth's atmosphere for more than 3 billion years. The ability in photosynthesis of cyanobacteria has attracted broad attention and made them a good candidate in the field of bioenergy and biotechnology [74–76]. Besides using the cyanobacteria as the biologics producer, it can also produce the carbon source and support the growth of other cells for the biologics production [77,78]. For example, Wang et al. developed the platform for the stable co-culturing of multiple species [63]. Especially, they created the phototrophic consortia comprising the engineered cyanobacteria *S. elongatus* PCC7942 that could secret the sucrose and *E. coli* that could digest the sucrose to support the growth. The pattern could be adopted to produce the biologics with no access to the nutrients. In the future, we hope to see more transdisciplinary research with teams of scientists and engineers to bridge the laboratory findings and ultimately practice for this emerging area.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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