Toward a Roadmap for Cell-Free Synthesis in Bioprocessing

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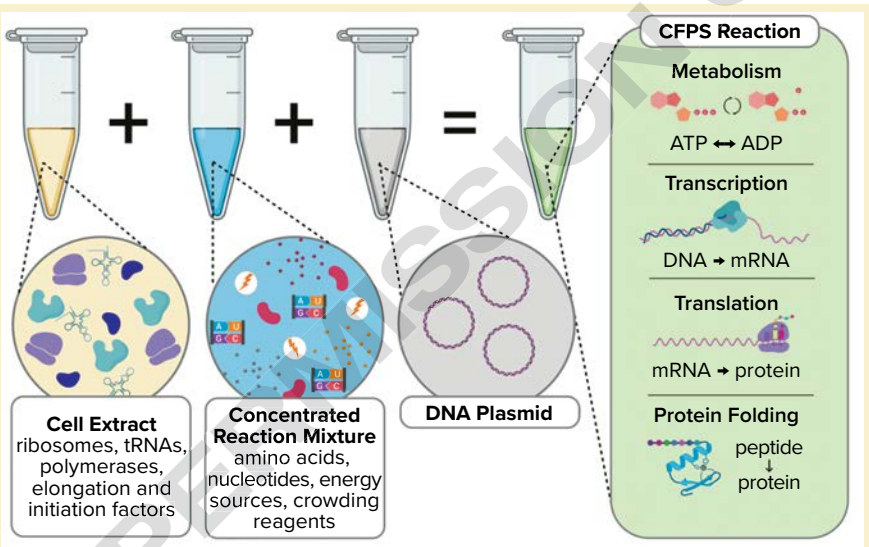
Cell-free synthesis (CFS), also known as *cell-free transcription and translation,* supplements cellular components (either a cell lysate or purified recombinant elements) with nucleotides, amino acids, metabolic intermediates, and salts to produce a nucleic acid or protein from a genetic template added to the reaction. This exciting technology has seen a substantial increase in both academic and commercial interest over the past decade (**1**). Interest stems in large part from the potential to democratize access to the machinery of biology by removing the need to engineer cells genetically (**2**). CFS has the potential to revolutionize healthcare in much the same way that personal computers did for information technology (**3**). However, our interest and that of the bioprocessing community at large comes from the potential to transform manufacturing for some healthcare products (**4**).

A growing area in which CFS platforms are recognized as a potentially enabling technology is in stratified approaches to facilitate distributed manufacturing of biological products (**5**). Although still developing as a manufacturing technology, CFS offers flexibility and potentially improved robustness over existing cell-based biotherapeutic manufacturing. The technology could reduce on-site footprints and infrastructure complexity and allow for robust process control combined with flexibility of output. In this context, University College London's biochemical engineering department — working with the Future Targeted Healthcare Manufacturing (FTHM) Hub supported by the United Kingdom's Engineering and Physical Sciences Research Council (EPSRC) — has undertaken a series of workshops (pictured herein). They focused on elucidating drivers and barriers to the use of this technology for stratified medicines manufacture and mapping out desirable future states of the technology.

Here we summarize the FTHM Hub specialist working group's discussions toward a roadmap for CFS platforms. We begin with a short history of CFS before proceeding to consider the motivations for commercial interest by looking at existing applications of the technology. Finally, we examine the technical challenges in applying CFS in bioprocessing and map the developmental stages toward a CFS-based device for distributed manufacturing.



Components of a cell-free protein synthesis reaction (extract, supplements, and a DNA template) with the key reactions that occur when they are combined

PRODUCT FOCUS: PROTEINS, VACCINES, AND NUCLEIC ACIDS

PROCESS FOCUS: PRODUCTION

AUDIENCE: PROCESS/PRODUCT DEVELOPMENT, MANUFACTURING

KEYWORDS: CELL EXTRACTS, QBD, AUTOMATION, PROCESS MONITORING AND CONTROL, PERSONALIZED MEDICINE

LEVEL: INTERMEDIATE

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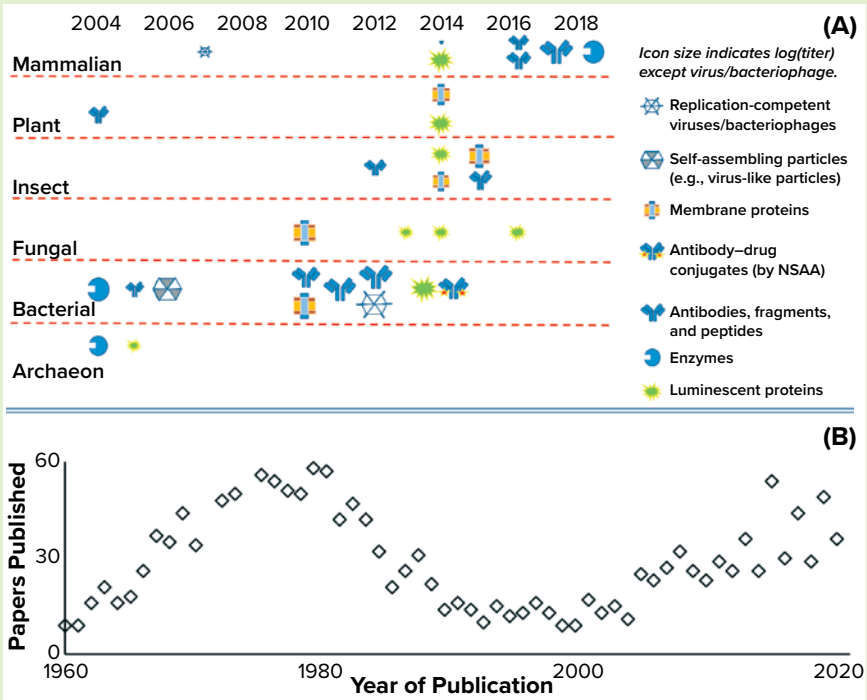
Figure 1: (A) Historic achievements in cell-free synthesis (CFS) across different cell types, with an indicative range of protein types and relative titers (log proportional to the icon size) achieved to date; the key achievement of incorporating nonstandard amino acids (NSAA) is highlighted. Another key achievement was cell-free glycosylation using Chinese hamster ovary (CHO) cell lysate in 2014. Bacterial extracts come from A19, Rosetta (DE3), BL21 (DE3), BL21, star (DE3), and ClearColi BL21 *Escherichia coli* strains that have been engineered to make them more suitable for manufacturing. Yeast extracts come from *Pichia pastoris* and *Saccharomyces cerevisiae* strains; mammalian extracts from CHO, human embryonic kidney (HEK), human epithelioid cervix carcinoma (HeLa) cells, and from blood-derived leukocytes; and plant extracts from wheat germ and tobacco. Results presented herein come from a range of research groups with limited or no standardization, so parameters such as the CFS reactor type (batch or substrate addition/inhibitor removal by dialysis), reaction mix and length do not match (see **13** and Further Reading). (B) The numbers of CFS papers published per year are based on a Scopus search for the terms *cell-free* and *synthesis* in article titles.



A Brief History

CFS is not a new technique. It was used first in 1961 and played an important role in understanding the genetic code and central dogma of modern biology: the link between DNA, messenger RNA, and protein expression (**6**). Figure 1B illustrates this history with a plot of the number of papers related to CFS showing a peak of interest in the early 1970s, followed by a substantial revival of interest over the past two decades. CFS activity follows the features of a classical hype curve for a new technology (**7**). As Figure 1A also shows, the number of extract sources and product types has expanded rapidly, with CFS used for the production of antigens, virus-like particles (VLPs), cytokines, antibodies, peptides, membrane proteins, viable bacteriophages and viruses, enzymes containing metal cofactors, proteins containing nonstandard amino acids (also known as nonnatural amino acids), and RNA. Applications also have expanded rapidly, including advancements in mobile biosensors (**8**) and manufacturing platforms (**5**).

Research to date has focused largely on optimization of the CFS reaction mix (nucleotides, amino acids, metabolic intermediates, and salts) and on cell-extract production, particularly source-strain modifications and use of different cell types. Those efforts have produced order-of-magnitude improvements in titer, with 2.3 mg/mL the maximum cited so far (**9**).

Figure 2 compares the rate of titer improvement achieved with the Chinese hamster ovary (CHO) manufacturing cell line with that achieved in CFS systems. This comparison brings us to a number of key observations. First, the titers currently achievable in a CHO-based system are an order of magnitude greater than those obtainable with CFS (**10**) — shown to be comparable from both *Escherichia coli* and CHO CFS systems in Figure 1A. However, that should be considered in the context of very differing levels of investment. Intensive process development for the CHO system has been ongoing for decades. By way of comparison, the number of papers published in CFS has reached about 40 per year, whereas for CHO it is about 200 per year and has been increasing linearly since the early 1970s.

Second, titers reported from CFS reactions show a wide scatter, reflecting the diversity of products and platforms (cell types) involved. In addition, titer variation may have been exacerbated by a lack of cross-laboratory good practice for reaction conditions used (**11**) and the fact that few researchers in the domain are focused on producing their protein of interest for a preparative or manufacturing application. In 2012, Carlson et al. (**12**) presented data on the trend in titers for chloramphenicol acetyltransferase (CAT) produced in CFS reactions, which represent the most complete set of data yet available charting historic improvements in cell-free titers for a single recombinant protein product. If we consider that trend, then progress in CFS titers has been substantial and sustained, and it compares well with the trajectory achieved so far with monoclonal antibody (MAb) expression in CHO cells. That could reflect the openness of the system and relative simplicity and speed with which CFS process modifications can be implemented.

Considering both the rapid rate of titer improvements achieved to date and the demonstrated scalability of the technology (**13**), the potential of CFS is clear. So why is adoption of the technology limited to so few companies in this domain to date, and what applications for CFS could drive growth (**11**)? To answer these questions, we begin with a few exemplar companies and their rationale for using CFS.

Exemplar Commercial Bioprocess Applications

Antibody–Drug Conjugates (ADCs): Worldwide more than 50 ADCs currently are in clinical trials (**14**), and 250 are

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Figure 2: Comparing the evolution of titer in glutamine synthetase (GS) selected Chinese hamster ovary (CHO) cells and cell-free production systems (**12, 13,** and Further Reading)

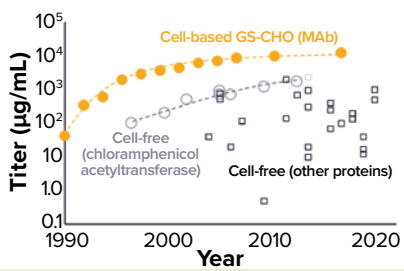

Table 1: Drivers for uptake and potential technical improvements that cell-free synthesis currently allows or may allow in the future

Challenges Driving CFS Uptake	Enabling Features of CFS
Pressure on development time and cost for manufacturing processes (particularly in pandemic control situations, for example, or for maximization of market share in small cohorts)	Ease of scale-up; reduced timelines for process development (high-throughput)
Distributed manufacturing of drug manufacture for increased drug stratification and/or political desire for geographical localization and/or industry desire to reduce logistics-related carbon footprint and complexity	Reduced infrastructure requirements and operator expertise; ease of automation; increased predictability based on critical process parameters (CPPs); improved reproducibility; reduced reliance on cold chain
Next-generation products such as antibody–drug conjugates with demanding, specific product quality attributes (PQAs)	Improved control of product quality
Toxicity of some products to cells	Reduced sensitivity to process environment
Toxicity of some products to operators	Ease of containment and/or reduced intervention

under development, with the market projected to be worth US$15 billion by 2030 (**15**). ADC product critical quality attributes (CQAs) often are more complex than those for therapeutic proteins alone, including attributes such as the ratio of conjugated drug to antibody (**16**). CFS offers the ability to incorporate nonstandard amino acids (NSAAs), which enables control of the location and number of conjugation sites (**17**), rapid prototyping of proteins, and subsequent scaling and incorporation of potent toxins as loads.

Based in South San Francisco, Sutro Biopharma was founded in 2003 and employs about 200 people. Its good manufacturing practice (GMP) production facilities use the proprietary Xpress CF+ cell-free platform to produce ADCs, bispecific antibodies, and cytokine-based therapies. The company's pipeline includes drugs in clinical trial stages from discovery to phase 1 that have been developed alone and in partnerships with Bristol-Myers Squibb, EMD Serono, and Merck using Sutro's platform (**18**).

Toxin Manufacture: Ipsen Biopharm is a global biopharmaceutical company with products in neuroscience, consumer healthcare, oncology, and rare diseases. One of its products is botulinum toxin used as a therapeutic for disorders caused by over-activity of muscles (**19**). The toxin is highly potent, so very low doses are required, and manufacturing is performed with relatively small-scale equipment. However, employees face substantial hazards during production (unusual for a biotherapeutic product) because of the extreme toxicity of the product. The challenge of managing those risks motivated this company's interest in CFS. The absence of cells allows for relatively little manual intervention during toxin synthesis, thus reducing the risk of containment loss. A collaboration among Ipsen Biopharm, Touchlight Genetics (which has a cell-free DNA synthesis technology), and the UK Centre for Process Innovation (CPI) is under way with the goal of developing a fully enclosed process for safely and securely producing this toxin (**20**).

RNA Products: Headquartered in Medford, MA, GreenLight Biosciences was founded in 2009 and employs about 100 people. The company produces RNA using the proprietary GreenWorX cell-free platform. The company's interests are in vaccine development, pandemic preparedness, and plant protection against disease and pests. Thus, the greater speed of response and lower cost compared with other methods for RNA production are key motivators for GreenLight to use CFS. Using RNA as an insecticide/fungicide is a novel technology that requires development (**21**), so the short prototyping turnover also is important. This company has partnerships with Bayer Crop Science, AgroSpheres, and Advanced BioNutrition.

Drivers for CFS Use

The case studies above show that uptake of CFS is driven by potential for

- improved control of product quality through standardized bulk manufacture of reagents as well as direct access to cellular machinery and mechanisms for protein engineering (e.g., introduction of NSAAs)
- the ability to make products that are difficult/impossible to express or make consistently using existing technology through removal of the need to keep cells alive and healthy, which broadens the available range of products, reaction conditions, and mechanisms for protein engineering
- ease of containment because of simplified equipment requirements in the absence of fermentation/cell culture and live genetically modified organisms
- increased speed of process development, enabling rapid-response manufacturing through shortened timelines for prototyping and subsequent scale-up (typical protein synthesis in a CFS system requires three to four hours rather than three to four weeks for CHO cell culture and two to three days for *E. coli* fermentation, excluding stable cell-line generation)
- increasing interest in mRNA-based therapeutics (**22, 23**) for which CFS/in vitro transcription is a leading manufacturing platform.

Within the context of the FTHM Hub, we were interested particularly in the applicability of CFS to personalized or stratified medicine. Intended to serve particular groups of patients identified by genetic screening and diagnostics, these drugs represent a more efficient use of limited resources and a better outcomes than might be available through the traditional model. It is anticipated that growing consumer knowledge and demand will increase the "market pull" for such medicines. That pull will come from both healthcare providers/funders and patients (**24**).

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To enable stratified CFS production of therapeutic proteins/nucleic acids, many experts envision centralized large-scale production of raw materials and localized production of the final therapeutic product, as outlined by Ogonah, et al., in 2017 (**4**). In principle, CFS is well suited for such localized manufacturing because of the reduced infrastructure and expertise required for protein production, making it suitable for automation and amenable to reproducibility/predictability improvements over the current biomanufacturing model.

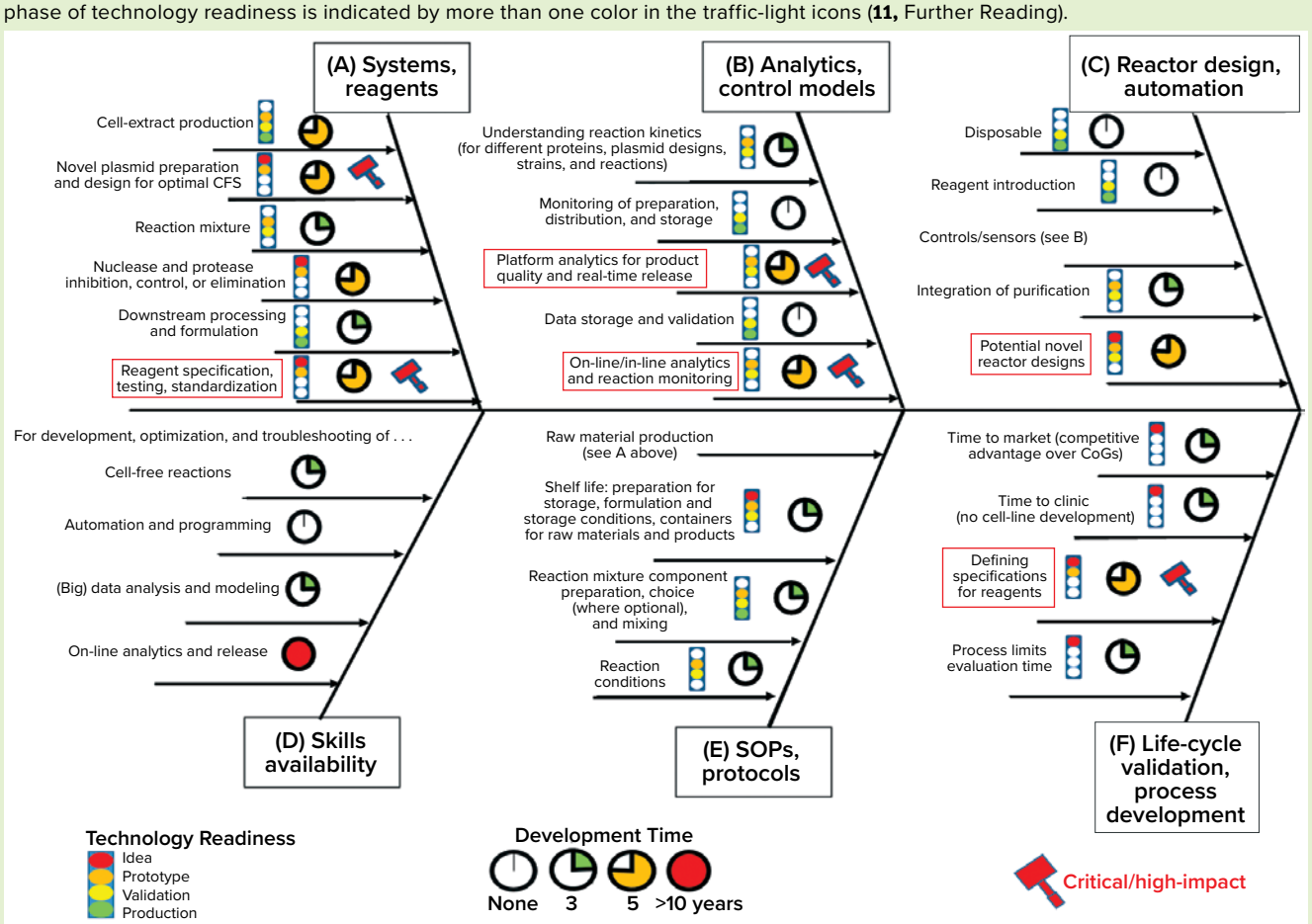
To date, cost of goods (CoG) reduction has not been a primary driving factor for the uptake of CFS, and it is unlikely to be so in the future. Indeed, analysis conducted within the FTHM Hub suggests that prokaryotic-based CFS currently is more expensive ($/mg) than CHO-cell–based processes, although only by a factor of about two (**25**). With protein biologics designed for small groups of patients, the route to reduced costs no longer can come from economies of scale, no matter the platform, which gives further impetus to reexamining our manufacturing paradigms. Some evidence suggests that at personalized or orphan-medicine scales, the economics may favor CFS (**10**). With its raw material components produced at scale, the goal can be minimal customization from product to product. It also is anticipated that costs will decrease substantially over time — e.g., by use of modified or alternative source strains (**26, 27**), with cell extracts currently representing a substantial proportion of the overall costs (**25**).

Challenges to CFS Uptake

Although the development rate for this technology is a function of both commercial and technical factors (**24**), here we focus only on the technical and regulatory barriers to uptake. First we consider the possibility that the same technical outcomes can be achieved through a competing technology. Then we assess the size of the technical obstacles to be overcome for arriving at a CFS-based device that will be acceptable to regulators for distributed manufacturing.

Competing Technologies for Stratified Medicines Production: Cell-based synthesis has been and continues to be the highly effective format of choice for most companies making or wishing to make recombinant proteins. We do not anticipate CFS to supplant cell-based manufacture entirely. However, as highlighted above and summarized in Table 1, CFS does offer a number of distinct advantages, particularly for distributed manufacturing. The costs, time, and resources required for development of a larger range of treatments, each with a naturally limited market, imply that stratified medicines will require streamlined development and manufacture. But can

Figure 3: This Ishikawa ("fishbone") diagram shows technical, organizational, and logistical challenges in achieving the objective of "making an injectable biotherapeutic without cells, using automated, on-demand, localized manufacture," as identified by the academic and industrial participants in the FTHM Hub (see Acknowledgments). Red boxes highlight critically important areas. An intermediate phase of technology readiness is indicated by more than one color in the traffic-light icons (**11,** Further Reading).



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existing technologies or alternatives in development match CFS?

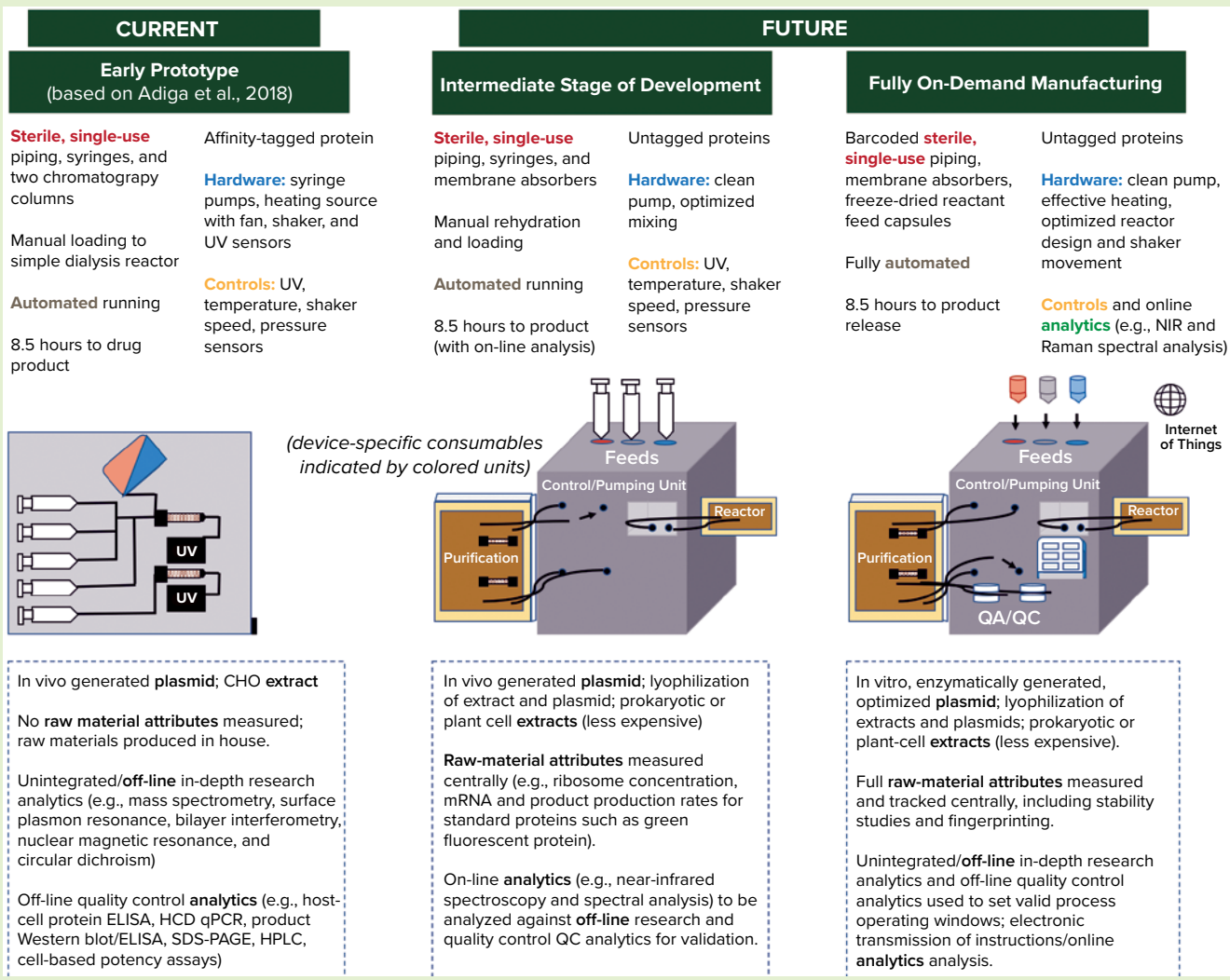
Such alternative approaches include

- decentralized manufacture using the currently predominant cell-based batch manufacturing
- intensified cell-based manufacture (e.g., continuous bioprocessing (**28, 29**))
- modular manufacturing units (**30**)
- use of alternative host organisms with simplified requirements (**31–33**)
- use of transient transfection approaches (**10**).

Within cell-based production, only intensified production with alternative hosts offers the possibility of relatively simple automation (without expensive robotics for handling cell culture processes/equipment) that might enable localized production of biotherapeutics (**33**). However, the complexity of the equipment and expertise required to control for variability in cellular responses must be resolved to achieve full automation (**34, 35**). For cell-based systems, maintenance of cell banks also will remain necessary, complicating distribution and storage of reagents.

A continuous production platform based on *Pichia pastoris,* an expression system capable of product secretion with low levels of harvest host-cell proteins (HCPs), has been proposed and trialed under the auspices of the Biologically-Derived Medicines on Demand (Bio-MOD) project of the US Defense Advanced Research Projects Agency (DARPA) (**33**). The bio-MOD program also funded a CFS-based tool for "battle-field medicine" (**36**). Considerable efforts have gone into engineering human-like glycosylation in the *P. pastoris* host, but it has yet to be fully resolved (**37**). Therefore, similar technologies also are in development using mammalian cells, which do require longer timelines with a more complex host cell but also offer an established method for direct human-like glycosylation of protein products. It could be argued that host types that produce aglycosylated proteins, either in cell-free or cell-based systems, might enable greater product quality control through subsequent enzymatic glycosylation (**38**).

Figure 4: Roadmap with development stages of prototype devices envisioned to achieve production of "an injectable biotherapeutic without cells, using automated, on-demand, localized manufacture," starting with a system based on that described by Adiga et al. (**5**), with additions from Pardee et al. (**47**) and Crowell et al. (**33**). Subsequent intermediate prototype and fully on-demand manufacturing are based on the technical development areas identified in Figure 3 and our understanding of which ones can and need to be addressed first. Device schematics and descriptions indicate distributed elements of the technology. Text below the schematics (in dashed boxes) indicates raw material and quality control (QC) development activities that might be completed elsewhere (e.g., at a centralized facility). Green text indicates the first appearance of a feature retained in the final device.



Other technologies exist or are in development for modification of cell-lines and acceleration of cell-line development for biotherapeutics. However, even at an accelerated rate, cell-line development and cell culture cannot match the development and production timelines achieved with CFS. It should be acknowledged that currently the CoG and (crude lysate) prepurification HCP/DNA levels would be higher for material produced in a CFS platform than for the proposed *P. pastoris*–based continuous platform. Nonetheless the potential of CFS remains clear set against its competitors.

Identifying Key Technical Hurdles: Having established an understanding of the current landscape and potential for cell-free technology, the FTHM Hub workshop team developed a problem statement. Its purpose was to help elucidate what would be required from a CFS platform to make it a viable manufacturing technology for stratified medicine. Here is the statement agreed upon: "What are the technical challenges to making an injectable biotherapeutic without cells, using automated, on-demand, localized manufacture?" Figure 3 summarizes the outcomes of this exercise.

As highlighted by the red boxes in Figure 3, the concerns of the industrial partners with an interest in CFS largely related to quality control and regulatory release. The challenge for CFS in this respect relates to

- complexity of the raw material (particularly cell extract) and characterization of its CQAs
- limited understanding of which raw-material attributes will influence reaction performance, which is plasmid/product-specific and therefore requires a process development strategy
- a need to establish appropriate in-process monitoring technologies
- a quality by design (QbD) approach to validation for the device to enable drug-substance release (**39**).

The importance of cell extract as a raw material is highlighted by the fact that two companies discussed in the case studies above stress their use of in-house developed and proprietary extracts and protocols. Furthermore, published literature dealing with the

question of extract preparation is extensive (although less so at the industrial scale) but diverse and still moving toward an accepted best practice (**40**). Even at laboratory scale, the sensitivity of cell-extract qualities to the production process is poorly understood, and such processes require systematization and rationalization to achieve robustness (**11, 41**). This issue was recently referred to by the CPI, which claims to have developed a scalable and simplified process for lysate production to address the issue (**42**).

After production of raw materials, CFS-reaction robustness and the connection between measured process parameters and final-product quality will need to be understood and ensured. The expectation is that tight control of raw-material attributes and characterization of the reaction's response to critical process parameters (CPPs, e.g., temperature and pH), will bring cell-free reactions close to the reproducibility seen in many small-molecule and enzymatic processes (without undue influence of stochastic or unidentified elements). To gather such understanding will require a combination of high-throughput experiments, for which CFS reactions are well suited, with appropriate modeling and control (**43**), such as through hybrid metabolic models like those proposed for cellular systems (**44**).

Finally, plasmid preparation (**10, 11**) and design (**45, 46**) are fields suggested by published literature and our own experience as likely to have a large impact on CFS titers.

Toward the Roadmap

Figure 4 shows a series of prototypes for a compact, localized CFS platform. On the left, the first prototype is based on systems described by Adiga et al. (**5**), Pardee et al. (**47**), and Crowell et al. (**33**). The latter developed a system using continuous cell-based production, however that overlaps with technology developments required for stratified processing, whether cell-based or cell-free, such that both technologies can be informed by each other (**43**). And recently reported collaborative work between CureVac and Tesla to generate an automated mRNA production platform also represents a technology from which a CFS platform could draw (**48, 49**).

Figure 4 also clarifies the importance of supporting technologies, particularly related to the centralized supply of tightly controlled raw materials and a system for on-board product quality measurement, control, and release. Centralized raw-material production will enable investment in robustness and reproducibility for economies of scale, with specifications set independently of a specific product and development of analytical techniques aided by consistency in raw materials.

Growing scientific understanding of disease pathology and the individuality of the treatment responses brings great potential for a revolution in biotherapeutic efficacy and safety. However, that will require a new paradigm in biotherapeutic production to sit in parallel with existing biomanufacturing of blockbuster drugs. The speed of development for this technology will depend on multiple factors — e.g., government interest/support, identification of early commercial applications, and vested interests in established technologies (**24**) — in order to secure sufficient investment to overcome the technical challenges highlighted herein. These technical challenges are considerable, as is the coordination and cross-disciplinary working that will be required to overcome them. But the history of MAb processes based on CHO cells shows what

can be achieved by a concerted effort when the potential of a technology is recognized. We hope that our analysis will inspire further debate, collaboration, and research toward realizing the potential of CFS in biomanufacturing.

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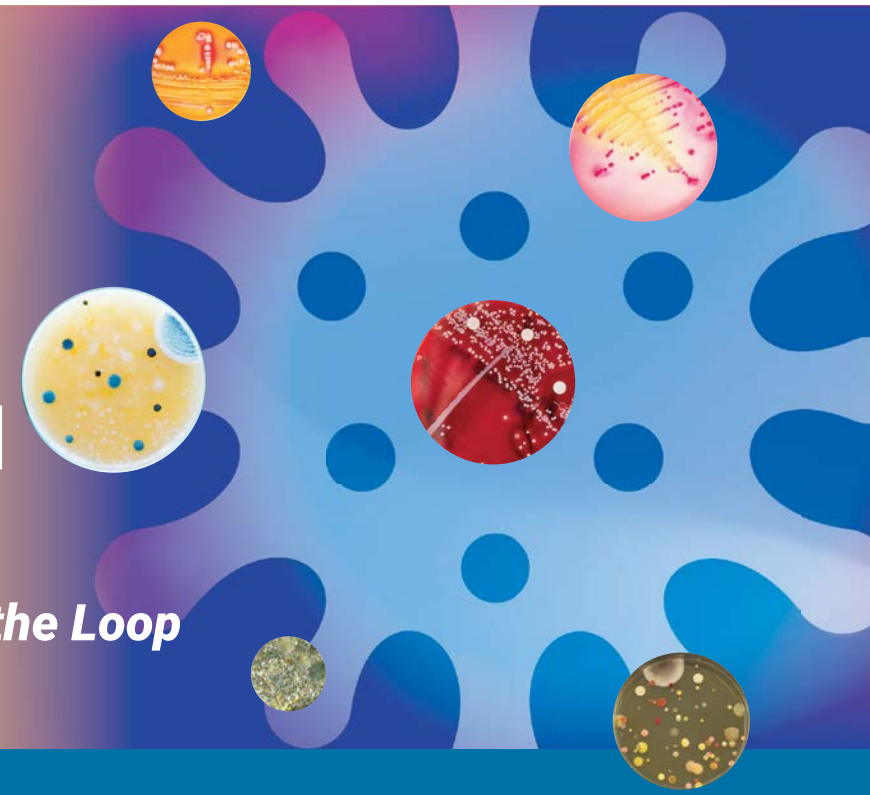

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