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## Cell-free biomanufacturing

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Since its early development and use to decipher the genetic code, *in vitro* or 'cell-free' systems have been used as an important research tool to understand biochemical mechanisms and metabolic pathways. More recently, due to important engineering advances the technology is rapidly becoming a biomanufacturing platform for protein therapeutics, vaccines, enzyme biocatalysts, fuels, and commodity chemicals. Here we report recent applications and advances in the cell-free biomanufacturing field and the potential of this emerging approach.

### Addresses

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### Introduction

Biomanufacturing has greatly improved humanity's quality of life from its earliest uses in fermentation which helped sterilize and preserve beverages to today's \$200 billion USD business that mass produces antibiotics, vaccines, protein therapeutics, fuels,

commodity chemicals, and biocatalysts (Evaluate-Pharma World Preview; URL: <http://www.evaluate.com/PharmaWorldPreview2017>). To achieve this success engineers have engaged in a great 'tug-of-war' with microorganisms; where engineers fight against an organism's natural programming that judiciously allocates energy toward maintenance and replication instead of producing the engineer's desired product. This has inspired the development of new technology to 'cut-the-rope' (Figure 1) by engineering *in vitro* or 'cell-free' systems that use the biomachinery harvested from the lysate of disrupted cells. The engineer now has unprecedented access to, control of, and real time knowledge about the biochemical machinery and reactions used in biomanufacturing without the constraints of keeping the cell alive and working against its metabolic programming [1,2].

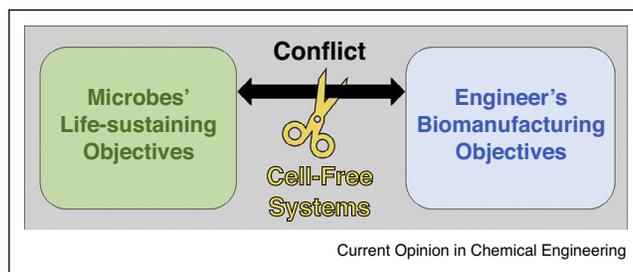
General advantages of Cell-free Biomanufacturing include:

- **Control/Access:** Biomanufacturing can be optimized to temperatures and chemical environments that may be cytotoxic [3]. Absence of a cell wall simplifies optimization by facilitating direct monitoring and the addition or removal of cofactors and enzymes (which are continuously produced and degraded at varying rates by living cells).
- **Scalability:** Biomanufacturing can be easily performed at very small scales (microliter) to very large scale (1000 L) with consistent results from the same stock pile of reagents [4] which facilitates large-scale responses to emergent threats as well as small, customized, on demand orders (personalized medicines).
- **Stability:** Biomanufacturing reagents can be stored in a shelf-stable lyophilized format that increases portability for 'just-add-water', distributed, on-site use [5–7].
- **Speed:** Time-sensitive products (medicines/vaccines against a pandemic threat) can be rapidly produced from active ribosomes, and the other necessary cellular machinery, previously harvested [8<sup>\*\*</sup>,9].

Cell-free biomanufacturing also come with its own set of challenges which include:

- **Risk:** Although Sutro Biopharma has, with FDA permission, begun clinical testing of a cell-free produced antibody-drug conjugate, no biopharmaceutical that

Figure 1



A simple representation of cell-free systems cutting the conflict or 'tug-of-war' between the microorganism's objective and the biomanufacturing engineer's objective.

uses this manufacturing approach has been approved [10<sup>\*</sup>]. In addition, biocatalysts and commodity chemicals have also not traditionally been produced in this manner.

- Standardization:** Independent research labs have developed both competing and complementary technology using various cell extract preparation procedures, different genetic strains, and different microorganisms/cells (*Escherichia coli*, yeast, CHO, Rabbit, Wheat germ, HeLa, and most recently human blood) [11,12] with significantly different results and costs (see Supplementary material for analysis). Biomachinery content and activity can also vary significantly within the same strain during different stages of growth, using different fermentation media, and potentially with different fermenters. In addition, 'cell-free' biomanufacturing can be performed in microfluidic [13,14], batch [4], fed-batch [6], and semi-continuous/continuous exchange [15,16] operational formats. Thus, while cell-free processes offer broad design flexibility, optimization for each product may be necessary.
- Cost:** Cell-free biomanufacturing is currently more expensive due to the energy and cofactors often required to activate the biomachinery after harvesting it from cells. This is particularly true for mammalian-based and the *E. coli*-based PURE system (each component is individually purified and then recombined). However, *E. coli*-based lysate systems that are inexpensive to produce and require less complex purification procedures are becoming cost-competitive [17–19].
- Post-translational Modifications:** While there have been some advancements with mammalian-based systems, it remains difficult to keep the endoplasmic reticulum and Golgi apparatus intact and functional for glycosylation in high-yielding cell-free systems [20].

As challenges increasingly are addressed, cell-free systems are being developed for more and more creative and impactful applications within the medical and chemical industries (Table 1). These applications include

production of vaccines, personalized and general biologics (protein therapeutics), next-generation biologics, biocatalysts, fuels, commodity chemicals, and even biosensors (Figure 2).

### Cell-free biomanufacturing: protein Therapeutic proteins

As some of the earliest cell-free protein synthesis systems were used to decode the fundamental genetic code, it is somewhat fitting that half a century later the tool is being increasingly repurposed as a protein biomanufacturing platform [2] (Figure 3). The achievements are predominantly due to advances that reengineer the system to produce more complex human proteins and to improve protein production yields (2 mg/mL in batch format [21] and 6 mg/mL in fed batch format [6,22] while lowering reagent costs for lysate-based systems (as low as \$1–\$2/mL for the least expensive batch system based on *E. coli* lysate when prepared in house and thus approaching \$1/mg protein; other systems based on CHO, HeLa, wheat germ, and purified *E. coli* proteins cost \$500–\$900/mL; see Supplementary for details). As large protein molecules can be reliably synthesized only with biomanufacturing, and therapeutic proteins have particularly high value (\$100/mg–\$1000/mg) [23], cell-free biomanufacturing of therapeutic proteins has received the most intense focus.

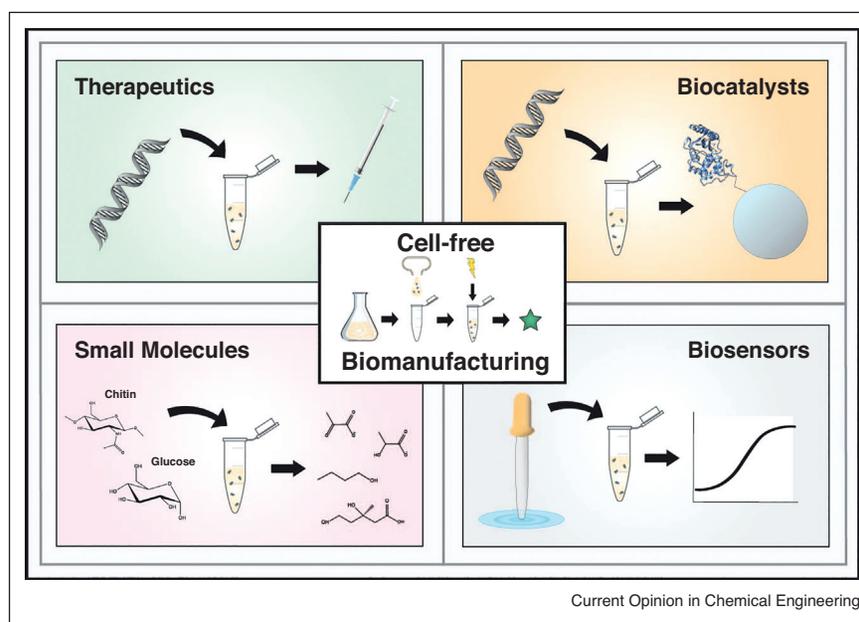
For reference, *in vivo* expression is typically more cost effective (~\$0.01/mg protein reagent cost when using *E. coli* [24]), however cell-free systems provide several advantages that justify this larger cost, which is still 2–3 orders of magnitude below the potential sales price of some therapeutic proteins. For example, cell-free system reagents can now be sterilized, lyophilized, and stock-piled which could enable large scale production of thousands of doses of a desired therapeutic protein in as little as one day [4,7]. A classic example for the need of such speed results from the varying effectiveness of the yearly influenza vaccines (which has varied from 10% to 60% effectiveness over the past 15 years with influenza remaining the 8th leading cause of death in the US). (CDC; URL: <https://www.cdc.gov/flu/professionals/vaccination/effectiveness-studies.htm>) The main reason for varying effectiveness is the challenge of predicting the three or four influenza strains that will be most prevalent nine months before the influenza season to allow enough time for manufacturing the vaccine. In contrast, stock-piled cell-free reagents could be used to produce high titers of vaccine in less than two weeks. Rapid biomanufacturing is also important for responding to unforeseen pandemic threats (which are considered one of humanity's greatest threats) as well as enabling personalized cancer therapeutics and medicines. Researchers have demonstrated the potential of cell-free technology for these applications by the rapid production of a personalized cancer vaccine [25]. Cell-free systems have also been

Table 1

## Advantages and challenges of cell-free biomanufacturing

Advantages	Challenges
Reduced toxicity concerns — no cells to keep alive	FDA has not approved a therapeutic produced using cell-free biomanufacturing
Direct access and control — no cell wall to prevent monitoring and inhibit transport to the biomanufacturing environment	Cell-free biomanufacturing reagents are more expensive than <i>in vivo</i> production and are prohibitive for some systems
Scalability from the microliter to the 1000 liter scale using the same reagents	Post-translational modifications are difficult to sustain in high-yielding cell-free systems
Shelf-stable stability and portability with lyophilized reagents	Cell-free systems could theoretically last much longer and produce more product than current systems and more engineering is needed to realize this potential
Rapid production in response to health crisis/disease or market demands	Acceptable product costs may require optimization of biomanufacturing reactor formats and lysate production

Figure 2



Biomanufacturing application of cell-free systems.

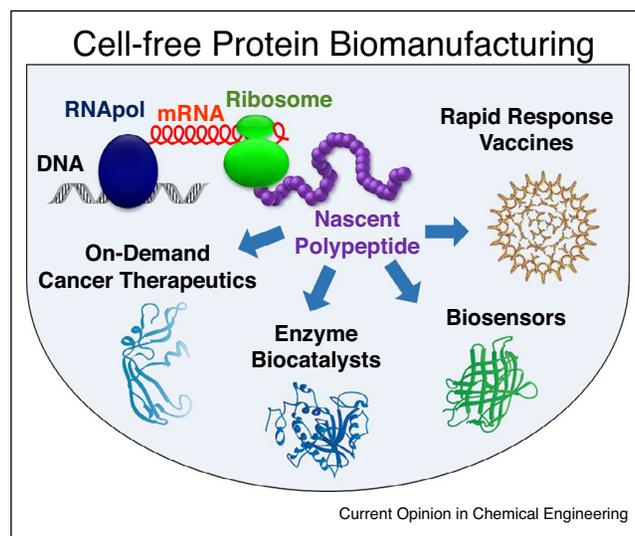
recently explored to produce other vaccines, including an influenza vaccine [26], as well as virus-like particles which have particular promise as a vaccines scaffold [3]. The use of cell-free systems for non-canonical amino acid incorporation also shows benefits to *in vivo* systems by removing transport rate limitations and enabling direct optimization of component concentrations.

In general, rapid, on-demand therapeutic production from sterile, shelf-stable, stockpiled reagents [7] shows great promise to break the cold-storage chain and potentially enable biosimilars (protein therapeutics off patent) to be produced at low cost nearly anywhere in the world. These advances could greatly expand the availability of life-saving therapeutics to humanity. To realize this goal,

researchers have developed suitcase-size automated technology [27<sup>••</sup>], simplified purification requirements [28], and very recently created an endotoxin-free *E. coli*-based cell-free system which could potentially increase the safety of protein therapeutics, simplify therapeutic purification, and reduce quality control needed [29]. For personalized medicine applications, researchers have recently demonstrated a cart-sized system that can synthesize a protein from digital sequence (combines DNA synthesis tools with cell-free protein expression) [30<sup>•</sup>].

The technology also shows great promise for the production of the next generation of protein therapeutics. For example, PEGylated (attachment of polyethylene glycol) protein therapeutics are commonly the 2nd generation

Figure 3



Applications of cell-free protein biomanufacturing.

therapeutic due to enhanced pharmacokinetics and stability. Cell-free researchers have recently demonstrated how integrating computation and cell-free screening can be successfully used to produce and screen a library of proteins site-specifically PEGylated at different locations to identify an optimally modified biotherapeutic [31<sup>\*</sup>]. The best candidate, having been produced with cell-free expression, could then be simply transferred to a cell-free enabled biomanufacturing platform. A number of researchers have also demonstrated how the open cell-free system enables facile incorporation of non-canonical amino acids, which expands sequence space and could enable the development of better protein therapeutics in the future [32–35]. Overall, recent developments illustrate the bright future of cell-free systems for the manufacturing of protein therapeutics [10<sup>\*</sup>].

### Enzyme biocatalysts

Beyond therapeutics, cell-free systems have also been utilized to manufacture enzyme biocatalysts. The continued reduction in cell-free reagent costs is particularly important in this area where enzyme biocatalysts, in general, have unit values only a small fraction of those for protein therapeutics. However, a niche exists for specialized biocatalysts and for more stable and recyclable enzymes which greatly increases their unit value [36]. Researchers have recently demonstrated that cell-free systems can be used to rapidly produce enzymes that can then be site-specifically attached to superparamagnetic carriers. These catalytic nanoparticles can then be screened to determine the optimal location of attachment for long-term stability and reuse [37]. In addition, the non-canonical amino acid incorporation capabilities can

be used in the future to expand sequence space and find faster, more stable, and more specific enzymes [38,39]. An additional feature that could be exploited is the rapid and scalable production capability of cell-free systems. This could enable a faster biomanufacturing response to market conditions, where cell-free systems could quickly produce enzymes for a different, more valuable biocatalytic pathway in response to daily market changes.

### Protein-based biosensors

A variety of cell-free-based biosensors has been developed in the past few years [40–44] where a protein reporter provides a signal by fluorescence (GFP), chemiluminescence (luciferase), or color (beta-galactosidase) based on regulation at transcription, translation, or even protein folding stages that responds to the presence of a specific analyte [45]. Toxins [40], pathogenic bacteria [44], viruses [41], and endocrine disrupting molecules [42,46] have been effectively detected in this way with nL and nL size cell-free protein synthesis-based biosensors. While these sensors were primarily designed for in-lab tests due to the need to freeze cell-free reagents, the ability to lyophilize cell-free systems [47] opens the door to portable use at low cost due to the small quantities of reagents required at the nL reaction scale [41]. Paper-based diagnostics are especially attractive for biosensors using cell-free protein synthesis [48,49].

### Cell-free biomanufacturing: small molecules

In addition to classical protein synthesis, cell-free systems are gaining momentum as small molecule biomanufacturing platforms as detailed in many recent reviews [50,51]. Briefly, lysate-based cell-free technology has been expanded beyond protein synthesis to other biochemical pathways [52]. Here genetic engineering requirements for metabolic engineering can be circumvented by mixing and matching different lysates enabling enrichment of limiting chemicals and enzymes [5]. In addition, there is no risk of an intermediate building up and being toxic to the cells, and the lack of cell wall, which provides direct monitoring and the ability to directly add, subtract, and control small molecule and protein concentrations, simplifies metabolic pathway optimization [53]. Because of these advantages, several small molecule pathways and products have been produced including: butanol [52], pyruvate [54], lactate [55], mevalonate [5], butanediol [56], and even natural products [8<sup>\*\*</sup>,57–59]. Small molecule manufacturing is particularly promising with cell-free systems engineered to use lower cost reagents such as glucose [60] and even chitin [54] as energy sources where cost is a major driver in bulk production of commodity, specialty, and fine chemicals.

### Future directions of cell-free biomanufacturing

With the exponential growth of research in cell-free systems [61], the future of cell-free biomanufacturing

has increasing promise. While diverse applications and products have been demonstrated by academic and industrial labs, the primary impediments to widespread use are FDA approval as a therapeutic production platform and the cost for lower margin biocatalyst, biosensing, and small molecule synthesis applications. Even for higher margin therapeutic applications, in-house preparation of cell-free reagents is necessary (see Supplementary). While, the *E. coli* lysate-based system appears to be the most cost-effective approach, it has so far been unable to perform some post-translational modifications such as human-form glycosylation. Thus progress is needed in engineering post-translational modifications such as glycosylation (with some initial progress reported by Jewett, DeLisa, and Mrksich [62–64,65\*\*]) or engineering CHO, HeLa, human blood, wheat germ, or other eukaryotic systems to be both higher yielding and available at a lower cost (e.g. less expensive reagents, streamlined preparation procedures). Decreasing the price further is especially important for industrial enzyme biocatalyst manufacturing and the majority of small molecule synthesis applications due to the very low margins of these commodities. Engineering to extend the life-time of these systems is also needed, which could be done by identifying and preventing the buildup of molecules that poison the cell-free system (such as phosphate as achieved by Kim *et al.* [66]) as well as inhibiting or removing nucleic acid and protein degradation pathways. Biosensors employing cell-free synthesis may soon gain commercial importance as the nL quantities required greatly lowers the cost and cell-free systems can be sterilized to essentially eliminate the release risk of a genetically modified organism.

As the above challenges are overcome, cell-free systems could greatly speed up customized biomanufacturing by integrating DNA synthesis with protein production for digital-to-protein synthesis in a matter of hours [30\*]. There is also potential for automated machine learning as integrating simulation with cell-free systems in optimization cycles has been reported [31\*]. In addition, cell-free systems are having transformational impacts in education, where relatively few biology classroom demonstrations have been practical. Very recently, lyophilized cell-free systems have been deployed to create safe, hands-on biomanufacturing classroom demonstrations [67,68]. Lyophilized shelf-stable cell-free systems are perhaps among the most exciting innovations because they break cold-storage chain requirements and enable the use of cell-free biomanufacturing potentially anywhere in the world. Thus cell-free systems are opening the door to distributed, real-time responses to humanity's biomanufacturing needs.

### Conflict of interest statement

Nothing declared.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.coche.2018.10.003>.

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