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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.

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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 (EvaluatePharma 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].

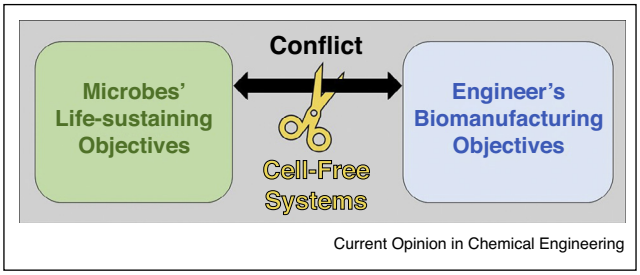
General advantages of Cell-free Biomanufacturing include:

- **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••,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•]. 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 biomachinary 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.

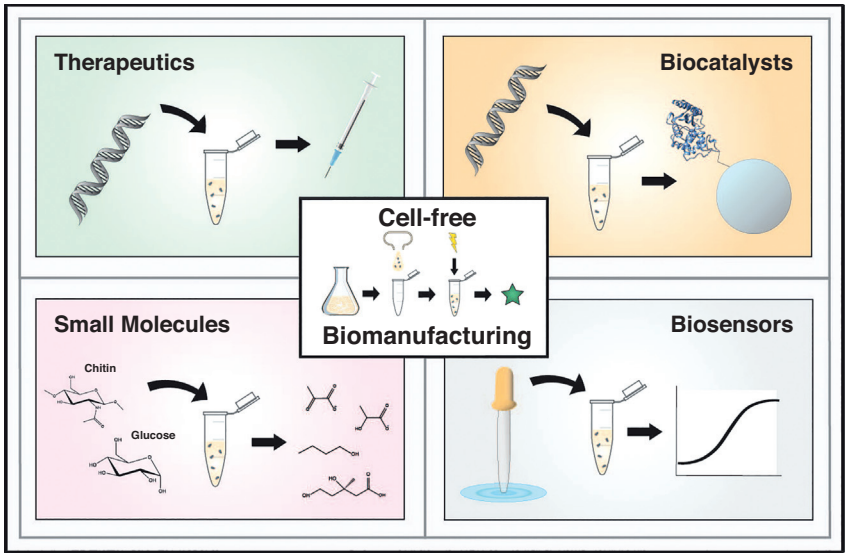
For reference, *in vivo* expression is typically more cost effective (~$0.01/mg protein reagent cost when using *E. coli* [24]), however cell-free systems provide several advantages that justify this larger cost, which is still 2–3 orders of magnitude below the potential sales price of some therapeutic proteins. For example, cell-free system reagents can now be sterilized, lyophilized, and stockpiled 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, stockpiled 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 biomanufacutring reagents are more expensive than *in vivo* 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 that 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



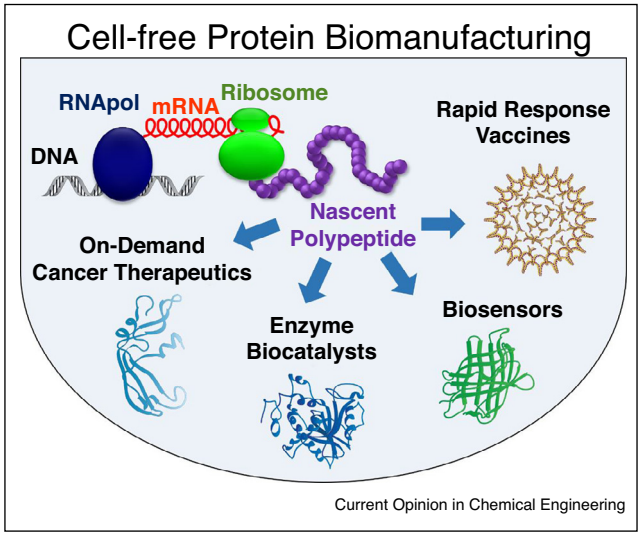
Biomanufacturing application of cell-free systems.

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••], 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•].

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•]. 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•].

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 mL 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••,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.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Swartz JR: **Transforming biochemical engineering with cell-free biology**. *AIChE J* 2012, **58**:5-13.

2. Carlson ED, Gan R, Hodgman CE, Jewett MC: **Cell-free protein synthesis: applications come of age**. *Biotechnol Adv* 2012, **30**:1185-1194.

3. Bundy BC, Swartz JR: **Efficient disulfide bond formation in virus-like particles**. *J Biotechnol* 2011, **154**:230-239.

4. Zawada JF, Yin G, Steiner AR, Yang J, Naresh A, Roy SM, Gold DS, Heinsohn HG, Murray CJ: **Microscale to manufacturing scale-up of cell-free cytokine production–a new approach for shortening protein production development timelines**. *Biotechnol Bioeng* 2011, **108**:1570-1578.

5. Dudley QM, Anderson KC, Jewett MC: **Cell-free mixing of Escherichia coli crude extracts to prototype and rationally engineer high-titer mevalonate synthesis**. *ACS Synth Biol* 2016, **5**:1578-1588.

6. Salehi ASM, Smith MT, Bennett AM, Williams JB, Pitt WG, Bundy BC: **Cell-free protein synthesis of a cytotoxic cancer therapeutic: onconase production and a just-add-water cell-free system**. *Biotechnol J* 2016, **11**:274-281.

7. Smith MT, Bennett AM, Hunt JM, Bundy BC: **Creating a completely "cell-free" system for protein synthesis**. *Biotechnol Prog* 2015, **31**:1716-1719.

8. •• Pardee K, Slomovic S, Nguyen PQ, Lee JW, Donghia N, Burrill D, Ferrante T, McSorley FR, Furuta Y, Vernet A *et al.*: **Portable, on-demand biomolecular manufacturing**. *Cell* 2016, **167**:248.
Demonstrates on-demand cell-free production of vaccine antigens, antibody analogs, antimicrobial peptides, as well as small-molecule biosynthesis.

9. Sullivan CJ, Pendleton ED, Sasmor HH, Hicks WL, Farnum JB, Muto M, Amendt EM, Schoborg JA, Martin RW, Clark LG *et al.*: **A cell-free expression and purification process for rapid production of protein biologics**. *Biotechnol J* 2016, **11**:238-248.

10. • Ogonah OW, Polizzi KM, Bracewell DG: **Cell free protein synthesis: a viable option for stratified medicines manufacturing?** *Curr Opin Chem Eng* 2017, **18**:77-83.

Provides insightful review of key issues regarding biotherapeutic supply chain development and regulation in the context of cell-free biomanufacturing.

11. Gan R, Jewett MC: **A combined cell-free transcription-translation system from Saccharomyces cerevisiae for rapid and robust protein synthesis**. *Biotechnol J* 2014, **9**:641-651.

12. Burgenson D, Gurramkonda C, Pilli M, Ge X, Andar A, Kostov Y, Tolosa L, Rao G: **Rapid recombinant protein expression in cell-free extracts from human blood**. *Sci Rep* 2018, **8**:9569.

13. Timm AC, Shankles PG, Foster CM, Doktycz MJ, Retterer ST: **Toward microfluidic reactors for cell-free protein synthesis at the point-of-care**. *Small* 2016, **12**:810-817.

14. Georgi V, Georgi L, Blechert M, Bergmeister M, Zwanzig M, Wustenhagen DA, Bier FF, Jung E, Kubick S: **On-chip automation of cell-free protein synthesis: new opportunities due to a novel reaction mode**. *Lab Chip* 2016, **16**:269-281.

15. Li J, Wang H, Kwon YC, Jewett MC: **Establishing a high yielding Streptomyces-based cell-free protein synthesis system**. *Biotechnol Bioeng* 2017, **114**:1343-1353.

16. Stech M, Quast RB, Sachse R, Schulze C, Wüstenhagen DA, Kubick S: **A continuous-exchange cell-free protein synthesis system based on extracts from cultured insect cells**. *PLoS One* 2014, **9**:e96635.

17. Calhoun KA, Swartz JR: **Energizing cell-free protein synthesis with glucose metabolism**. *Biotechnol Bioeng* 2005, **90**:606-613.

18. Shrestha P, Smith MT, Bundy BC: **Cell-free unnatural amino acid incorporation with alternative energy systems and linear expression templates**. *New Biotechnol* 2014, **31**:28-34.

19. Jewett MC, Calhoun KA, Voloshin A, Wuu JJ, Swartz JR: **An integrated cell-free metabolic platform for protein production and synthetic biology**. *Mol Syst Biol* 2008, **4**:10.

20. Gurramkonda C, Rao A, Borhani S, Pilli M, Deldari S, Ge X, Pezeshk N, Han TC, Tolosa M, Kostov Y *et al.*: **Improving the recombinant human erythropoietin glycosylation using microsome supplementation in CHO cell-free system**. *Biotechnol Bioeng* 2018, **115**:1253-1264.

21. Caschera F, Noireaux V: **Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-free transcription-translation system**. *Biochimie* 2014, **99**:162-168.

22. Klammt C, Schwarz D, Loehr F, Schneider B, Doetsch V, Bernhard F: **Cell-free expression as an emerging technique for the large scale production of integral membrane protein**. *FEBS J* 2006, **273**:4141-4153.

23. Ecker DM, Jones SD, Levine HL: **The therapeutic monoclonal antibody market**. *mAbs* 2015, **7**:9-14.

24. Chrast L, Chaloupkova R, Damborsky J: **Gram-scale production of recombinant microbial enzymes in shake flasks**. *FEMS Microbiol Lett* 2018, **365** http://dx.doi.org/10.1093/femsle/fnx265.

25. Kanter G, Yang J, Voloshin A, Levy S, Swartz JR, Levy R: **Cell-free production of scFv fusion proteins: an efficient approach for personalized lymphoma vaccines**. *Blood* 2007, **109**:3393-3399.

26. Lu Y, Welsh JP, Swartz JR: **Production and stabilization of the trimeric influenza hemagglutinin stem domain for potentially broadly protective influenza vaccines**. *PNAS* 2014, **111**:125-130.

27. •• Adiga R, Al-adhami M, Andar A, Borhani S, Brown S, Burgenson D, Cooper MA, Deldari S, Frey DD, Ge X *et al.*: **Point-of-care production of therapeutic proteins of good-manufacturing-practice quality**. *Nat Biomed Eng* 2018, **2**:675-686 http://dx.doi.org/10.1038/s41551-018-0259-1.

Achieves on-demand manufacture and continuous purification of several biotherapeutics using lyophilized and reconstituted CHO cell lysate in a portable device.

28. Fan Y, Miozzi J, Stimple S, Han T-C, Wood D: **Column-free purification methods for recombinant proteins using self-cleaving aggregating tags**. *Polymers* 2018, **10**:468.

29. Wilding KM, Hunt JP, Wilkerson JW, Funk PJ, Swensen RL, Carver WC, Christian LM, Bundy BC: **Endotoxin-free E. coli-based cell-free protein synthesis: pre-expression endotoxin removal approaches for on-demand cancer therapeutic production**. *Biotechnol J* 2018 http://dx.doi.org/10.1002/biot.201800271.

30. • Boles KS, Kannan K, Gill J, Felderman M, Gouvis H, Hubby B, Kamrud KI, Venter JC, Gibson DG: **Digital-to-biological converter for on-demand production of biologics**. *Nat Biotechnol* 2017, **35**:672-675.

Integrates process automation with biotherapeutic production. Virus particles, antibody polypeptides, and whole bacteriophages are produced in a fully automated biomanufacturing unit from digital DNA sequence input.

31. • Wilding KM, Smith AK, Wilkerson JW, Bush DB, Knotts TA, Bundy BC: **The locational impact of site-specific PEGylation: streamlined screening with cell-free protein expression and coarse-grain simulation**. *ACS Synth Biol* 2018, **7**:510-521.

Integrates simulation and cell-free protein synthesis to screen for optimal sites for protein PEGylation. Demonstrates the impact of simulation and the potential for integrating machine learning toward optimizing cell-free products.

32. Oza JP, Aerni HR, Pirman NL, Barber KW, ter Haar CM, Rogulina S, Amrofell MB, Isaacs FJ, Rinehart J, Jewett MC: **Robust production of recombinant phosphoproteins using cell-free protein synthesis**. *Nat Commun* 2015, **6**:8168.

33. Albayrak C, Swartz JR: **Cell-free co-production of an orthogonal transfer RNA activates efficient site-specific non-natural amino acid incorporation**. *Nucleic Acids Res* 2013, **41**:5949-5963.

34. Salehi ASM, Smith MT, Schinn SM, Hunt JM, Muhlestein C, Diray-Arce J, Nielsen BL, Bundy BC: **Efficient tRNA degradation and quantification in Escherichia coli cell extract using RNase-coated magnetic beads: a key step toward codon emancipation**. *Biotechnol Prog* 2017, **33**:1401-1407.

35. Zimmerman ES, Heibeck TH, Gill A, Li XF, Murray CJ, Madlansacay MR, Tran C, Uter NT, Yin G, Rivers PJ *et al.*: **Production of site-specific antibody-drug conjugates using optimized non-natural amino acids in a cell-free expression system**. *Bioconjug Chem* 2014, **25**:351-361.

36. Grimaldi J, Collins CH, Belfort G: **Towards cell-free isobutanol production: development of a novel immobilized enzyme system**. *Biotechnol Prog* 2016, **32**:66-73.

37. Wu JCY, Hutchings CH, Lindsay MJ, Werner CJ, Bundy BC: **Enhanced enzyme stability through site-directed covalent immobilization**. *J Biotechnol* 2015, **193**:83-90.

38. Martin RW, Des Soye BJ, Kwon Y-C, Kay J, Davis RG, Thomas PM, Majewska NI, Chen CX, Marcum RD, Weiss MG *et al.*: **Cell-free protein synthesis from genomically recoded bacteria enables multisite incorporation of noncanonical amino acids**. *Nat Commun* 2018, **9**:1203.

39. Schinn S-M, Bradley W, Groesbeck A, Wu JC, Broadbent A, Bundy BC: **Rapid in vitro screening for the location-dependent effects of unnatural amino acids on protein expression and activity**. *Biotechnol Bioeng* 2017, **114**:2412-2417.

40. Pellinen T, Huovinen T, Karp M: **A cell-free biosensor for the detection of transcriptional inducers using firefly luciferase as a reporter**. *Anal Biochem* 2004, **330**:52-57.

41. Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, Ferrante T, Ma D, Donghia N, Fan M *et al.*: **Rapid, low-cost detection of zika virus using programmable biomolecular components**. *Cell* 2016, **165**:1255-1266.

42. Salehi ASM, Tang MJS, Smith MT, Hunt JM, Law RA, Wood DW, Bundy BC: **Cell-free protein synthesis approach to biosensing hTRβ-specific endocrine disruptors**. *Anal Chem* 2017, **89**:3395-3401.

43. Jang YJ, Lee KH, Yoo TH, Kim DM: **Complementary cell-free translational assay for quantification of amino acids**. *Anal Chem* 2017, **89**:9638-9642.

44. Wen KY, Cameron L, Chappell J, Jensen K, Bell DJ, Kelwick R, Kopniczky M, Davies JC, Filloux A, Freemont PS: **A cell-free biosensor for detecting quorum sensing molecules in P.**

aeruginosa-infected respiratory samples. *ACS Synth Biol* 2017, **6**:2293-2301.

45. Soltani M, Davis BR, Ford H, Nelson JAD, Bundy BC: **Reengineering cell-free protein synthesis as a biosensor: biosensing with transcription, translation, and protein-folding**. *Biochem Eng J* 2018, **138**:165-171 http://dx.doi.org/10.1016/j.bej.2018.06.014.

46. Salehi ASM, Yang SO, Earl CC, Shakalli Tang MJ, Porter Hunt J, Smith MT, Wood DW, Bundy BC: **Biosensing estrogenic endocrine disruptors in human blood and urine: a RAPID cell-free protein synthesis approach**. *Toxicol Appl Pharmacol* 2018, **345**:19-25.

47. Hunt JP, Yang SO, Wilding KM, Bundy BC: **The growing impact of lyophilized cell-free protein expression systems**. *Bioengineered* 2017, **8**:325-330.

48. Slomovic S, Pardee K, Collins JJ: **Synthetic biology devices for in vitro and in vivo diagnostics**. *PNAS* 2015, **112**:14429-14435.

49. Pardee K, Green AA, Ferrante T, Cameron DE, DaleyKeyser A, Yin P, Collins JJ: **Paper-based synthetic gene networks**. *Cell* 2014, **159**:940-954.

50. Wilding KM, Schinn S-M, Long EA, Bundy BC: **The emerging impact of cell-free chemical biosynthesis**. *Curr Opin Biotechnol* 2018, **53**:115-121.

51. You C, Zhang YHP: **Cell-free biosystems for biomanufacturing**. In *Future Trends in Biotechnology*. Edited by Zhong JJ. 2013:89-119.

52. Karim AS, Jewett MC: **A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery**. *Metab Eng* 2016, **36**:116-126.

53. Karim AS, Heggestad JT, Crowe SA, Jewett MC: **Controlling cell-free metabolism through physiochemical perturbations**. *Metab Eng* 2018, **45**:86-94.

54. Honda K, Kimura K, Ninh PH, Taniguchi H, Okano K, Ohtake H: **In vitro bioconversion of chitin to pyruvate with thermophilic enzymes**. *J Biosci Bioeng* 2017, **124**:296-301.

55. Ye X, Honda K, Sakai T, Okano K, Omasa T, Hirota R, Kuroda A, Ohtake H: **Synthetic metabolic engineering-a novel, simple technology for designing a chimeric metabolic pathway**. *Microb Cell Fact* 2012, **11**:120.

56. Kay JE, Jewett MC: **Lysate of engineered Escherichia coli supports high-level conversion of glucose to 2,3-butanediol**. *Metab Eng* 2015, **32**:133-142.

57. Kakkar N, Perez JG, Liu WR, Jewett MC, van der Donk WA: **Incorporation of nonproteinogenic amino acids in class I and II lantibiotics**. *ACS Chem Biol* 2018, **13**:951-957.

58. Goering AW, Li J, McClure RA, Thomson RJ, Jewett MC, Kelleher NL: **In vitro reconstruction of nonribosomal peptide biosynthesis directly from DNA using cell-free protein synthesis**. *ACS Synth Biol* 2017, **6**:39-44.

59. Toogood HS, Ni Cheallaigh A, Tait S, Mansell DJ, Jervis A, Lygidakis A, Humphreys L, Takano E, Gardiner JM, Scrutton NS: **Enzymatic menthol production: one-pot approach using engineered Escherichia coli**. *ACS Synth Biol* 2015, **4**:1112-1123.

60. Korman TP, Opgenorth PH, Bowie JU: **A synthetic biochemistry platform for cell free production of monoterpenes from glucose**. *Nat Commun* 2017, **8**:15526.

61. Smith MT, Wilding KM, Hunt JM, Bennett AM, Bundy BC: **The emerging age of cell-free synthetic biology**. *FEBS Lett* 2014, **588**:2755-2761.

62. Schoborg JA, Hershewe JM, Stark JC, Kightlinger W, Kath JE, Jaroentomeechai T, Natarajan A, DeLisa MP, Jewett MC: **A cell-free platform for rapid synthesis and testing of active oligosaccharyltransferases**. *Biotechnol Bioeng* 2018, **115**:739-750.

63. Kightlinger W, Lin L, Rosztoczy M, Li W, DeLisa MP, Mrksich M, Jewett MC: **Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases**. *Nat Chem Biol* 2018, **14**:627-635.

64. Martin RW, Majewska NI, Chen CX, Albanetti TE, Jimenez RBC, Schmelzer AE, Jewett MC, Roy V: **Development of a CHO-based cell-free platform for synthesis of active monoclonal antibodies**. *ACS Synth Biol* 2017, **6**:1370-1379.

65. •• Jaroentomeechai T, Stark JC, Natarajan A, Glasscock CJ, Yates LE, Hsu KJ, Mrksich M, Jewett MC, DeLisa MP: **Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery**. *Nat Commun* 2018, **9**:2686.
Enable*E. coli* cell-free biomanufacturing of glycoproteins using *E. coli* cell lysates enriched in oligosaccharyltransferases and lipid-linked oligosaccharides.

66. Kim DM, Swartz JR: **Prolonging cell-free protein synthesis with a novel ATP regeneration system**. *Biotechnol Bioeng* 1999, **66**:180-188.

67. Huang A, Nguyen PQ, Stark JC, Takahashi MK, Donghia N, Ferrante TC, Dy AJ, Hsu KJ, Dubner RS, Pardee K *et al.*: **BioBits™ Explorer: a modular synthetic biology education kit**. *Sci Adv* 2018, **4** http://dx.doi.org/10.1126/sciadv.aat5105.

68. Stark JC, Huang A, Nguyen PQ, Dubner RS, Hsu KJ, Ferrante TC, Anderson M, Kanapskyte A, Mucha Q, Packett JS *et al.*: **BioBits™ Bright: a fluorescent synthetic biology education kit**. *Sci Adv* 2018, **4** http://dx.doi.org/10.1126/sciadv.aat5107.