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Escherichia coli-based cell-free extract development for protein-based cancer therapeutic production

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ABSTRACT Cell-free protein synthesis has been around for decades but it has never been close to becoming a robust tool for the production of biotherapeutic agents. In this review, we focus on how *Escherichia coli*-based cell-free protein synthesis can be modified in various ways to produce challenging, complex anticancer biotherapeutics. Here we report progress in extract preparation and its relation to cell-free cancer research. The future prospects of cell-free technology and its potential in various areas of cancer therapeutics production are also highlighted.

KEY WORDS: *cell-free protein synthesis, cell extract, cancer research, anticancer biotherapeutics*

Introduction

From 1991 to 2012 the cancer death rate dropped by 23 percent constituting nearly 1.7 million lives saved (Siegel *et al.*, 2015). Much of this success could be contributed to the advances in targeted therapies such as Monoclonal antibody therapeutics (Sanchez-Garcia *et al.*, 2016, Sommerfeld and Strube, 2005) and anticancer peptides (Boohaker *et al.*, 2012, Tyagi *et al.*, 2015). Still, cancer remains the second leading cause of death in the United States and is projected to overtake heart disease in the next few years (Group, 2013) with a projected 1,685,210 new cancer cases and 595,690 deaths in 2016 (Siegel *et al.*, 2015).

The development of targeted oncological therapies in the field of protein biologics has revolutionized our ability to treat cancer. To date, all FDA-approved anticancer protein biologics have been produced *in vivo* (Sanchez-Garcia *et al.*, 2016) where 69% of those have been produced recombinantly in *E. coli* (Sanchez-Garcia *et al.*, 2016). As successful as *in vivo* production has been and continues to be, there are still many drawbacks to the closed, transport-limited *in vivo* environment including 1) inability to produce cytotoxic proteins at high yields, 2) transport inhibition of non-natural components, 3) a walled in environment that complicates direct *in situ* monitoring, control, and dynamic optimization of required reagents (e.g cofactors, redox, translation elements), and 4) a crowded environment that can inhibit the correct folding of complex proteins (Smith *et al.*, 2014c, Swartz, 2006, Swartz, 2012). The open, non-living environment provided by cell-free technology overcomes these limitations allowing the production of cytotoxic proteins (Salehi *et al.*, 2016, Smith *et al.*, 2012), unnatural amino acid (uAA) incorporation (Smith *et al.*, 2014c, Zimmerman *et al.*, 2014), and the rapid synthesis of personalized medicines (Kanter *et al.*, 2007).

In this review, we will discuss the promise of *E. coli*-based cell-free extract development for protein-based cancer therapeutic production. We will discuss the advances that have allowed cell-free protein synthesis (CFPS) to become the exponentially expanding field that it is (Smith *et al.*, 2014c). CFPS advances have been detailed in a number of broad-reaching reviews (Rosenblum and Cooperman, 2014, Smith *et al.*, 2014c, Swartz, 2012); in this paper we will focus our discussion to a few particularly poignant advances in extract development as well as a few emerging and important technologies in anticancer research in which cell-free technology may be particularly impactful. We hope to review these ideas in an effort to give the reader a broader prospective of the past, present, and future of extract development and cancer therapeutic production.

Cell extract for cell-free protein synthesis

Extract preparation

Previously, robust and active cell extracts for cell-free protein synthesis (CFPS) required particular equipment that was costly and labor intensive. This inhibited entrance into the field and slowed the rate of progress. Fortunately, our lab and other researchers introduced the utilization of basic equipment commonly found in biotechnology labs such as incubator shakers and sonicators to produce active cell extract (Kwon and Jewett, 2015, Shrestha *et al.*, 2012). This has increased participation in the field and is promoting

Abbreviations used in this paper: CFPS, cell-free protein synthesis; uAA, unnatural amino acid.

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a widespread use of CFPS in biotechnology.

Potentially any organism could be used to produce the cell extract for CFPS and when selecting a source one should consider the biochemical nature of the protein and downstream applications (Katzen *et al.*, 2005). However, *E. coli* is most commonly used because it is inexpensive, simple to ferment, rapidly produces protein, and is commercially available (Carlson *et al.*, 2012). Also, only a few steps are required to prepare *E. coli*–based extract for CFPS as illustrated in Fig. 1. There are many resources, including videos, available describing protocols and methods of performing CFPS (Spirin and Swartz, 2008, Sun *et al.*, 2013, Swartz *et al.*, 2004). Below we highlight advances in the field.

Strain selection

The first step to prepare cell extract is to choose the right strain. Applications often define which strain should be used or should be engineered. The common strain that our lab and others use is *E. coli* BL21 Star ™ (DE3) (Kim *et al.*, 2006, Smith *et al.*, 2014b). This strain is a generic strain with T7 expression system and mutation in RNaseE gene, which promotes the high mRNA stability and the rapid mRNA production necessary for high protein yields. The strain is particularly attractive as it is commercially available for all users.

There are a number of engineered strains for CFPS reported in literature which improve the protein production yield or have specific purpose (Calhoun and Swartz, 2006, Hong *et al.*, 2013, Knapp *et al.*, 2007). Dr. James Swartz at Stanford University has led this effort and has engineered an *E. coli* strain to better control the redox potential in CFPS by deleting the gene coding for glutathione reductase (Gor), and adding hemagglutinin tag to the thioredoxin reductase (TrxB) gene (Knapp *et al.*, 2007). These two reductases complicate controlling the redox potential in favor of disulfide bond formation and their removal enabled better production of disulfide bond containing proteins. Extract from this strain has the ability to use the glucose as an energy source and requires less iodoacetamide (IAM) to inhibit reductases. This improves the economics of producing correctly folded disulfide bond containing proteins using CFPS such as secreted mammalian protein and vaccines (Knapp *et al.*, 2007). The Swartz lab has also developed other *E. coli* strains with deletions to prevent the use of amino acids by metabolic pathways other than protein synthesis. Strains have been developed with nuclease deletions/modifications to facilitate the stabilization and use of linear DNA to template cell-free protein production (Calhoun and Swartz, 2006, Michel-Reydellet *et al.*, 2004, Michel-Reydellet *et al.*, 2005). Additional efforts by other labs include the development of a genomically recoded organism where 321 UAG stop codons in *E. coli* MG1655 were substituted with the UAA stop codon and the release factor 1 gene was deleted (Lajoie *et al.*, 2013). CFPS with extract produced from this strain resulted in improved production yields of proteins containing unnatural amino acids encoded by the UAG stop codon (Hong *et al.*, 2013).

Cell lysing method

A prior bottleneck in cell extract preparation is lysing cells, due to the higher capital cost of needed equipment. Recent advances decreased the cost associated dramatically especially at laboratory scale. Here we briefly review a few lysing methods.

High-pressure homogenization

Using either a French press or impinge-style homogenizer, suspended cells are passed through several times at high pressure (about 20,000 psi) (Kim *et al.*, 2006). The small eddies created by the high pressure shears the cells apart, exposing the inner soluble

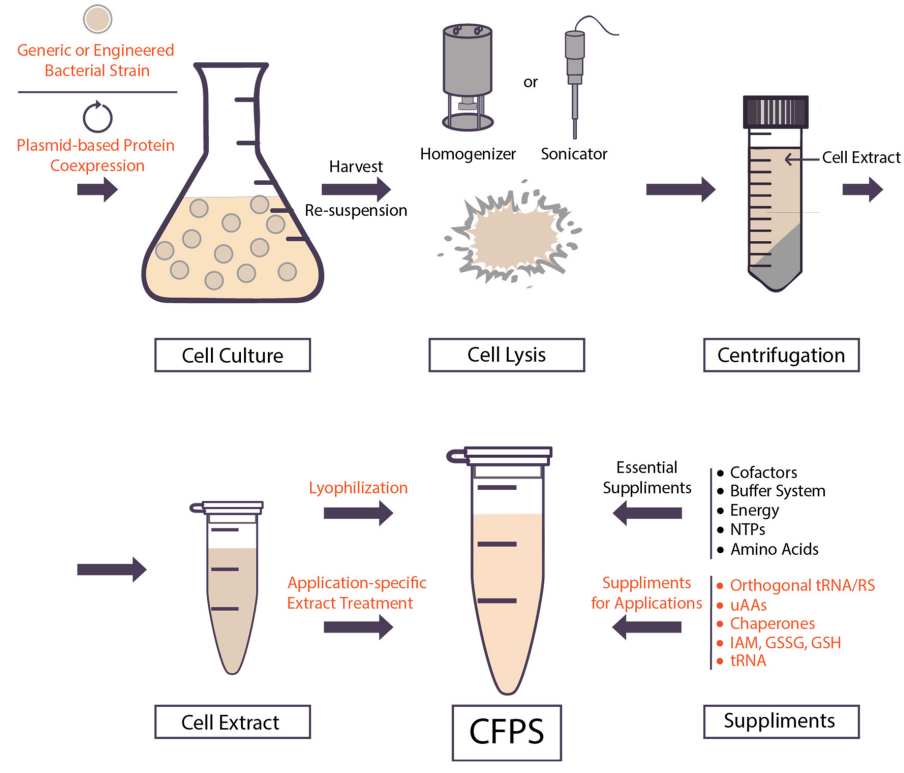

Fig. 1. Schematic diagram of *E. coli* cell-extract preparation and cell-free protein synthesis steps with potential modifications. *Briefly, the appropriate strain is chosen based on the desired application. E. coli cells are cultured and harvested in exponential phase. The cells are lysed using a homogenizer or sonicator and the lysate is clarified by centrifugation to remove the cell wall and genomic DNA. The resulting supernatant is separated as "cell extract" and used in CFPS reaction with additional essential and optional components. The procedures may not be the same for other organisms' cell-free system such as rabbit reticulocytes, wheat germ, or insect cells. Text in red describes variables to engineer depending on the applications. Abbreviations: uAAs, unnatural amino acids; IAM, iodoacetamide; GSSG, oxidized glutathione; GSH, reduced glutathione; NTPs, nucleoside triphosphates; RS, tRNA synthetase.*

material. The homogenized lysate is then centrifuged removing the larger, less-soluble cell wall fragment, and genomic DNA. Homogenization has proved effective in cell extract preparation but requires a significant capital investment. Commercial homogenizers are available with various capacities from the milliliter to the commercial thousand liter scale and are thus ideal for large scale extract preparation.

Sonication

The sonication process requires a smaller capital cost, and the equipment is common to most biotechnology labs. By using the correct protocol, the lysis efficiency is nearly identical to high-pressure homogenization and is the recommended method for small-scale extract preparation. A small probe is placed into a sample of suspended cells, and ultrasonic sound energy is sent through the probe. This ultrasonic energy is converted into mechanical energy that vibrates the solution, causing cavitation that agitates the cells and causes them to rupture (Shrestha *et al.*, 2012). Once again, the insoluble cell material is removed after centrifugation. In addition to the lower capital cost, the ability to perform small volume cell extract preparation, on the order of 100 μl, facilitates high-throughput testing of genetically modified strains for CFPS performance (Kwon and Jewett, 2015).

Other lysing method

There are other methods available to lyse cells, but not all of them are practical for making cell extract. Methods such as freeze-thawing or lysozyme treatment can efficiently lyse cells, but the lysate did not retain protein synthesis capability (Shrestha *et al.*, 2012). Another method developed for lysing prokaryotic cells is using bead vortex mixing. Though this method produces less protein and at greater variability of yield than homogenization and sonication, a simple table-top vortexer and glass beads are all that is needed (Shrestha *et al.*, 2012).

Cell-free scaling: Laboratory to industry

While small scale extract production systems have benefited CFPS research, a scalable, efficient, and reproducible system is required for industrial CFPS production. For scaling up the CFPS volume, the cell extract preparation method including cell culture and lysing are not limiting steps due to decades of industrial practices developed for *in vivo* production of biocatalysts and biologics. However, an initial barrier to industrialize this technology was optimizing the cell-free reaction chamber due to complicated factors such as the need for oxygen for ATP regeneration, and inhibition of CFPS by byproducts such as inorganic phosphate (Caschera and Noireaux, 2015, Swartz, 2006, Swartz, 2012). Sutro Biopharma Company, a pioneer in industrializing the cell-free technology, demonstrated in 2011 the viability of a scaled up cell-free reaction from the microliter to a 100 liter scale with negligible changes in reaction kinetics and protein production yields. At the 100L scale, they consistently produced high yields (700mg/L) of granulocyte macrophage colony-stimulating factor (rhGM-CSF) (Zawada *et al.*, 2011). This was an important advancement in industrializing the cell-free technology.

Essential supplements for cell extract

To perform CFPS and thus activate transcription/translation metabolic process, the cell extract needs a few essential supplements, including energy sources, amino acids, NTPs, cofactors, and a buffer system. The importance of these elements has been studied in some detail (Alexandrov and Johnston, 2014, Spirin and Swartz, 2008, Swartz *et al.*, 2004). Here we briefly discuss energy sources as this element is one of the most expensive components in cell-free reactions.

Energy resources and cost associated

Cell-free transcription and translation requires ATP regeneration throughout the reaction, with a detailed description reported in a previous review (Swartz, 2006). Traditionally this required external enzymes, co-factors, and phosphate donor molecules (Caschera and Noireaux, 2015). Conventionally these systems use acetyl phosphate and acetate kinase, phosphoenolpyruvate and pyruvate kinase, and/or creatine phosphate and creatine kinase with each exploiting a unique metabolic pathway (Kondo *et al.*, 1983, Shrestha *et al.*, 2014).

During the past 15 years, engineering research focused on using alternative energy sources such as glucose and maltose has helped decrease the energy source cost by up to 1000-fold (Calhoun and Swartz, 2005, Jewett *et al.*, 2008, Kim *et al.*, 2011). In addition, the yield of active protein production improved by 2-fold in some cases due to reuse of inhibitory byproduct such as inorganic phosphate to generate ATP. For instance, Caschera et. al. demonstrated a cost-effective ATP regeneration system utilizing hexametaphosphate (HMP) (Caschera and Noireaux, 2015). HMP efficiently fuels protein synthesis when coupled to a carbon source (such as maltose or maltodextrin found in E. coli cell extract) and creates an ATP regeneration system that exploits endogenous enzymes from E. coli extract. Also maltodextrin (Kim *et al.*, 2011), glutamate (Jewett *et al.*, 2008), and glucose (Calhoun and Swartz, 2005) in combination with potassium phosphate monobasic are energy sources, which cost less than a dollar per gram. The development of less expensive and high-yielding energy sources continues to made cell-free systems a more economical alternative to produce potential biotherapeutics.

Extract preparation for specific applications

Due to less complex nature of *E. coli* compared to eukaryotic cells, cell-free expression of exogenous proteins requiring post-translational modifications is often challenging with standard *E. coli* cell extract. Also, for some applications such as site-specific unnatural amino acid incorporation, it is necessary to add new functionalities of cell-free systems. There are many efforts to expand the capability of *E. coli* cell extract by modifying or adding exogenous elements. Here we will mention some of the main advances in extract modification.

Unnatural amino acid incorporation

Unnatural amino acid (uAA) incorporation into proteins has found its pass into many applications specifically in therapeutics. For instance, the PEGylated version of drugs has shown promising improvement in pharmacokinetic properties (Pelegri-O'Day *et al.*, 2014). One of the key technologies to incorporate PEG molecules site-specifically into protein is using uAA incorporation (Deiters *et al.*, 2004). In addition, uAAs can be used site specifically in antibody-drug conjugations for targeted chemotherapeutic cancer treatment (Axup *et al.*, 2012, Zimmerman *et al.*, 2014). There are many advances in incorporating uAAs via the cell-free system, one

particularly promising technology was developed based on the *in vivo* work by the Schultz lab engineering the orthogonal tRNA/tRNA-synthetase pairs to incorporate uAAs at amber stop codons (Wang *et al.*, 2001). This technology was adjusted to cell-free systems to incorporate different uAAs including p-propargyloxyphenylalanine, and p-azido-L-phenylalanine, which can be selectively and covalently conjugated with copper-catalyzed and copper-free click chemistries (Bundy and Swartz, 2010, Goerke and Swartz, 2009). In earlier cell-free systems, synthetases were expressed and purified separately and then added to the cell extract. Optimization of the plasmid expressing the tRNA and tRNA synthetase has enabled sufficient expression during cell growth for extract preparation such that uAAs can be incorporated in CFPS without the need to purify and add supplemental components (Ozawa *et al.*, 2012, Shrestha *et al.*, 2012, Smith *et al.*, 2013, Young *et al.*, 2010). More details on advances in uAA incorporation using cell-free systems and its promise in therapeutic development and production can be found in the recent reviews (Des Soye *et al.*, 2015, Quast *et al.*, 2015).

Disulfide bonds and proper folding

Many therapeutic proteins including mammalian secreted proteins, antibodies, and fusion proteins have a complex structure with multiple disulfide bonds and are challenging to correctly fold in *E. coli*. In contrast, the open nature of cell-free system provides greater accessibilty to optmize the redox potential and chaperone concentration to facilitate correct protein folding. These optimization efforts include 1) using iodoacetamide (IAM) and/or strain development to inactivate/remove reductases for redox potential stabilization in cell extract (Kim and Swartz, 2004, Knapp *et al.*, 2007); 2) adding specified ratios of oxidized glutathione (GSSG) and reduced glutathione (GSH) to achieve the optimal redox poential for disulfide bond formation (Bundy and Swartz, 2011); 3) adding the disulfide bond isomerase DsbC to break incorrect disulfide bridges that form prior to proper folding (Bundy and Swartz, 2011, Knapp and Swartz, 2007). Examples of using these techniques to produce proteins with disulfide bonds include the Qβ virus-like particle (Bundy and Swartz, 2011), *Candida antarctica* Lipase B (Park *et al.*, 2009), and murine granulocyte macrophage-colony stimulating factor (Knapp *et al.*, 2007).

Additional optimization efforts have focused on facilitating protein folding using chaperones and cochaperones. Examples include expressing during cell extract preparation or directly adding the GroEL/GroES chaperones to facilitate correct folding of the industrial enzyme CalB (Park *et al.*, 2009), Fab fragment of a catalytic antibody 6D9 (Jiang *et al.*, 2002), and *E. coli* DapA protein (Shimizu *et al.*, 2005). Also using the endoplasmic reticulum (ER) resident Hsp70 chaperone, BiP, in fusion form with protein trigger factor showed improvement on soluble yield production of secreted eukaryotic proteins (Welsh *et al.*, 2011). In another attempt, amphiphilic polysaccharide nanogels were used as artificial chaperones in CFPS to prevent aggregation of proteins and improve folding properties (Sasaki *et al.*, 2011).

Lyophilized cell extract

The recently developed lyophilization capabililty of *E. coli*-based CFPS reagents is an important tool to expanding the applicability of CFPS technology. Lyophilized extract has been found to maintain ~20% of its protein producing activity after storage at room temperature for 90 days and ~30% of its activity after storage at 4ºC for one year (Smith *et al.*, 2014a). This facilitates facile transportation by avoiding ultralow temperature required for long-term storage of liquid form of extract. It also simplifies stockpiling large quantities for an emergency such as rapid vaccine or biotherapeutics production in response to epidemic disease or bioterrorism. Our lab demonstrated that we can efficiently produce a cytotoxic anticancer therapeutic, onconase, with a year old lyophilized cell extract (Salehi *et al.*, 2016). Also, to create a portable CFPS platform to produce biotherapeutics and specially personalized/portable medicine and diagnostics, we reported how lyophilization can be used to sterilize and decontaminate cell extract without losing protein production capability (Smith *et al.*, 2015). Additionally, a group of researchers recently reported a lyophilized, paper-based, *in vitro* system for synthetic gene network, which can expend the applications of the system into different areas including the clinic and global health (Pardee *et al.*, 2014). They reported the use of this system as a glucose sensor and strain-specific Ebola virus sensor, which demonstrates the capability of the system to be employed in user-friendly manner in diagnostic and biosensing.

CFPS for Cancer research development

The open nature and facile manipulation of the cell-free environment allows for greater control, monitoring, and high-throughput screening techniques for improved protein evolution compared to the *in vivo* method (Carlson *et al.*, 2012, Casteleijn *et al.*, 2013, Smith *et al.*, 2014c, Yan and Xu, 2006). While standard *E. coli*-based cell-free systems lack the ability to perform glycosylation, the potential of using hybrid cell-free lysates and the addition of exogenous components has the promise of overcoming this limitation (Panthu *et al.*, 2015, Zárate *et al.*, 2010). All of these aspects combined with the high toxicity tolerance of CFPS makes this system a compelling platform for rapidly developing, screening, and producing difficult to express anticancer biotherapeutic proteins. As potential anticancer therapeutic treatments are diverse (e.g. chemotherapy (Schnipper *et al.*, 2015), targeted therapy (Sanna *et al.*, 2014), immunotherapy (Untch *et al.*, 2013), photodynamic therapy (Sanna *et al.*, 2014)), we will limit our discussion to a few emerging and important technologies in anticancer research and treatment in which cell-free technology may be particularly impactful.

CFPS for personalized anticancer biotherapeutics

One emerging application in cancer research where cell-free technology is vital is the synthesis of personalized vaccines to more quickly and efficiently treat certain types of cancers. Previous work reported that vaccine proteins for anti-cancer therapeutics could be produced rapidly in *E. coli*-based cell-free systems (Yang *et al.*, 2005). In that study, a complex fusion of GM-CSF and B-lymphocyte Id scFv with different arrangements were produced successfully with CFPS (Kanter *et al.*, 2007). Importantly cell-free reagents can be stockpiled and then scaled for reliable consistent production at the microliter, milliliter, or liter scale. Thus the major limitation becomes the time required for synthesizing the DNA that templates CFPS and custom DNA can now be economically synthesized in as little as a day. While the implementation of customized immune therapy soon after diagnosis on a large scale requires further research and streamlining; simple, rapid and economical

production of personalized anti-cancer therapeutics may soon be practicable using cell-free technology (Kanter *et al.*, 2007).

CFPS for challenging to express cytotoxic anticancer biotherapeutics: Onconae case study

Recently we published a work demonstrating the ability of CFPS to produce a hard to express anticancer biotherapeutic, onconase, which was recently in phase IIIb of clinical trials (Ardelt *et al.*, 2008). Onconase is particularly challenging as it is a tRNase that in its active form degrades the machinery necessary to make it. The main purpose of the work was to show how CFPS can be adapted to produce a cytotoxic protein in completely active form and high yield. There have been many attempts to express and screen mutants of onconase using *E. coli in vivo* system, but most of them were limited to study of two to three mutants at the same time (Hacke *et al.*, 2013, Notomista *et al.*, 1999, Turcotte and Raines, 2008). The reason being aggregation of onconase and inclusion body formation. It is thus time-consuming, order of weeks, to produce the active form of the protein. By using modified CFPS, which was supplemented with tRNA throughout the reaction, onconase was produced at a yield near 2 mg/ml and solubility of more than 95%. The observed anticancer activity of CFPS made onconase was 60 times more than *in vivo* refolded onconase, which is attributed to the inefficiency of the refolding process. Furthermore, the CFPS platform made it possible to directly test onconase anticancer activity against a cancer cell line without the need to purify the protein. Using this system, the time needed to express and test the protein was reduced to one day which is ideal for high-throughput screening. A combination of this modified cell-free method and linear template DNA, which has been studied and engineered by a number of researchers (He and Taussig, 2001, Woodrow *et al.*, 2006, Yang *et al.*, 1980), could facilitate rapid screening for a mutant onconase with increased potency against cancer cells. More information about using linear DNA templates and high-throughput CFPS can be found in recent reviews and papers (Murray and Baliga, 2013, Schinn *et al.*, 2016, Yabuki *et al.*, 2007).

Future prospects: Additional application of CFPS in cancer research

While many proteins of oncological value are produced recombinantly *in vivo* (Sanchez-Garcia *et al.*, 2016) cell-free technologies could contribute to improving the effectiveness and economics of these and future therapeutics. For example, the cell-free technologies of protein evolution with ribosome display can improve the solubility and activity of protein therapeutics (Buchanan *et al.*, 2012, Carlson *et al.*, 2012, Yan and Xu, 2006). Another important technology in oncological therapies is that of anticancer peptides. Anticancer peptides in many cases have higher target specificity, lower intrinsic toxicity, and greater ease of modification compared to full-length proteins used in chemotherapy (Barras and Widmann, 2011, Li and Cho, 2012, Tyagi *et al.*, 2015). Peptides can also be fused to cell penetrating moieties to better target and treat cancer (Barras and Widmann, 2011). Some of these modification schemes include attaching radionuclides, hormones, vaccines, or other drugs to a nascent peptide (Thundimadathil, 2012). One great challenge in the development of clinically viable therapeutic anticancer peptides is improving delivery, minimizing non-specific toxic effects, and a greater understanding of pharmacokinetic properties (Boohaker *et al.*, 2012).

While peptides are traditionally produced by chemical synthesis or *in vivo* via nucleotide sequences, these methods have significant drawbacks in time scale and cost (Lee *et al.*, 2010). Cell-free technologies offer a compelling platform that could be explored to rapidly and more efficiently to study, screen, and produce similar peptides on an industrial level (Lee *et al.*, 2010, Smith *et al.*, 2014c, Swartz, 2006). For example, Lee et. al. developed a strategy for rapid cell-free expression and recovery of multiple peptide molecules (Lee *et al.*, 2010). They optimized peptide production by developing a DNA construct with an enhanced sequence for improved translation efficiency, protease resistance, purification recovery, and cleavage efficiency. Continued engineering of cell-free technologies such as these demonstrates how cell-free systems could be utilized for the automated, rapid production of clinically viable pure proteins and peptides that have significant oncological value.

Conclusion

The open nature of the cell-free system makes this technology highly adaptive for new applications including cancer research. Progress during the last few decades has transformed this technology from simply a research tools to a potential industrial alternative

TABLE 1

ADVANTAGES AND DISADVANTAGES OF USING CELL-FREE SYSTEMS FOR THERAPEUTICS PRODUCTION

Advantages	Disadvantages

• Open Nature
  ▪ Ease of monitoring (Smith *et al.*, 2014b)
  ▪ Ease of optimization (Smith *et al.*, 2014b)
  ▪ No transport limitation (Swartz, 2012)
• Higher toxicity tolerance (Casteleijn *et al.*, 2013)
• Ease of Manipulation
  ▪ uAA incorporation (Zimmerman *et al.*, 2014)
  ▪ Disulfide bond formation (Bundy and Swartz, 2011, Goerke and Swartz, 2008)
  ▪ Folding using chaperones (Spirin and Swartz, 2008)
  ▪ Addition of exogenous materials (Swartz, 2012)
• Fast protein synthesis (Zawada *et al.*, 2011)
• Ability to use linear DNA template (Schinn *et al.*, 2016)
• High-throughput screening (Woodrow *et al.*, 2006, Woodrow and Swartz, 2007)
• Scalable (Zawada *et al.*, 2011)
• Platform for personalized medicines (Kanter *et al.*, 2007)
• Lyophilization capable
  ▪ Ease of stockpiling reagents (Smith *et al.*, 2014a)
  ▪ Ease of transportation (Smith *et al.*, 2014a)
• Sterilization method (Smith *et al.*, 2015)
	• Not yet an FDA-approved Process
	• Higher reagent cost compared to in vivo systems (Swartz, 2006)
	• Largest industrial scale fermentation demonstrated is 100 L (Zawada *et al.*, 2011)
	• Limited protein synthesis life time (typically 3-12 hours in batch format) (Michel-Rydellet *et al.*, 2004)
	• Lack of cell-free specific bioreactor development reported in literatura

for the production of many biotherapeutics. The advantages of this system, as summarized in Table 1, have and will likely continue to be an important answer to producing some hard to express and complex anticancer biotherapeutics. Most importantly, cell-free technology could significantly reduce cancer-therapeutic development and production times and facilitate the realization of personalized cancer therapeutics.

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