Review

BioTechniques

Cell-free protein synthesis: advances on production process for biopharmaceuticals and immunobiological products

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ABSTRACT

Biopharmaceutical products are of great importance in the treatment or prevention of many diseases and represent a growing share of the global pharmaceutical market. The usual technology for protein synthesis (cell-based expression) faces certain obstacles, especially with 'difficult-to-express' proteins. Cell-free protein synthesis (CFPS) can overcome the main bottlenecks of cell-based expression. This review aims to present recent advances in the production process of biologic products by CFPS. First, key aspects of CFPS systems are summarized. A description of several biologic products that have been successfully produced using the CFPS system is provided. Finally, the CFPS system's ability to scale up and scale down, its main limitations and its application for biologics production are discussed.

KEYWORDS:

biologics ● biopharmaceutical ● cell-free protein synthesis ● high throughput ● lyophilization ● on demand

Biopharmaceutical and immunobiological products (also referred to as biologics) are basically recombinant therapeutic proteins obtained from biotechnological processes. In 1982, insulin (Humulin, by Eli Lilly) was the first biotechnological product to be launched commercially [1]. Before this accomplishment, insulin was extracted and purified from dead animal tissues. Thanks to recombinant DNA technology, today a large amount of insulin is globally produced. Recombinant DNA technology has enabled production of many biologically active proteins used in a wide range of treatments and prevention of diseases, such as hematopoietic growth factors, growth factors, hormones, cytokines and the interferon family, blood factors, recombinant enzymes, recombinant vaccines, and monoclonal antibodies [2]. Over the years, biologics have become more target-specific, with fewer side effects.

Biologics represent a growing share of the global pharmaceutical market, totaling US$228 billion in global sales in 2016 [3]. The last biopharmaceutical benchmarks (2018) [1] showed a cumulative sales value of $652 billion from 2014 to 2017, a value that exceeds the reported gross domestic product (GDP) of three quarters of the economies included in the World Bank GDP ranking database. Recently, many of these biologics are losing their patent protection and other exclusivity rights, and thus, many companies are in a competition to produce biosimilars and fight for a share of the market [3]. Biosimilars require less investment in research and development, offering a lower cost alternative to expensive biopharmaceuticals therapies and expanding patient access. Approximately 400 million people worldwide are dependent on such proteins and, often, the treatment lasts for a lifetime. Therefore, development toward these noninnovative versions of such molecules, including improved manufacturing processes, is a compelling need.

Currently, there are three strategies to synthesize therapeutic proteins: chemical synthesis, cell-based expression and cell-free protein synthesis (CFPS) [4]. In the first case, chemical reactions, mass transfer, and the hydrodynamic phenomena that take place inside the reactor were not completely understood in the beginning [5]. More recently, further advances were achieved, and the possibility of online monitoring together with the ability to add reagents directly to the reactor enabled effective real-time process control for further process improvement [5]. However, chemical synthesis has limited application because it is not capable of producing large peptides or proteins [5].

Cell-based synthesis overcame the size limitation of the molecule to be synthesized [4]. The reactions take place inside a cell in the cytoplasm, and reagents can be absorbed and expelled by the membrane, associated with transporters. The concentration of thousands of chemical components is cell-controlled and often changes drastically during the batch process [4]. The processes were developed to optimize the survival of organisms because they oppose overproduction and release of a single product [4,5]. Despite important advances with recombinant DNA technology and metabolic engineering, it is impossible to control the enormous set of reactions within the cells. Formation of inclusion body, degradation of the protein of interest, loss of the DNA template and incompatibility with cytotoxic product production are examples of problems associated with cell-based expression [4–7].

To tackle these issues, a new platform has been explored: CFPS. This review aims to describe this system and the related advances made in the production process for biologic products. First, we present a short historical outline of the development of CFPS and summarize some of its main characteristics. We then describe some biologic products that have been successfully produced using the CFPS system. We conclude by discussing the ability of CFPS to be scaled up and scaled down and its application for biologics production.

CFPS systems

Cell-free systems explore biological processes without using living cells. The cell is lysed, and the cytoplasmic content, full of active biomolecules, is capable of performing many cellular functions, such as transcription and translation [4,5,8]. The system has been used for decades, mainly to study biological functions, and was used by Matthaei and Nirenberg [9] to decipher the genetic code in 1961. In the late 1960s and early 1970s, CFPS was used to elucidate the operons of lactose [10] and tryptophan in *Escherichia coli* [11]. Initially the platform was used only for scientific purposes, but in recent years, propelled by the CFPS system's increasing capacity of rapid and high throughput protein production, the system has attracted interest of pharmaceutical companies for implementation on an industrial scale [12–14].

In CFPS, the absence of cell membrane contributes to protein folding, to the synthesis of difficult-to-express proteins and to high throughput production. Because there is no need to maintain cells alive, all available resources are channeled toward expressing the gene of interest. This feature leads to a simpler purification process because the majority of the protein produced is expressed from the gene of interest. Thus, the loss of target proteins during the purification process is reduced, and higher protein yields can be obtained. In addition, the burden caused in the cell by overexpressing an heterologous and single product is no longer a problem in CFPS system, and, as a result, negative feedback is not of concern [4,5,15]. The open environment allows for the addition and removal of substrates, as well as the online monitoring of the reaction for greater control of the process. It is possible, for instance, to add enzymatic substrates, adjust the DNA template concentration, remove waste molecules, increase cofactor concentrations and add nonnatural reagents [15]. These advantages allow difficult-to-express protein production, common among biopharmaceuticals and immunobiological products. Examples of these proteins are cytotoxic proteins, misfolded proteins and nonsoluble proteins. Cytotoxic proteins are suitable for synthesis in CFPS because the cell is no longer alive, and consequently, the toxicity of the protein is not a problem. Restriction endonucleases, cytolethal distending toxins and human microtubule binding proteins are some examples of proteins that interfere with cellular metabolic pathways and inhibit cell division and therefore are hard to express with high yields in cell-based systems [16]. As an example, Salehi and colleagues [17] reported the rapid production of the difficult-to-express cytotoxic protein onconase in its soluble and active form. The authors pointed out that the open nature of the reaction environment allows for direct and immediate downstream characterization without the need of purification.

Incorrectly folded proteins may either accumulate or be degraded in the cytoplasm of cell-based systems, and host cells become overloaded with an inactive translation product. The CFPS system allows medium supplementation with components that optimize the redox potential and prevent the formation of undesirable disulfide bonds [18]. To control the formation of sulfide bounds, Yin and colleagues [19] report the preparation of a genome engineered *E. coli* extract with a RF1 mutant strain that enhances suppression efficiency during cell-free protein synthesis. The reported extract allows nonnatural amino acids incorporation at previously intractable sites of an IgG1 and at multiple sites in the same polypeptide chain designed for efficient tumor killing. In a similar way, Dopp and Reuel [20] reported the production of an extract from a commercially available *E. coli* strain, the T7 SHuffle strain from New England Biolabs (MA, USA) that allows the production of proteins with efficient disulfide bonds formation. This extract evinced the ability to produce enzymes with more than three disulfide bonds, such as hevamine, endochitinase A and periplasmic AppA. Goerke and Swartz [21] also reported the production of disulfide bonded therapeutic protein at 710 μg/ml, antibody fragment at 230 μg/ml and a vaccine fusion protein at 300 μg/mL using an engineered *E. coli* extract and minimal iodoacetamide concentration to stabilize the oxidizing environment.

Supplementation with chaperones can assist correct folding of membrane proteins, avoiding proteins being confined in an intermediate minimum energy state and encouraging correct formation of multiple natural disulfide bonds [18,22,23]. For proteins that tend to form inclusion bodies, the aggregation occurs mainly due to noncovalent hydrophobic interactions [24]. This is common among membrane proteins, which represent 60% of approved drug targets [16]. The flexibility of cell-free expression allows addition of components that prevent or reduce aggregation, such as polyethylene glycol, polysaccharide nanogel, ethanol, choline and amino acids such as Arg, Pro and Glu [24]. Another strategy recently studied is an *in situ* removable fusion partner, which increases its expression and solubility [25]. Likewise, because there is no cell membrane, cell-free technology bypasses cell culturing steps of *in vivo* methods, accelerating production. In addition, because the protein is not synthesized inside the cell, the environment in which it is formed is less crowded (often 10–20 times more diluted) [24]. This improves protein folding, reduces undesirable reactions and improves diffusion rates.

Several cell hosts can be the origin of cell-free systems, such as bacteria, protozoa, plants, insects and mammals. There are advantages and disadvantages of each expression system. The main difference between prokaryotic and eukaryotic expression systems is the ability to perform posttranslational modifications (PTMs) on *de novo* synthesized molecules [26]. However, the peculiarities go well beyond, and each CFPS system has its own features. To choose the host cell, the protein to be synthesized must be considered. The first CFPS system studied was prepared from *E. coli* extracts. This is an attractive system because it is well established, able to rapidly produce high yields of proteins, is scalable and is cost effective to prepare [5,27]. *E. coli* extract has been extensively studied and developed by many researchers [26–34]. It is the most characterized expression system and has a wide application in therapeutic

production, for example, antibodies as described, vaccines [35] and cytokines [36]. Also, in the diagnostics field, *E. coli* extract have been used to produce highly specific biosensors for Zika and Ebola diagnostics [37]. However, because it does not perform PTMs naturally, eukaryotic cells have been attracting increasing interest [38]. Despite the lower yield and higher costs compared with prokaryotic extracts, eukaryote extracts perform glycosylations, phosphorylations and give correctly folded proteins, also allowing the synthesis of membrane proteins [26,38]. An intermediate alternative that provides a relatively high yield and performs PTMs, correctly folds proteins and can synthesize CG-rich gene-encoding proteins are yeast extracts such as *Saccharomyces cerevisiae* [26,39]. Plant extracts such as wheat germ have been developed to synthesize proteins to discover novel malaria vaccines candidates [40,41]. Mammalian cell extracts have lower productivity; however these cells are genetically closer to human cells and provide proteins similar to humans [26,42]. Chinese hamster ovary (CHO) cells are the most frequently used cells for complex therapeutic protein production, and for this reason, CHO extracts have been studied as CFPS systems. However, proteins produced in nonhuman cells can include non-human proteins which may cause adverse effects during therapeutic use. For this reason, human cell lines, such as HeLa and HEK293 cells, have been explored and one of the benefits is the use of natural codons that facilitate the synthesis of high molecular weight human proteins [26,43]. Recent advances in CFPS technology involving the production of oligosaccharyltransferases [44] and the enrichment of cell extracts with glycosylation components [45] indicate that CFPS extracts from *E. coli* may be adapted to efficiently produce site-specific glycosylation of target proteins [46].

Still, there are some obstacles remaining regarding the CFPS system that need to be addressed. One of them is the short reaction duration in conventional batch reaction because it does not allow for removal of coproducts. For extended protein production, some other reaction formats have been studied, such as continuous-flow cell-free (CFCF), continuous-exchange cell-free (CECF), hollow fiber and a bilayer format [4,47]. Optimization of compartment volume for high-order reaction [48], and development of bioreactors in CECF format by assessing small molecule mass transfer effects [43] are some examples of format improvements that prolong reaction time. Another drawback of cell-free expression is high cost. The main component that increases overall cost of CFPS is the need for energetic molecules (e.g., ATP, GTP and others). However, there are some energy regeneration systems being studied that seek cheaper sources of energy for cell-free reaction [4,49]. In addition, the high yields of the CFPS system may counterbalance its price. Another aspect that contributes to the CFPS system's investment is the unnecessary maintenance of expensive biosafety areas because the system does not use genetically modified organisms (GMOs). In cell-based expression, the production of proteins is achieved by manipulating GMOs into which the DNA coding the desired protein was introduced. This manipulation requires a laboratorial infrastructure with adequate biosafety precautions (biosafety level 2 or higher) and is under specific laws that regiment GMOs management. An industrial biosafety area level 2 dedicated to GMOs costs approximately $7000 US/m^2 to construct and has a maintenance cost of $1100 US/m^2/year [50]. Because CFPS systems do not require GMOs to produce the cell extract, the biosafety area precaution requirements are unnecessary.

The evolution of the CFPS system has been remarkable over the years. With the potential to improve protein synthesis with higher-throughput process, simpler process purification, the possibility to produce difficult-to-express proteins, reduction of the cost, development of novel reaction formats and more studies in process optimization, the system has become feasible for industrial applications for biopharmaceutical and immunobiological products manufacture. Several works have demonstrated the synthesis of therapeutic proteins using CFPS systems for both, high yield and on-demand production.

Biopharmaceutical & immunobiological products in high-throughput production

CFPS platforms have many applications but only recently, with the discovery of the potential CFPS systems for high-throughput production, the system has attracted the attention of pharmaceutical companies. For instance, Jérôme *et al.* [51] compared the synthesis yield of recombinant human bone morphogenetic protein in both cell-based and cell-free expression systems. This glycoprotein induces *de novo* bone formation, and it was synthesized in mammalian cells and mammalian extracts because the PTMs are essential for this protein activity. Recombinant production in stably transfected CHO cells was compared with transient expression in human embryo kidney (HEK) cells and cell-free synthesis in CHO cell lysates. The concentrations achieved were, respectively, 153 pg/ml, 280 ng/ml and 40 ug/ml. Cell-free platform achieved much higher yield within just 3 h, which was only reached with prokaryotic cells incapable of processing PTMs. One of the factors that may cause low yield is negative feedback interactions between recombinant protein and the cells [52]. This is avoided in the CFPS platform, which does not activate inhibitory signaling pathways and proteins do not accumulate within the cells.

Antibodies and monoclonal antibodies are an important class of biopharmaceuticals, widely used for treatment of cancer, autoimmune and inflammatory disorders, due to their high specificity, low immunogenicity and long serum half-life. Typically, antibodies are conventionally synthesized in CHO cell-based transient expression systems, but it can be time-consuming and highly expensive [53]. Therefore, antibodies have been synthesized in CHO extracts, as demonstrated by Martin *et al.* [54]. The authors used CHO extracts commercially available for rapid cell-free expression of monoclonal antibodies. Some modifications were implemented, such as the setup of a proper redox environment for disulfide bridges formation and temporal addition of heavy chain and light chain plasmids for intact monoclonal antibodies production. Testing these modifications, the group achieved a yield of more than 100 mg/l. In comparison with transient or stable transfection in cell-based expression systems, which require at least 7 days, CFPS enabled setup and execution within 2 days. This work also demonstrated that CFPS is well suited for automation; another study evinced the possibility of on-chip automation of CFPS for cytotoxic protein Pierisin production in a microfluidic reaction format [55]. Another work presented

by Stech *et al.* [56] using CHO extracts demonstrated the synthesis of complex antibodies such as IgG and single-chain variable fragment Fc fusion (scFv-Fc). To mimic the environment for protein assembly and folding, antibody genes were fused to an endoplasmic reticulum-specific signal sequence. The researchers determined that signal-peptide induction for antibody polypeptide chain translocation to the microsome lumen is essential for antibody assembly and functionality. They also accomplished a rapid synthesis in batch reaction mode as well as in continuous flow format. In the same year, Thoring *et al.* [57] developed other optimizations in CECF format for difficult-to-express protein synthesis (membrane proteins and single chain variable fragments) and were able to obtain yields up to 980 μg/ml.

Despite the increasing number of studies using CHO extract for therapeutic protein synthesis, they are not the only extract used for antibody production. The trastuzumab IgG, for example, was synthesized in *E. coli* extracts with a yield of up to 1 g/L [58]. Further modification into CFPS reaction described by Cai *et al.* [59] indicated the ability to maintain high throughput production with cost reduction of 95%. This reaction configuration was also tested for the synthesis of IgG, Fab, scFv and other proteins. All of them demonstrated improvements in productivity. Another kind of antibody production has proven possible with theCFPS system are the bispecific 'knob into holes' antibodies, described by Xu *et al.* [60]. These antibodies are capable of recognizing two targets and are potentially a promising research field for oncology therapies and infectious diseases. Glycoproteins also have been successfully synthesized in CFPS using eukaryotic cell extracts such as insect cell extract for erythropoietin production [61], avoiding batch-to-batch variations in its glycoforms.

Immunobiologic products synthesized in CFPS systems include vaccines of virus-like particles (VLPs). VLPs are nanostructures that resemble viral structure and can trigger a high humoral and cellular immune response. A crucial factor related to safety of these vaccines is the lack of viral genomic material, which improves safety during manufacture and administration [62]. VLP synthesis is advantageous in the CFPS system because they are cytotoxic proteins [63]. Botulinum toxin, for instance, is a cytotoxic protein successfully synthesized in the CFPS system, with a yield of up to 1 g/l [64]. Human norovirus (HuNoVs), the most common cause of viral gastroenteritis, is another example of a cytotoxic protein produced in the CFPS system [65]. The first and only vaccine candidate against this virus is in clinical trials by the company Takeda Pharmaceuticals. Its synthesis in several cell-based expression systems has a low yield that is not high enough to meet vaccine demand. Sheng *et al.* [65] developed a cell-free expression of HuNoVs. In this study, the particle could be synthesized in *E. coli* lysate in just 4 h (compared with 50 h in cell-based systems). A cost analysis was performed, and it was concluded that price could be reduced to <5 cents per μl with a yield of 1 g/l. Assuming a similar dosage to the candidate manufactured by Takeda, the cost of a dose would be in the range of $2.50–5.00 [65]. Given the simplification of downstream purification process conferred by the CFPS platform, this technology could be promising to produce norovirus vaccines.

Natural products are also a target for CFPS systems. These products, such as polyketides and nonribosomal peptides, have many biological activities (e.g., antibiotics, immunosuppressive, anticancer) and more than 50% of the new drugs available in the pharmaceutical market are from this class of products [66]. Like other difficult-to-express proteins, natural products are synthesized in cell-based systems but suffer from low yields (caused by metabolic burden that inhibit host cell growth), incorrect folding, the lack of PTMs and the unavailability of precursors in heterologous hosts [66]. Therefore, the CFPS system appears as an alternative for natural products synthesis. An example is the production of gramicidin S which, even without extract optimization, yielded a protein titer higher than the previously reported cell-based expression [67]. Dopp and colleagues [68] report a system able to produce assayable quantities of custom sequence proteins within 24 h from receipt the DNA fragment from vendors. With this system they were able to produce seven fluorescent proteins, three enzymes (including subtilisin), a nanobody and two antimicrobial peptides (BP100 and CA(1-7)M(2-9)). Another product category extracted from natural sources is venoms. There are several venom peptides with therapeutic potential for the treatment of pain, diabetes, multiple sclerosis and cardiovascular diseases [69]. These peptides need to be stable to not degrade in the tissue of the prey or the patient. Also, several PTMs and/or disulfide bonds may be present. The CFPS system could be a strategy to synthesize venom peptides because it can provide a better environment to assemble the correct form of the peptide. Moreover, the CFPS system can overcome the problem of limited venom resources and enable its synthesis at a large scale. This would enhance characterization studies [70] and could create the possibility of using them for pharmaceutical applications, such as crotalphine, which was demonstrated to be a potent analgesic peptide [71]. Thus, both plant enzymes and venom peptides are no longer damaging the environment, and there is no need for extensive animal breeding to explore its natural sources.

The diversity of proteins that have been synthesized in the CFPS platform is impressive (refer to Table 1 for a brief comparison of CHO and *E. coli* extract yields). Yet, many others can be explored in this free-membrane system. The emergence of epidemics in southern hemisphere countries such as Zika, dengue, chikungunya, yellow fever, Ebola and especially the recent COVID-19 pandemic require the development of therapeutics in high yields and at high speed. The CFPS platform meets these requirements and can be used for the production of these new drugs. On the opposite side, the production of therapeutics for orphan diseases, personalized therapies and point-of-care do not necessarily require the use large reactors because they target a small number of patients. Consequently, it is usually not cost effective for pharmaceutical companies to manufacture these medicines. The CFPS system may provide a solution to this problem.

From high-throughput to on-demand production

As detailed above, several proteins have been synthesized in the CFPS system in yield production. However, some biologics do not have a vast consumer market and need to be manufactured at a smaller scale, such as treatment for orphan diseases, personalized medicines

Table 1. Comparison of reports on production of protein by cell-free protein synthesis indicating yield, duration, extract source, production format and protein produced.

Study (year)	Yield up to (mg/l)	Duration (h)	Extract	Format	Protein
Jerôme *et al.* (2017)	0.4	3	CHO	Batch	Recombinant human bone morphogenetic
Martin *et al.* (2017)	1	48	CHO	Semicontinuous	Monoclonal antibodies
Steck *et al.* (2017)	250 and 500	24	CHO	Batch and continuous-exchange	IgG and scFv-Fc
Thoring *et al.* (2017)	980	48	CHO	Continuous exchange	EGFR
Cai *et al.* (2015)	10	14	*E. coli*	Batch	Immunoglobulin
Xu *et al.* (2015)	1300	14	*E. coli*	Batch	Bispecific antibodies
Kanter *et al.* (2016)	10	4	*E. coli*	Batch	GM-CSF
Zawada *et al.* (2011)	7	10	*E. coli*	Batch	GM-CSF
Sheng *et al.* (2017)	620	4	*E. coli*	Batch	HuNoVs

CHO: Chinese hamster ovary.

and point-of-care medical products. These on-demand medicines are not economically feasible for pharmaceutical companies, and they will not be cost effective until there are systems available to enable production of single doses or small-scale, made-to-order products for individual needs that meet regulatory criteria for human use [72]. Current cell-based technologies are only amenable for large-scale productions and need expensive industrial production facilities. The CFPS system creates possibilities to produce biologics in smaller scale and opens the horizons for on-demand production. Besides manufacturing products at point-of-care, the CFPS system can design new manufacturing centers, with 'multipurpose' facilities in a single area with small-scale reactors able to produce different proteins in different extracts according to consumers need. Bundy's research group [73], for instance, report the production of an US FDA-approved L-asparaginase (crisantaspase) in an on-demand, self-stable and low-cost *E. coli* platform device indicating a no-too-distant future when CFPS systems will be used to diagnose, treat and monitor treatment of diseases in a clinical setting.

Streptokinase were synthesized on a small scale using instrumented mini-bioreactors in 2.5 h of reaction by Tran and colleagues [74]. The authors also tested two affinity tags and compared them with a tag-free self-cleaving intein capture technology. The intein purification method provided an increase in product recovery and a gain in activity, compared with conventionally tagged proteins. Another kind of personalized medicines are lymphoma vaccines [35]. With CFPS capacity, it is possible to synthesize proteins from a cloned patient's lymphoma-specific Ig V genes within hours. The traditional production through stable transfected mammalian or insect cell lines can take months to prepare, whereas the CFPS platform can produce it in a matter of days. This may make it possible to use individualized therapy as frontline treatment because the vaccine could be available for use soon after diagnosis and before the use of immunosuppressive chemotherapy [35]. The CFPS system is also amenable for stratified medicines [75], which are medicines to treat preselected patients based on their response to a diagnostic test.

For biologics to become feasible for point-of-care treatments, it is important not only to develop a platform suitable for small and rapid synthesis but also a portable device. For instance, a microfluidic exchange bioreactor was already developed, and protein yields and rates of protein synthesis were improved by changing the flow parameters through the feeder channel and modulating membrane permeability [76]. Pardee and colleagues [77] report the development of a portable, on-site, on-demand manufacturing device for therapeutics and biomolecules. The reported system was able to produce antimicrobial peptides (B1CTcu1, PEP3, CA(1-7)M(2-9), BP100, magainin 2, cecropin P1, cecropin B, Bac7(1-35), tachystatin A1 and opistoporin 1), vaccine antigens (for botulinum, anthrax and diphtheria) and combinatorial antibody analogs (DARPins and nanobodies). Another microfluidic device able to produce proteins in different extracts in 24 h was developed by Sullivan *et al.* [72]. This rapid and end-to-end technology demonstrated potential to be fully automated. A recent work [78] developed a suitcase-like device that offers an automated and portable medicines on-demand production. Using extracts from reconstituted lyophilized CHO cells and a continuous purification system, the researchers could synthesize a variety of proteins in small scale on a timescale of hours. This technology stands out for its end-to-end good manufacturing practice quality manufacturing process and shows potential for FDA approval. Applications are wide and may enable rapid manufacturing of biologics at the point-of-care, ranging from the patient's bedside, doctor's office, local pharmacy, battlefield, endemic diseases treatment, disaster areas or very remote areas.

Extract lyophilization is also an exciting advancement for underdeveloped communities and remote areas, which are challenged by the need for uninterrupted cold storage to maintain stored protein activity. For cell-based expression, maintaining cell viability until use is not practical. A cell-free system overcomes this limitation, allowing a set of reagents to be lyophilized and with no need for cold chain distribution networks [79]. *E. coli* extracts, for instance, were lyophilized and enabled a high-density storage [80]. This allowed a more economical storage, simplified transport conditions and a simple just-add-water protein synthesis system. Some of the applications are pharmacy-on-a-chip microfluidic devices for rapid on-the-site treatment and rapid large-scale vaccine or therapeutic protein production from stockpiled extract. Wheat-germ extracts have also been lyophilized successfully [81]. As described previously, for on-demand therapeutic protein production, extract lyophilization is of paramount importance. An onconase from a lyophilized *E. coli* extract, for

instance, was synthesized by only adding water and the cell machinery was restored. This system remained viable after being stored above freezing for up to 1 year [17].

Conclusion

The CFPS system is a robust and reliable system. Chemical synthesis and cell-based expression have limitations that the CFPS system can overcome. There are some obstacles, but researchers continue working to develop and optimize the platform. As a result, many proteins have been already synthesized in various types of cell extracts, both in high-yield production and on an on-demand scale. Improvements to reduce the costs and to extend reaction time are allowing the CFPS system to become increasingly suitable for industrial use. Lyophilization expands horizons to increase shelf-stability and creates the possibility for portable devices and to take the treatment to the patient's bedside, the doctor's office, the local pharmacy, the battlefield, or areas needing endemic disease treatment, suffering disasters or that are remote.

Nowadays, with the rapid development of next-generation of DNA sequencing, more proteins can be synthesized instead of requiring extraction from its natural resources. Their synthetic production could avoid flora and fauna exploitation and become more environmentally friendly, sustainable and cost effective. Several other new products can be produced in this platform. In addition, the CFPS system can also be used for drug discovery, with microarray analysis, to synthesize natural products for pharmaceutical biomaterials applications, for discovery of CRISPR enzymes, to synthesize PETase, and so on. Another outlook that the CFPS system can offer is decentralization of manufacturing facilities. Because the cell is no longer alive, there is no GMO that hinders its transportation and enables the DNA to be shipped separately from the extract. The CFPS system is a promising technology with vast application fields that may break paradigms and solve health and environmental problems.

Future perspective

CFPS possibilities are rapidly advancing with the development of different cell extracts and techniques that favor the production of complex proteins including those with different PTM requirements and functionalities. Due to its several advantages, it is easy to foresee this technology becoming the standard protein production platform in years to come. However, there are obstacles that need to be addressed before this technology can achieve its full potential. One of these important obstacles is the definition of critical process parameters, the production under Good Manufacturing Practices and compliance with International Council for Harmonisation (ICH) Q5, ICH Q8, ICH Q9 and ICH Q10 guidelines, a requirement for market approval of the synthetized proteins in Europe, the USA, Brazil, Japan, Canada, China and many other countries. This obstacle is currently being addressed by several research groups, such as Rao's group at the Center for Advanced Sensors at the University of Maryland, Baltimore County (USA) and may soon be resolved. The other obstacle that hinders CFPS is the overall cost of its components. The primary issue is the energy system required for protein production, namely the energetic molecules such as ATP. This issue is also being addressed, and new energy regenerating mechanisms are under investigation.

Author contributions

CH Chiba, MC Knirsch, AR Azzoni, AM Moreira and MA Stephano contributed equally to the conception and design of the article, the bibliography acquisition and critically revising the content.

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