BioTechniques Spotlight

eBook: Recombinant protein production

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Sino Biological

eBook: Recombinant protein production

CONTENTS

Introduction

Recent developments in protein engineering technologies, such as cell-free methods, have brought the field into a golden era of innovation, creating new opportunities and ingenious products that can be designed for a host of therapeutic, diagnostic or investigative applications. One group of products that have benefitted from these developments are recombinant proteins, in particular recombinant antibodies.

However, as these recombinant proteins become more complex, the processes utilized in their development can become challenging; varying expression levels can lead to issues of aggregation and toxicity associated with the recombinant product can damage the expression systems designed to produce them, such as mammalian cells. This makes the selection of expression systems and the utilization of effective and reliable techniques essential in the production of recombinant proteins.

In this eBook, we discuss the advantages of recombinant antibody production over more traditional hybridoma-based technologies for the generation of monoclonal antibodies and the expression systems used to create them. Get an overview of the many formats of recombinant antibodies available and their applications. Dive deep into the use of *E. coli* and baculovirus-insect expression systems and the cell-free expression systems available for recombinant protein and antibody production.

With this interactive eBook, you can also hear from two experts on the evolution of recombinant proteins and antibodies, the most exciting developments in their production and some of the most interesting applications of these molecules in the research space.

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Ensuring quick, efficient CRO services for recombinant protein and antibody production

Yutao Lin (right) is the Head of Customer Service and Project Management at Sino Biological. Here, she shares her insight into the advantages of recombinant antibodies over hybridoma-based monoclonal antibodies, the expression systems involved in their production and the upcoming improvements in the development and production of these essential molecules.

Q) Could you provide a brief overview of Sino Biological?

Sino Biological is a leading manufacturer of biological research reagents, offering a comprehensive set of premium quality recombinant proteins, elite antibodies, genes, ELISA kits, and other products. All reagents are developed and produced in-house. The company also offers superior and integrated CRO services, including recombinant protein production, antibody production & development, and bioanalytical assay services.

Powered by its in-house developed technology platforms and proprietary reagents, Sino Biological has rapidly grown into one of the largest protein vendors worldwide. During the past 10 years, the company has successfully developed over 6,500 catalog protein products, and completed thousands of custom protein & antibody development and production projects.

Q) Can you explain what recombinant antibodies are?

Recombinant antibodies are monoclonal antibodies produced rapidly by recombinant DNA technology. Owing to their high specificity, sensitivity, and reproducibility, recombinant antibodies are widely used in human therapeutics, diagnostics, and scientific research. In addition to full-length formats, recombinant antibodies can also be produced in other formats, including chimeric antibodies, bispecific antibodies, and smaller antigen-binding fragments, such as fragment antigen-binding (Fab) and single-chain fragment variable as well as single-domain antibodies. In addition, they can be generated in multiple expression systems, including bacteria, insect cells, yeast, and mammalian cells.

Recombinant antibodies can be produced in mammalian systems using either transient or stable expression approaches. With the help of an efficient transient transfection platform, starting from antibody gene sequences, one could produce 10-100 grams of high-purity antibodies in just a few weeks. The human embryonic kidney (HEK) 293 cell lines have been widely used for transient protein expression due to the fact that expression vector introduction into these cells is highly efficient and results in significant yields of high-quality antibodies.

Q) Can you describe the advantages of recombinant antibody manufacturing?

A recombinant antibody is a type of monoclonal antibody that is generated *in vitro* from a synthetic gene without the need for animal immunization or cell culture. The production involves *in vitro* genetic manipulation. After cloning the antibody gene into an expression vector, this vector is subsequently introduced into an appropriate host cell line for antibody expression. Mammalian cell lines are most commonly used for recombinant antibody production given their high yields and accurate reproduction of antibody bioactivity, although cell lines of bacterial, yeast, or insect origin are also suitable.

Recombinant antibodies can be cloned from any

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species of antibody-producing animals. Once the sequence is cloned, it is possible to modify it for enhanced or altered function – this is one of the major advantages of recombinant antibodies. For example, Fc fragments from a species can be exchanged with another species to produce a chimeric antibody, and the variable chain on the Fab fragment can be mutated to alter binding specificity or affinity. Mutation of cloned antibodies is an alternate way to obtain the desired specificities, which can be implemented, if needed, for diagnostic and therapeutic applications.

In addition to the above advantages of ease and rapid engineering, recombinant antibodies have several advantages when compared to hybridoma-based systems for producing monoclonal antibodies. The first is the increased reliability and reproducibility, due to the fact that recombinant antibody gene sequences are known and fully characterized, guaranteeing a long-term supply. Perhaps most importantly, recombinant technology decreases antibody production time to just a few weeks in most cases. The fast production and the diversity of the resulting antibody products constitute the second key advantage. Finally, unlike traditional methods for antibody production, recombinant approaches avoid the need to use animals.

Q) What can the use of a baculovirus-insect cell system provide that other cell systems cannot?

Insect cells are versatile expression hosts for a range of recombinant proteins. The accurate three-dimensional protein folding capability and relatively high culture density of insect cells resulting in considerable final yields make them excellent choices for the expression of complicated intracellular proteins and virus proteins. In addition, proteins that would normally be toxic to mammalian cells may be manufactured in insect cells. They may also be used for the manufacture of therapeutic recombinant antibodies or proteins. In 2007, Cervarix, an HPV vaccine produced by an insect cell line in the format of virus-like particles (VLPs) was approved for human use.

Besides potentials in therapeutic and vaccine development, highly active proteins produced in insect cells are widely used in a variety of disciplines in biophysics and biochemistry for structure elucidation, drug design, assay establishment, and diagnostic reagent development. Sino Biological employs insect cells extensively for recombinant protein product development and contract research projects.

Q) Can you explain how Sino Biological is improving its research and development capabilities and what advantages this confers to the consumer?

Sino Biological's core strength is its in-house developed mammalian cell culture and recombinant expression platforms. The platforms employ several proprietary reagents that are specially optimized to enhance the expression level of recombinant proteins and antibodies in HEK293 and CHO mammalian cells. Sino Biological's proprietary high-density cell culture technology, combined with proprietary transfection reagents and medium formulations, and high-efficiency expression vectors, maximize the cells longevity and increase the expression success rate, thereby saving clients considerable costs.

Sino Biological offers a variety of different packages for customers who need antibody expression services depending upon their specific requirements. High-throughput gene to antibody production service offers clients a rapid and cost-effective solution to quickly express a large number of antibodies at small scale. The service utilizes our expertise in high-throughput gene synthesis, vector construction and optimized transient antibody expression technology to manufacture high-quality recombinant antibodies with high purity and low endotoxin levels. For larger scale production requirements, Sino Biological's production capacity is considerable, via the use of over 80 bioreactors ranging from 2L to 1500L.

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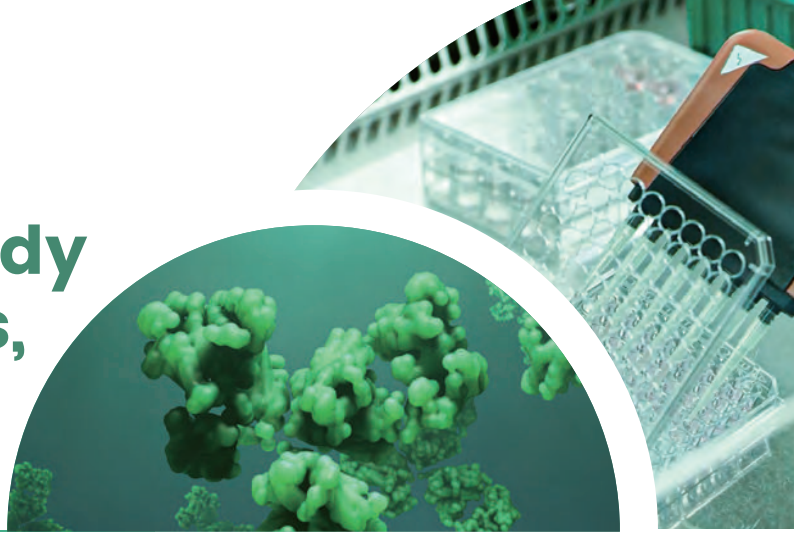
Recombinant Antibody Formats, Expressions, and Applications



Introduction

Recombinant antibodies (rAbs), also known as genetically engineered antibodies, are generated by *in vitro* cloning of the antibody heavy and light chain DNA sequences. Compared to monoclonal antibodies produced using traditional hybridoma techniques, rAbs offer advantages, such as high lot-to-lot consistency, animal-free manufacturing, engineering advancement possibilities, and continuous supply. Given the importance, recombinant antibodies are becoming indispensable tools for basic research, diagnostics, and clinical applications.

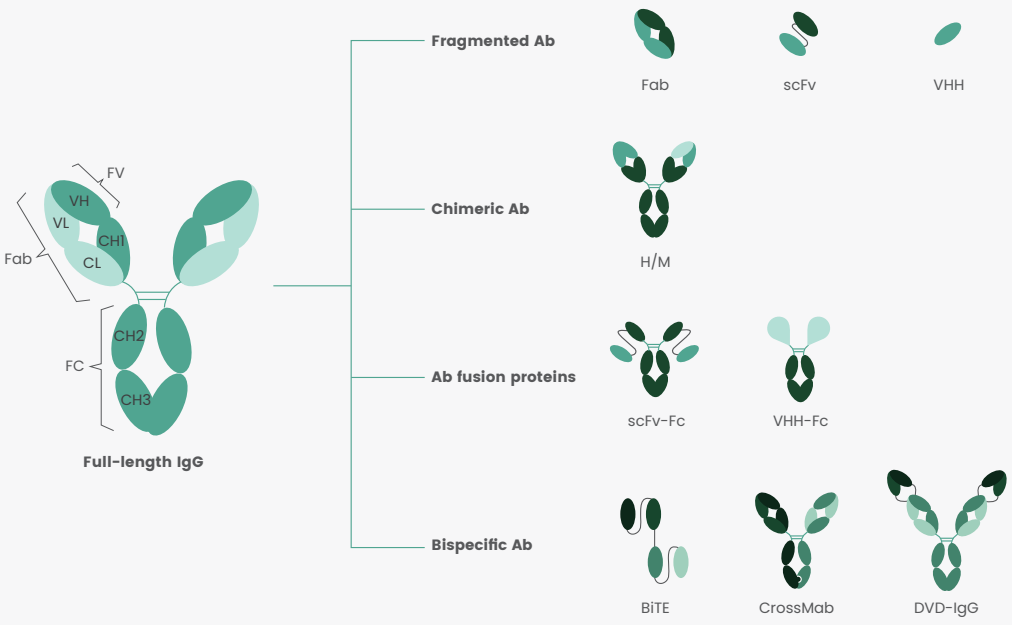


Figure 1. Some Recombinant Antibody Formats at a Glance

Chimeric Antibodies

Antibody research and clinical development were revolutionized by the discovery of hybridoma technology in 1975. However, for therapeutic purposes, the efficacy of murine-derived antibodies is limited by human anti-mouse antibody responses, in which the murine antibodies are identified as foreign molecules by the human immune system. In 1984, the first chimeric antibody, also recognized as the first version of recombinant antibodies, was constructed by genetic engineering to reduce the immunogenicity of murine antibodies in humans. A total of 30%–35% of the molecules are derived from mouse antibody sequences and 65%–70% are from human antibody sequences. The resulting chimeric antibodies retain the antigen-binding ability of the parental mouse antibodies. Antibody chimerization is the first step in developing therapeutic humanized antibodies. Using complementarity-determining region grafting technology and computer-aided molecular modeling, Sino Biological provides high-quality monoclonal antibody humanization services that enable a high degree of successful humanization (>90%).

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Antibody Fragments

Each full-length immunoglobulin (IgG) molecule contains two heavy and two light chains linked by disulfide bonds (Figure 1). Antibody fragments, such as Fab, scFv, and VHH, have a small size, providing better penetration of tissues or tumors than their full-length counterparts. This gives them a promising future in immunotherapy, especially in solid tumors. Furthermore, they also have a short half-life, which is useful as radioactive imaging agents. However, due to the lack of Fc regions, they cannot elicit Fc-mediated antibody effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

Enzymatic digestion was initially used to fragment IgG antibodies. Pepsin cleaves the IgG heavy chains in the hinge regions after the disulfide bonds, creating a bivalent Fab fragment known as $F(ab')_2$. Then, the fragment can be cleaved into two identical Fab fragments by papain. However, this enzymatic cleavage method is limited by the types of antibody fragments generated. Besides, it is unsuitable for industrial antibody production and purification. Thanks to advances in antibody engineering techniques, these problems can be solved by producing antibody fragments recombinantly. After successfully cloning and sequencing the antibody genes, antibody fragments can be expressed in microbial expression systems, such as *E. coli* and mammalian systems (i.e., HEK293 cells), through transient transfection.

With rich experience and expertise in recombinant production, Sino Biological has built a high-throughput (HTP) VHH expression platform (Figure 2) that delivers numerous VHH antibody production projects, with an overall success rate of more than 90%. In addition to common VHH formats, we can express dual- and multi-targeting VHHs (Figure 3). Furthermore, Sino Biological can express various other fragments with high specificity and affinity, such as scFv and Fab.

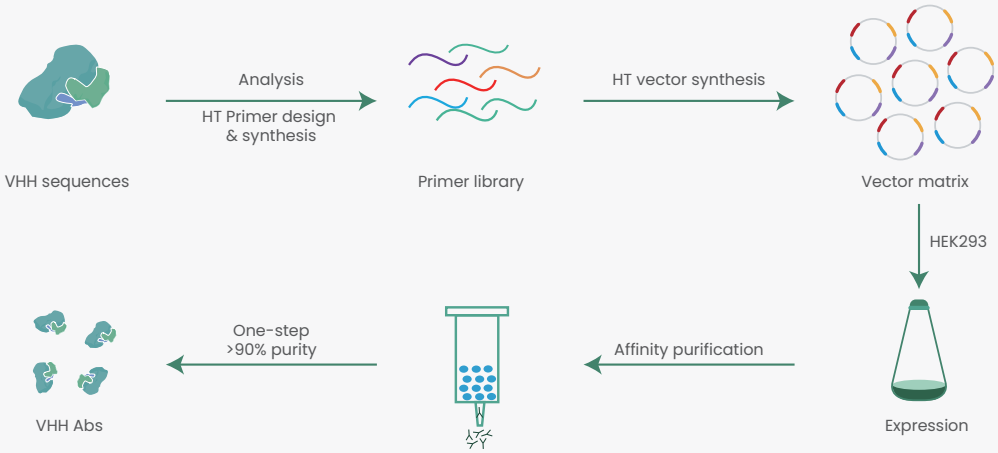

Figure 2. High-throughput (HTP) VHH Expression Platform

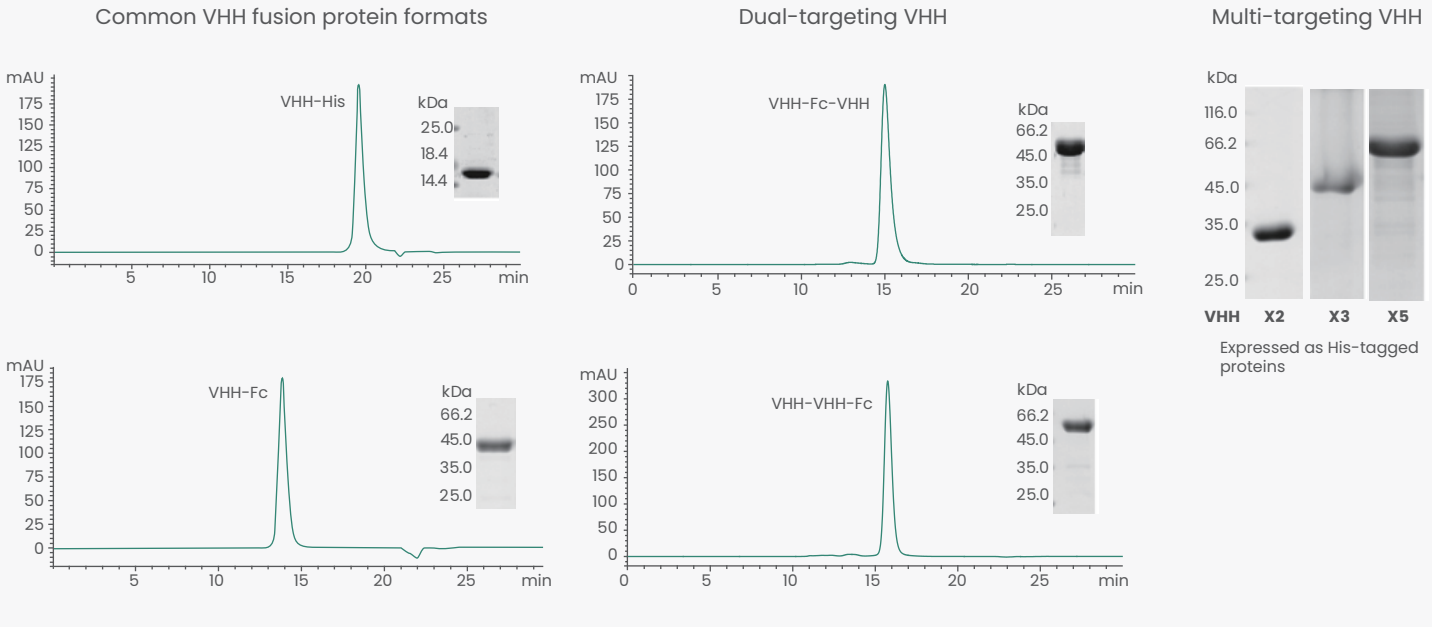

Figure 3. Expression of Various VHH Formats

Bispecific Antibodies

Unlike conventional monoclonal antibodies, bispecific antibodies (bsAbs) are those with two binding sites that can recognize two different antigens or epitopes on the same antigen. Due to this unique feature, bispecific antibodies have attracted much attention from researchers and the drug industry. To this date, four bsAb drugs have been approved by the Food and Drug Administration (FDA), and over 160 bsAbs currently undergoing clinical trials for cancer, diabetes, Alzheimer's disease, and other diseases.

Initially, bispecific antibodies were generated by quadroma technology, but it poses a significant challenge to downstream antibody manufacturing and purification. Following the development of recombinant DNA technology in the last 20 years, several bispecific antibody formats have emerged to suit the desired target–product profile. To solve the heavy chain mismatching problem, Genentech first proposed the "knob-into-hole" (KiH) technology, which involves engineering CH_3 domains to create either a "knob" or a "hole" in each heavy chain to induce heterodimerization. Similarly, other technologies, such as common light chain and CrossMab are employed to tackle the light chain mispairing problem. Expressing bispecific antibodies is predominantly generated in mammalian cells. Due to various structural similarities between monoclonal and bispecific antibodies, many established purification processes for conventional mAbs are compatible with bispecifics. (See another article: "Bispecific Antibodies: Rising Stars in Antibody Therapeutics")

Fc-Fusion Proteins

Fc-fusion proteins (also known as Fc chimeric fusion proteins, Fc-Ig's, and Fc-tag proteins) are homodimers consisting of an IgG-Fc domain fused to a protein of interest, such as a ligand, peptide, and enzyme. Although monoclonal antibodies are at the focal point of therapeutic biologics development, Fc-fusion proteins are also a successful class of biopharmaceutical products, with at least thirteen drugs approved by the European Medicines Agency (EMA) and FDA. In addition to therapeutic applications, Fc-fusion proteins serve as detection reagents in basic research, including flow cytometry, immunohistochemistry, and protein binding assays. In fact, linkage to the Fc domain can improve the solubility and stability of some binding partners. Given the size and need for glycosylation (most are glycoproteins), Fc-fusion proteins are mainly produced in mammalian expression systems.

Concluding Remarks

Recently, advancements in antibody engineering technologies have greatly enhanced the generation of recombinant antibodies in various formats as therapeutic agents. There are already more than 100 antibody-based drugs approved by the FDA, and numerous antibodies are currently in their late-stage clinical studies. Moreover, engineered recombinant antibodies can be used in many research applications: western blotting (WB), immunohistochemistry (IHC), immunofluorescence (IF), flow cytometry (FC), and surface plasmon resonance (SPR).

As molecular biological and immunological technologies are continuously developing, recombinant antibodies will be widely used in basic scientific research and disease prevention, diagnosis, and treatment, thereby providing strong support for scientific research and pursuing a healthier future for humans.

Find out more at **sinobiological.com/services/recombinant-antibody-production-service**

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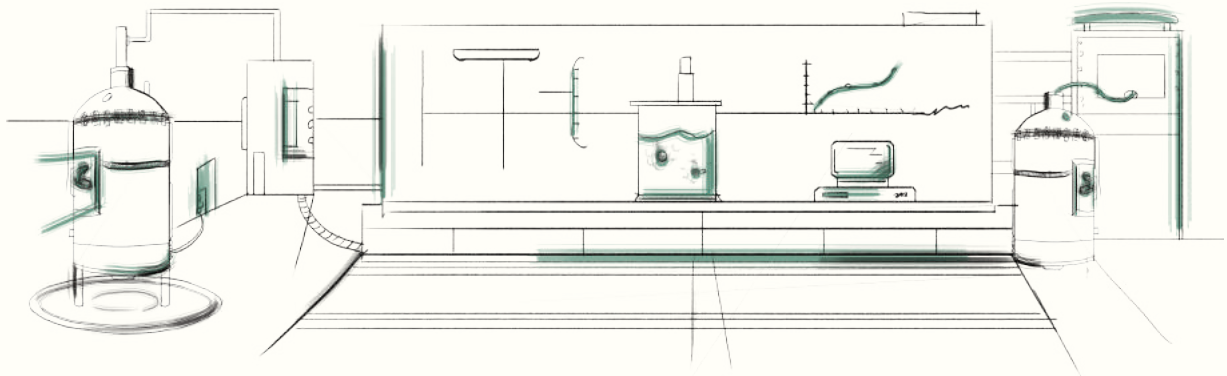
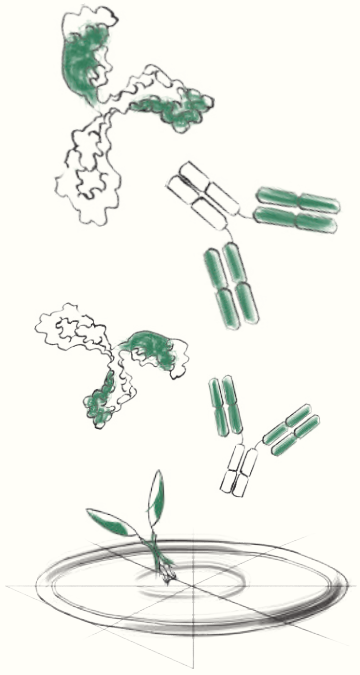
Sino Biological specializes in recombinant production of proteins and antibodies. The company routinely conducts high-throughput projects of up to 1,000 per batch. Sino Biological's manufacturing facility can also handle large-scale production at gram level.



Diverse Antibody Formats

scFv, Fab, VHH, chimeric, bispecific, Fc-fusion proteins, or full-length IgG, IgM, IgA, and IgE antibodies



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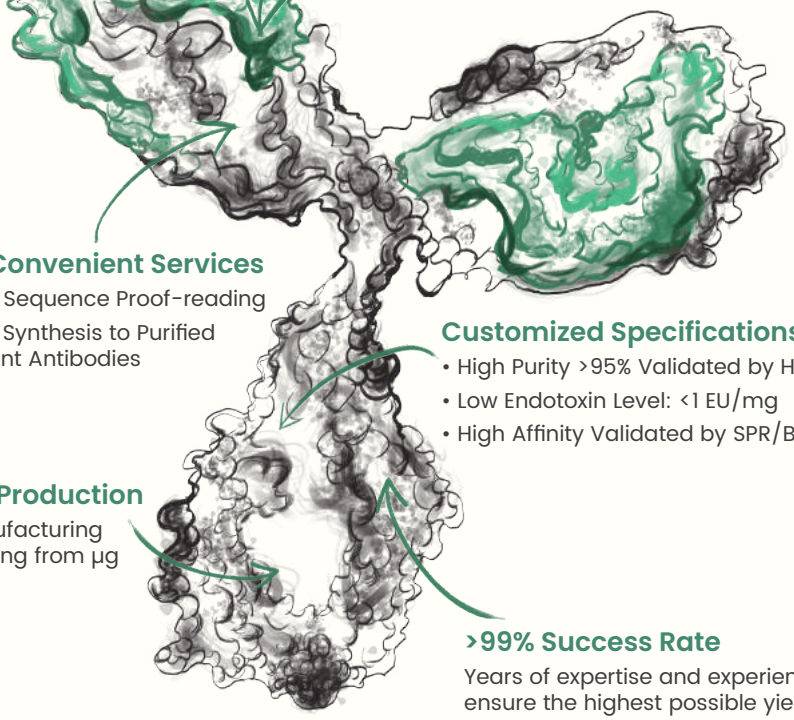

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Benchmark

BioTechniques

High-yield expression of periplasmic single-chain variable fragments by solid *Escherichia coli* cultures

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ABSTRACT

High-yield expression of quality antibody fragments is indispensable for research and diagnosis. Most recombinant antibody fragments are expressed in *Escherichia coli* using liquid cultures; however, their yields and quality are often poor. Here the authors expressed a single-chain variable fragment in *E. coli* cultivated on the wet surface of a solid support. Compared with a liquid culture, the authors obtained 2.5-times more single-chain variable fragments with membrane-cultivated *E. coli*. This method has two important advantages: it enables high yields of periplasmic single-chain variable fragments compared with liquid culture and offers simple and rapid expression and extraction.

METHOD SUMMARY

Here single-chain variable fragments are expressed in a simple and rapid manner by cultivating *Escherichia coli* on the wet surface of a solid support. This method enables good oxygenation from the air and nutrient transfer from the medium, thus overcoming existing limitations of conventional liquid and solid cultures.

KEYWORDS:

antibody ● *E. coli* ● expression ● periplasm ● scFv ● solid culture

Antibodies are indispensable for therapeutic, diagnostic and research purposes. Various recombinant antibody fragments, such as Fabs, single-chain variable fragments (scFvs) and bispecific antibodies, are generated from whole antibodies and utilized for specific applications [1]. ScFvs consist of a single polypeptide (25 kDa) whose variable regions of the light and heavy chains of IgG are joined by a flexible linker [2]. Owing to their small size, scFvs are widely used for screening antibody libraries and show good penetration into tissues, rapid clearance and low immunogenicity. Their small size and lack of glycosylation allow for the production of functional scFvs via prokaryotic expression systems, which are easier and cheaper to manipulate than insect cell lines or mammalian expression systems [3–5]. *Escherichia coli* is the standard laboratory host for the expression of proteins [6]. Its main advantages include easy handling, short life cycle, simple cultivation conditions, well-known genome and metabolism and wide array of expression vectors.

The yield and quality of the produced scFvs are important for subsequent applications; hence, there is a strong need to improve these parameters [7]. Expressing scFvs in *E. coli* is hampered by low expression, formation of inclusion bodies, cell lysis and proteolytic degradation. Several efforts have been made to overcome these issues, including codon optimization, swapping promoters, changing host strains, adding a solubilization tag and fine-tuning cultivation conditions, yet yields have not improved substantially [3].

Commonly, lab-scale recombinant protein expression in *E. coli* relies on batch cultivation in shake flasks containing liquid Luria–Bertani (LB) medium rich in yeast extract and peptone. However, the growth of *E. coli* in LB medium stops once usable carbon sources are depleted and the pH has become too alkaline following peptone metabolization. Additionally, culture in shake flasks suffers from limited oxygen transfer, leading to a decrease in protein production [8]. Improved media, such as Terrific Broth (TB) and Super Broth, with richer nutrient compositions, buffer components and supplemental carbon sources have been developed. These media promote fast, exponential growth in the initial phase but prevent growth to a high cell density owing to nutrient and oxygen exhaustion, unfavorable pH and production of growth-inhibitory substances through overflow and anaerobic metabolism. Enzyme-based glucose release technology was also developed to achieve very high cell densities [8–10]. Although fed-batch cultivation enables prolonged growth of bacteria and better yield of target proteins by controlling nutrients, pH and oxygenation during protein expression [11], it requires labor- and time-consuming optimization for high-yield expression.

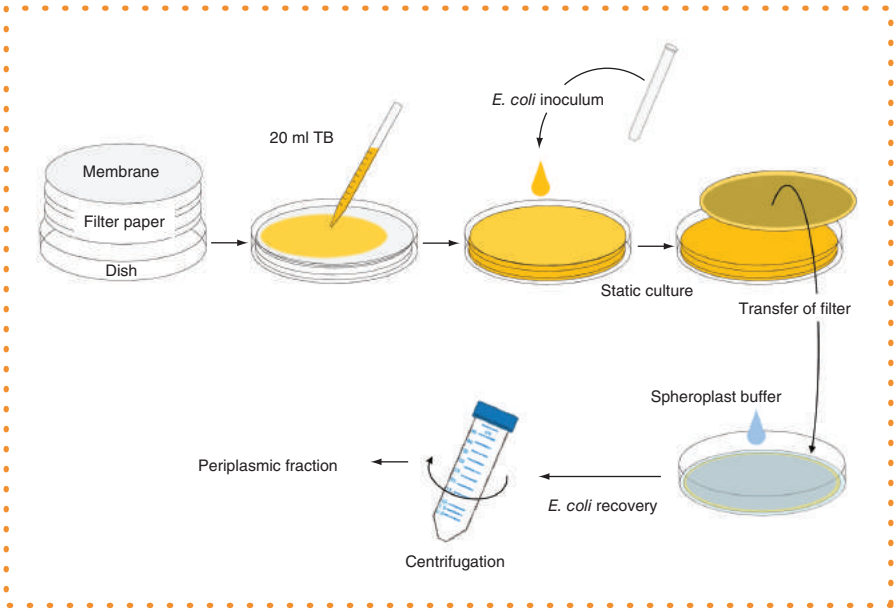

Figure 1. Schematic diagram of membrane culture outlining the procedure for expression and extraction of periplasmic single-chain variable fragments.
TB: Terrific broth.

Here the authors describe the periplasmic expression of functional scFvs in *E. coli* grown on solid support. Antigen-binding scFvs were successfully expressed on the wet surface of a hydrophilic membrane followed by their simple and rapid extraction. The volumetric yield of periplasmic scFvs was 2.5-times greater with the solid culture than with a shake-flask culture.

Anti-rabbit IgG scFv (A10B) was inserted in a pET-22b expression vector and transformed into *E. coli* BL21 (DE3) cells [12]. Transformants were cultivated in a shake flask (shake-flask culture) or on a membrane (membrane culture) and scFv expression between the two methods was compared. The expression and harvesting procedures for the membrane culture are summarized schematically in Figure 1. Inocula were prepared by adding 100 μl high-cell-density glycerol stocks (OD_{600}: 20–30) containing scFv-expressing clones to 3 ml LB medium and incubating for 4 h at 37°C and 200 rpm in a shake flask.

For solid cultivation, a hydrophilic membrane was placed on filter paper soaked with 20 ml TB medium and fitted in a 90-mm dish (Figure 1). Inoculum (100 μl) was suspended in 100 μl of an auto-induction solution containing glucose and lactose [13], spread on the membrane and grown at 26°C in a humidified incubator. Then, the filter was transferred to a new dish. The periplasmic fraction was extracted by cold osmotic shock directly from the cells on the filter. To measure cell density, all cells were scraped from the filter and suspended in 20 ml LB. For shake-flask cultivation, the overnight culture was inoculated in a 200-ml flask containing 20 ml TB medium and incubated at 37°C and 200 rpm. At OD_{600} of 0.5, expression was initiated by the addition of IPTG to a final concentration of 0.5 mM. The cells were incubated overnight at 26°C, harvested by centrifugation at 6000 × *g* and 4°C and washed with phosphate-buffered saline, and the periplasmic fraction from the pellet was extracted by cold osmotic shock. The shake-flask method required continuous shaking to ensure adequate oxygen supply, optical density measurements to properly induce protein expression with IPTG and centrifugation to collect cells after expression. In comparison, the solid method relied on an incubator for growth and required no optical density monitoring, and collection was achieved by simply transferring the membrane. It took only 25 min to harvest the cells from dishes and collect the periplasmic fraction, whereas it took 50 min in the case of the shake-flask culture.

The antigen-binding activity of scFvs obtained from both culture methods was measured by ELISA. Rabbit IgG was used to coat a 96-well ELISA plate, and the periplasmic fraction was applied. Binding to the antigen was detected by horseradish peroxidase-labeled anti-His tag antibody. The results are shown in Figure 2. EC_{50} values were calculated using Prism (GraphPad Software, CA, USA) with nonlinear regression fit. The periplasmic fraction from the membrane culture showed higher activity, with a 2.4-times greater EC_{50} (594) compared with the shake-flask culture (EC_{50}: 245).

For a quantitative comparison of scFv expression between shake-flask and membrane cultures, scFvs were purified with a Ni column. Cell growth, volumetric yield and relative yield were analyzed (Table 1). Cultures grown in 20 ml TB medium reached an OD_{600} of 8.0 at 24 h after induction. Cells grown on the membrane for 24 h at 26°C were collected and resuspended in 20 ml LB medium, at which point OD_{600} was 7.2. The similar OD_{600} values indicated good oxygenation in the shake-flask culture. Although the number of cells was almost identical in both cases, the volumetric yield was 2.5-times larger and the relative yield (i.e., scFvs expressed per cell) was 2.7-times larger in the membrane culture, indicating a significantly higher expression per cell in the latter. Given the similar improvements in antibody yield and activity (EC_{50}), the authors concluded that the expressed and extracted scFvs were functional.

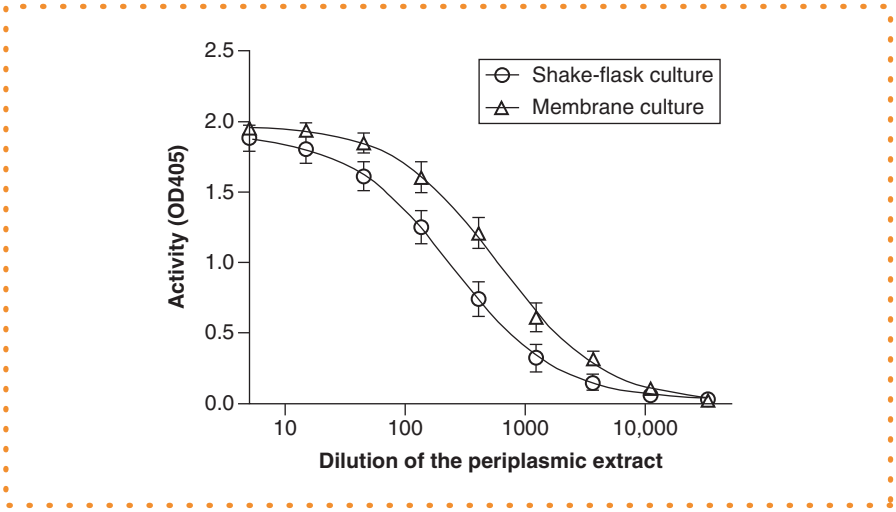

Figure 2. Comparison of antigen-binding performance by shake-flask and membrane culture methods. The sigmoidal binding curves obtained by ELISA describe concentration-dependent antigen-binding of each periplasmic fraction. Data represent the mean of three replicates. Error bars represent the standard deviation.

Table 1. Comparison of cell growth and volumetric/relative yields for A10B–single-chain variable fragments obtained with shake-flask liquid culture and membrane culture.

	OD_{600} at harvest	Volumetric yield (mg/l)	Relative yield (mg/l/OD)
Shake-flask culture	8.0	1.7	0.21
Membrane culture	7.2	4.2	0.58
Ratio, membrane:shake flask	0.9	2.5	2.7

OD: Optical density.

Table 2. Comparison of volumetric and relative yields for various single-chain variable fragments obtained with shake-flask liquid culture and membrane culture.

	Volumetric yield (mg/l)			Relative yield (mg/l/OD)			Ref.
	Shake flask	Membrane	Ratio	Shake flask	Membrane	Ratio	
Anti-rabbit IgG scFv	2.1	4.0	1.9	0.26	0.56	2.1	[14]
Anti-human IgG scFv	2.0	6.2	3.1	0.33	1.03	3.1	[15]
Anti-rabbit IgG scFv from chicken	4.2	6.3	1.5	0.6	1.17	1.9	[12]
Anti-*Gaussia* luciferase scFv	1.5	3.2	2.1	0.19	0.44	2.3	[12]

OD: Optical density; scFv: Single-chain variable fragment.

The authors also compared the expression among various scFvs [12,14,15] obtained from membrane and liquid cultures (Table 2). All scFvs obtained by membrane culture exhibited significantly larger volumetric and relative yields of soluble proteins. The strongest increase was observed for anti-human IgG, the volumetric yield of which was augmented 3.1 times, from 2.0 to 6.2 mg/l. Because cell density did not differ between the two culture methods, the observed increase in relative yield was due to higher soluble scFv expression.

Expressing scFv in the periplasmic space of *E. coli* grown on the wet surface of a solid support ensured a greater yield of recombinant protein with a shorter, lower cost and labor-saving procedure compared with existing liquid-based protocols. In the case of shake-flask cultures, nutrients are well supplied, but oxygenation is insufficient. In a conventional solid culture, oxygen supply is sufficient, but nutrient diffusion in a solid support such as agar is restricted. To overcome this limitation, the authors cultivated *E. coli* on the wet surface of a nylon membrane placed on medium-soaked filter paper. In this way, oxygen was supplied effectively through the air and nutrients were well supplied from the liquid medium beneath the nylon membrane. These conditions might explain the high yield of periplasmic scFvs obtained, which were likely the result of strong protein synthesis and efficient periplasmic transport and disulfide bond formation. It was found that avoiding stress during cultivation was critical for elevated expression in *E. coli*, and oxygen supply was a critical factor in this sense [16–18]. The authors' system could reduce stress by improving oxygenation to the cells.

The growth rate in the authors' system was comparable to that of a well-aerated shake-flask culture in which the medium accounted for 10% of the flask volume [19]. Thus, the authors obtained almost the same amount of *E. coli* from a membrane culture on 90-mm dishes as from a liquid culture in 200-ml flasks. Although the shake-flask culture could be easily scaled up by increasing the volume, the process is not as straightforward for the membrane culture. However, the membrane culture system is suitable for large sample numbers and small scales because it does not require a temperature-controlled shaker and expression and purification are relatively simple and easy, allowing for a high-throughput screening approach. As the membrane culture and recombinant protein production process developed here is a time- and cost-saving method, scaling up would be beneficial at the industrial level. To achieve this, a large surface area for culture should be secured. An important feature of membrane culture is the wettability of the filter surface. Dehydration or excess liquid should be avoided to ensure good cell growth, and the system should be under saturated humidity. Some parameters, such as incubation period, temperature, membrane material and medium composition, may be further optimized to ensure even better performance.

In summary, the authors describe a high-yield expression system using the pET vector and periplasmic scFvs in membrane-grown *E. coli*. The effectiveness and wider applicability of membrane culture should also be tested with other antibody fragments and proteins as well as combinations of hosts and vectors. The increase in scFv expression in the periplasm by the membrane culture may be caused by the increase in total expression, decrease in inclusion body formation or increase in transport to the periplasmic space. To elucidate the mechanism, further quantitative analysis is required. By using SHuffle cells (New England Biolabs, MA, USA) [20] with the authors' system, a higher expression of cytosolic proteins containing disulfide bonds could be expected. Other vectors, such as rhamnose-induced or cold-shock vectors [21,22], that do not require IPTG, which is toxic to *E. coli* in some cases, would reduce cell lysis during expression, thereby increasing the expression yield.

Author contributions

Y Hanyu designed the study and wrote the manuscript. M Kato performed the experiments and analyzed the data.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

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Recombinant Protein Expression Using a Baculovirus-insect Cell System

Introduction

Proteins, the key products of the central dogma of molecular biology, are the basic building blocks of life. Proteins perform various functions such as creating cellular matrices, catalyzing biochemical reactions, and forming signaling pathways to respond to external stimuli. Studies of the structure and function of proteins hold the key to understanding the "meaning of life," so they are pursued by scientists across multiple disciplines. However, because most proteins of interest are difficult to obtain, it is important to establish sources that could potentially provide researchers with unlimited supply of proteins. Recombinant protein expression is the process in which a gene encoding a protein-of-interest is cloned into an expression vector (usually a plasmid) and transferred into a host cell for protein production by harnessing its intrinsic protein synthesis machinery. Several host cell systems have been established for recombinant protein production and the selection of the optimal host for any given protein is a major factor for its successful expression. The advantages and limitations of commonly used expression hosts are summarized in Table 1.

Table 1. Commonly used host cells for recombinant protein expression

Expression Host	*E. coli*	Yeast	Insect	Mammalian
Category	Prokaryote	Eukaryote	Eukaryote	Eukaryote
Culture density	High	High	High	Medium
Culture duration	Short (1~2 days)	Short (1~2 days)	Medium (2~4 days)	Long (5~7 days)
Protein folding	Limited	Yes	Yes	Yes
PTM	None	Glycosylation	Glycosylation, phosphorylation...	Glycosylation, phosphorylation...
Suitable proteins	Proteins with low MW	Secreted, intracellular	Secreted, intracellular	Secreted
Cost	Low	Low	High	High
Example Cell Lines	BL21(DE3), Rosetta...	Pichia pastoris	sf9, sf21, High-five	HEK293, CHO
Note	Inclusion bodies	High mannose glycan	Low MW glycan	

Baculovirus-insect Cell System (BEVS)

To deliver a target gene into the cells and achieve protein expression, insect cells require baculovirus as an intermediate. Baculoviruses represent a diverse group of DNA viruses that are capable of infecting various (>600) insect cells. They serve as a shuttle for the introduction of a target gene into a given host cell. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-characterized baculovirus for this purpose and it is widely used for insect cell-mediated protein expression. A flow chart of this process is presented in Figure 1. Briefly, a gene encoding the protein-of-interest is inserted into a primary vector, which is subsequently cloned into a secondary vector known as Bacmid. Bacmid is transferred into a bacteria strain (commonly *E. coli*) for preliminary virus production and assembly to obtain generation 1 baculovirus (P1). The P1 virus is amplified in an insect cell (e.g., sf9) to achieve a suitable titer (P2), which is then used to infect the same or a different insect cell line (e.g., High-five) for protein expression. This "Bac-to-Bac" system has been adapted for the expression of a wide variety of proteins as secreted, intracellular, or membrane-bound molecules. Of note, there are many commercially available methods for baculovirus generation.

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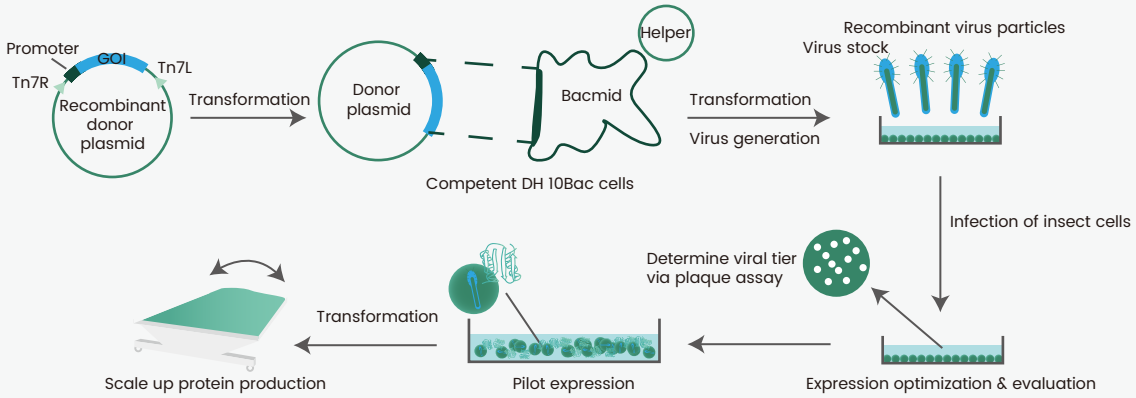

Figure 1. Flowchart for recombinant protein expression in insect cells.

Application of BEVS

Insect cells are versatile expression hosts for a range of recombinant proteins. They are excellent choices for the expression of complicated intracellular and viral proteins because of their robust protein folding capability and relatively high culture density. In 2007, Cervarix, a human papilloma virus vaccine produced using insect cell line in the format of virus-like particles was approved for human use. Highly active proteins produced in insect cells are used in various disciplines including structure elucidation, drug design, assay establishment, and diagnostic reagent development. To obtain fully functional recombinant proteins, a systematic design is required for each step from construct design and culture optimization to protein purification and protein formulation. Here, we describe two cases to demonstrate key features of recombinant protein expression using BEVS.

(1) Core region fusion

Insect cells are often used to produce large molecular weight (MW) proteins (MW > 150 kDa) because of their superior folding and posttranslational modification capabilities. One major downside of this endeavor, however, is the structural complexities of such proteins, which often results in relatively low yields (Fig. 2, left). One alternative approach is to express a domain-of-interest rather than the entire protein; however, in the case presented here, the direct expression of two enzyme domains of human fatty acid synthase (FASN) was not feasible because of low protein yield and heavy degradation (Fig. 2, middle). By extrapolating and fusing the sequences encoding the methyltransferase and ketoreductase domain with a linker, we successfully obtained a high-yield construct (Fig. 2, right). The Elute 1 and 2 of this construct were pooled and further purified to yield a final fusion protein with >90% purity.

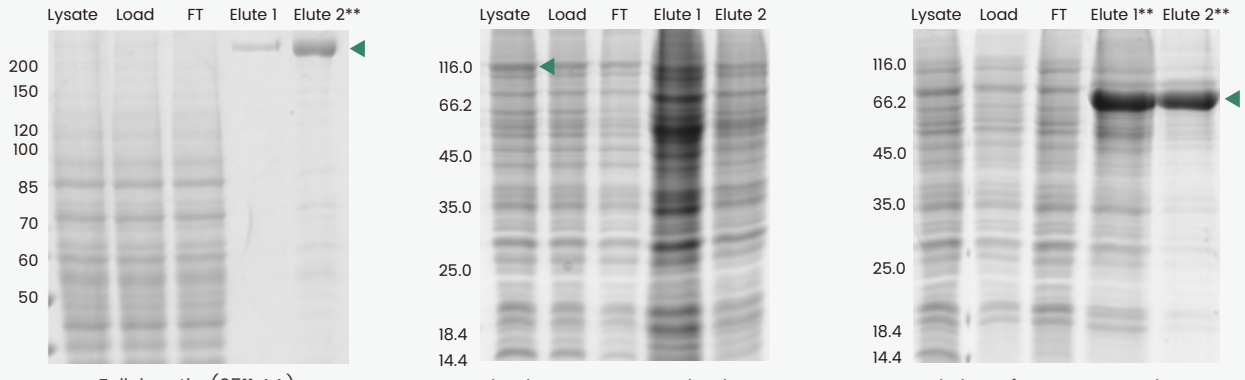

Figure 2. Expression of full-length (left), truncated (middle), and methyltransferase plus ketoreductase domain fusion (right) constructs of human FASN. The full-length protein was prepared as reported by Hardwicke *et al.* (2014, doi:10.1038/nchembio.1603), whereas the domain fusion construct was prepared as reported by Lu *et al.* (2018, doi: 10.1016/j.bmcl.2018.05.014).

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(2) Obtaining protein with the correct oligomeric status

Some proteins require certain oligomeric formations to be functional. For example, the hemagglutinin proteins of influenza virus and the spike protein of SARS-CoV-2 exhibit a trimeric format, whereas the human prolyl endopeptidase, FAP, is an intrinsic dimer. Caution should be exercised during the purification steps to track the protein fractions with the correct oligomeric status to ensure proper protein activity. In this study, a his-tagged protein was expressed using BEVS and the Ni-affinity eluate contained a mixture of its monomer and dimer (Figure 3.). Because the protein is active as a dimer, a second gel-filtration purification step was required to enrich the protein dimer. The dimeric conformation of the final product was confirmed by SEC-HPLC and the protein exhibited reproducible enzymatic activity between two different batches using the established method.

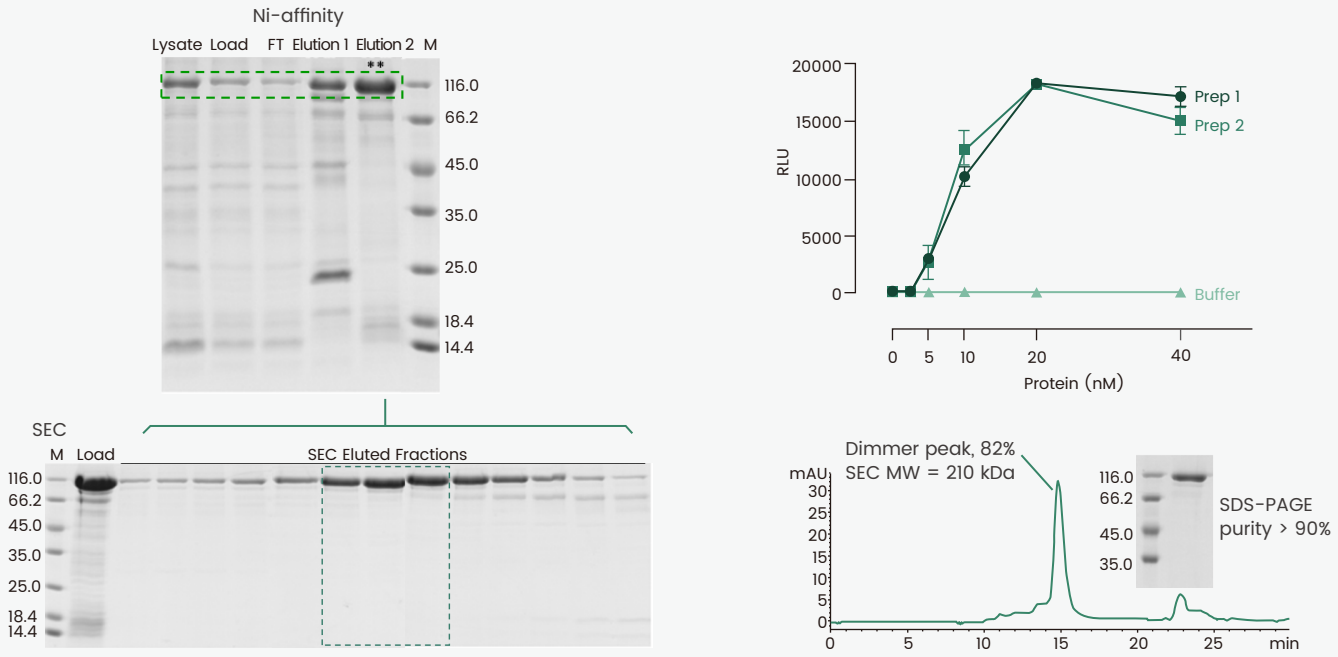

Figure 3. Expression and purification of an active protein dimer. The Ni-affinity Eluate 2 was subjected to gel-filtration chromatography to enrich for the protein dimer. The dimeric status of the protein was assessed by SEC and the activity was determined using the corresponding enzymatic assay.

Conclusive Remarks

Recombinant proteins are fundamental to the study and development of biologics. Insect cells are a superior choice as an expression host because they enable correct protein folding and posttranslational modification, and are suitable for high-density cell culture. They can produce both secreted and intracellular proteins of various species. A systematic design and optimization approach is essential to obtain high-quality recombinant proteins using insect cells.

Find out more at **sinobiological.com/services/recombinant-protein-expression-service**

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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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