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Cell-Free Protein Synthesis: Pros and Cons of Prokaryotic and Eukaryotic Systems

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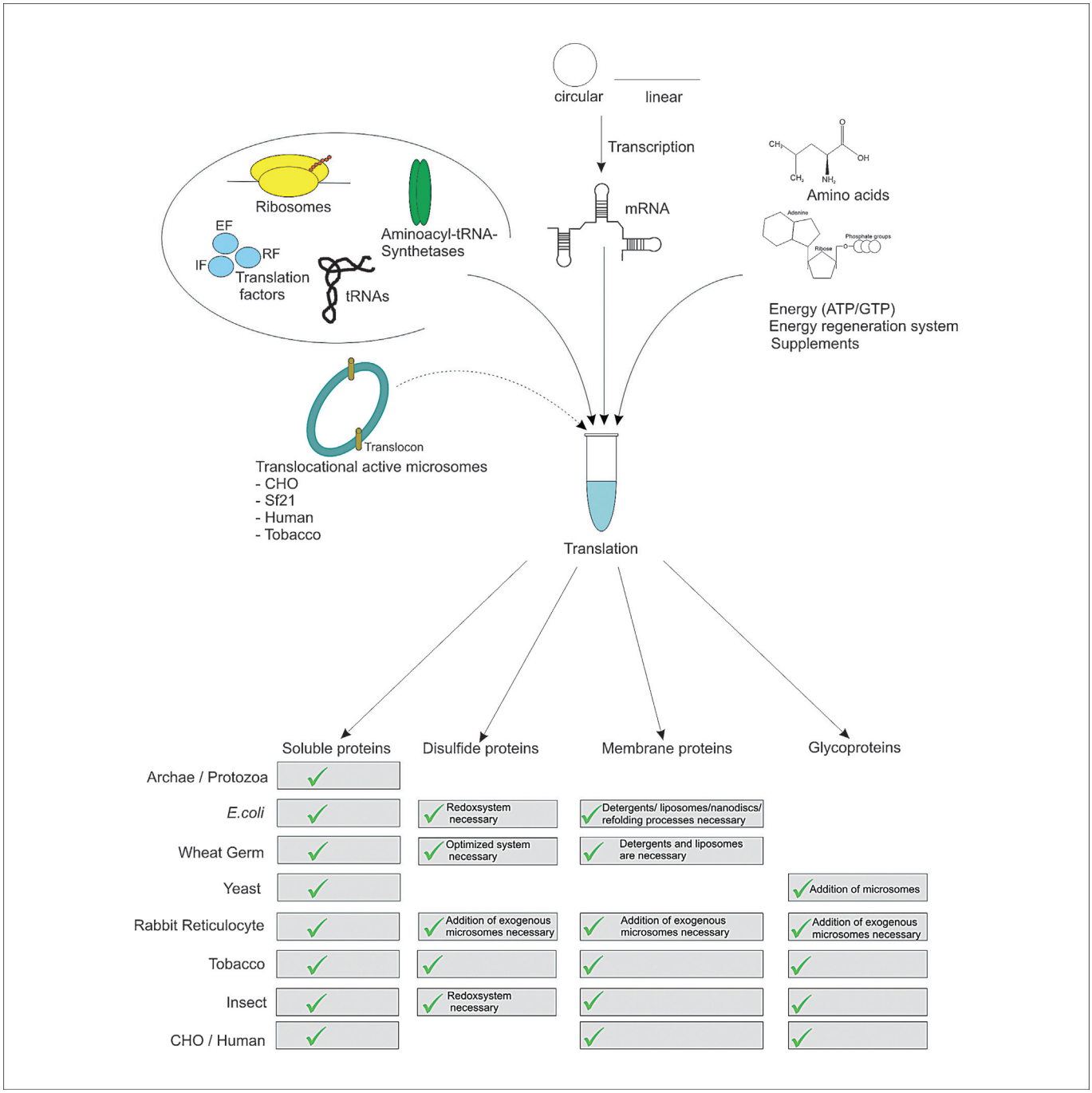

From its start as a small-scale in vitro system to study fundamental translation processes, cell-free protein synthesis quickly rose to become a potent platform for the high-yield production of proteins. In contrast to classical in vivo protein expression, cell-free systems do not need time-consuming cloning steps, and the open nature provides easy manipulation of reaction conditions as well as high-throughput potential. Especially for the synthesis of difficult to express proteins, such as toxic and transmembrane proteins, cell-free systems are of enormous interest. The modification of the genetic code to incorporate non-canonical amino acids into the target protein in particular provides enormous potential in biotechnology and pharmaceutical research and is in the focus of many cell-free projects. Many sophisticated cell-free systems for manifold applications have been established. This review describes the recent advances in cell-free protein synthesis and details the expanding applications in this field.

Introduction

Cell-free protein synthesis (CFPS) has become a fast-growing research area with high potential for industrial protein production.[1] The basic principle of cell-free systems was introduced by Eduard Buchner, developed not primarily to synthesize proteins but to convert sugar to ethanol and carbon dioxide in yeast extract.[2] More than 60 years later, Nirenberg and Matthaei developed a CFPS system based on *Escherichia coli*, primarily to study translational processes.[3] This system paved the way for the multitude of sophisticated CFPS systems that are available today.

CFPS systems can be distinguished in many ways, for example, the organism from which they are prepared. Various cell-free systems are available, originating from Archaea, prokaryotes, fungi, plants, insects, and mammals.[1] In most cases, the basic principle of the cell-free reaction is the same. Crude extracts are generated from cultured cells and depleted of endogenous DNA and mRNA, and the lysate is subsequently supplemented with energy components and free amino acids. The translational process is initiated by the addition of a suitable template (linear or circular DNA or mRNA),[4,5] and carried out at an appropriate temperature for the chosen system. Reaction mixtures that are supplemented with DNA templates are referred to as "coupled reactions", where transcription and translation are performed simultaneously; reactions supplemented with purified mRNA are termed "linked reactions". The origins and the manufacturing processes of the crude cell extracts lead to significant differences in terms of the quality and quantity of the de novo synthesized protein. CFPS conditions differ from protein to protein to account for protein complexity, folding, and post-translational modifications. Different cell-free reaction formats have been developed, from classical batch reactions with short reaction times, limited protein yields, but easy handling and scalability,[6] to more complex dialysis systems known as continuous-flow cell-free (CFCF)[7] and continuous exchange cell-free (CECF)[8,9] Prolonged reaction lifetime and higher protein yield (up to several mg mL^{-1}) are achieved by integrated dialysis systems.[7] CFPS systems are of growing interest particularly for the in vivo production of difficult-to-express proteins, such as toxic and membrane proteins, because internal cell metabolism does not have to be preserved and no cellular barriers restrict the translation control.[10,11] In contrast to living cells, CFPS systems can be easily adapted and modified by the addition of a multitude of supplements, such as chaperones,[12,13] radioisotope labels,[14] nanodiscs,[15] and microsomes.[16,17] Therefore, CFPS is easily adaptable to the translational requirements of a particular target protein, and the synthesis conditions can be adjusted for a desired subsequent analytical setup. Moreover, because of the simple handling of liquids and the easy scalability of cell-free reactions, novel automated high-throughput systems are being developed.[18–21,4]

In addition, the absence of the cellular membrane allows the synthesis of modified proteins with statistically as well as site-specifically embedded non-canonical amino acids[22,23] Methods have been developed with tRNA chemically and enzymatically connected to non-natural amino acids, and specifically designed tRNA/tRNA-synthetase pairs have been added with the corresponding free non-natural amino acids.[24,25] This research field is highly beneficial in the pharmaceutical context and might lead to novel therapeutic concepts based on bioconjugate chemistry.

In this review we present the diversity of CFPS systems with a focus on their future, their potential, and their applications in bioproduction.

Prokaryotic Cell-Free Systems

E. coli extracts

One of the first CFPS systems was based on *E. coli* cell extracts,[3] and developments of this system have aimed at enhancing the yields of de novo synthesized proteins. The direct connection between protein yield and reaction life-time has led to the development of reaction methods that remove inhibitory byproducts such as inorganic phosphates by continuous flow[7] or passive dilution (CECF system).[8] Efficient ATP regeneration for energy-consuming protein synthesis reactions was a challenging task. Usually, energy regeneration was performed by supplementation with the high-energy phosphate

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compound phosphoenolpyruvate (PEP). However its rapid degradation into pyruvate and inorganic phosphate by phosphatases in the lysate resulted in the development of alternative ATP regeneration systems,[6] such as the use of glucose-6-phosphate as the secondary energy source.[26] However, the initial protein yield with glucose-6-phosphate-dependent energy regeneration was substantially lower than comparable synthesis with the PEP/pyruvate kinase system.[26] After pH stabilization and optimization of the phosphate concentration, the protein yields in cell-free translation reactions using glucose and glucose-6-phosphate were equivalent to those by PEP reactions. The relative product costs were reduced by factors of 2.2 (glucose-6-phosphate) and 2.4 (glucose).[27] The search for an ideal sugar as an energy source was picked up again in 2007. Instead of glucose-6-phosphate the glycolysis intermediate fructose-1.6-bisphosphate was applied to a cell-free reaction, and because of the cheaper energy source, the cost of the synthesized protein was reduced.[28] Nevertheless, as well as the established creatine and acetate kinase systems, PEP-based systems are still widely used in cell-free systems.[29–31] In addition to optimization of the energy regeneration system over the past 40 years, several attempts have been made to improve the quality of the translation components: purified soluble components,[32] purified precharged aminoacyl-tRNAs, purified translation factors,[33] and purified aminoacyl-tRNA synthetases[34] have been developed. The most successful improvement was achieved by Shimizu et al. in 2001 by using fully purified recombinant proteins for translation.[35] This system is known as PURE (protein synthesis using recombinant elements). Addition or subtraction of translation components can direct protein synthesis in a desired direction. For example, the reduction of release factor one (RF1) resulted in highly efficient incorporation of non-canonical amino acids into the protein by using amber stop codons.[36,37] The presence of RF1 in cell extracts often leads to truncated proteins that are prematurely terminated at the amber stop codon UAG.[35] Non-canonical amino acids can be used to incorporate post-translational modifications at particular positions in a protein. In this context Chalker et al. clicked an N-acetyl glucosamine to an introduced azido tag.[38] Post-translational modifications for functional proteins are hugely restricted in *E. coli* cell-free systems, as only limited modifications are possible.[39]

The lack of a natural membrane impedes the synthesis of membrane proteins. Various synthesis methods have been established to enhance the correct folding and solubility of transmembrane proteins. These include supplementation with membrane-mimicking structures such as micelle-forming detergents, nanodiscs, liposomes, or exogenous microsomes.[40,41] Initially, the synthesis of membrane proteins in the absence of membrane-mimicking structures resulted in a precipitated product with steady yields.[42] With these systems, additional laborious protein purification and re-solubilization is necessary in order to obtain soluble membrane proteins. In addition, this procedure can negatively influence the protein's characteristics.[42] To circumvent the refolding problem, detergents were screened for suitability during protein synthesis. Brij and Tween derivatives, as well as DDM, Digitonin, and Triton X-100

were identified to fit with cell-free systems and to form micelles at defined concentrations in order to enclose the membrane protein.[43] However, some detergents can interfere with downstream analysis and therefore have to be displaced. Improved membrane protein folding and functionality has been achieved by a hydrophobic artificial environment composed of nanodiscs and liposomes. Nanodiscs consist of a phospholipid bilayer surrounded by membrane scaffold proteins.[44] Nanodiscs provide several advantages, including increased stability of integrated membrane proteins. Because of the randomly orientated incorporation into the bilayer, membrane-embedded proteins are accessible from both sides of the nanodiscs. Nanodisc technology is as a powerful tool for measuring quantitative binding affinities and kinetics for membrane proteins interacting with their ligands. However, the random orientation of membrane proteins is often a limitation of nanodiscs in certain cases, for example, when studying transporter proteins. Processes such as the regulated passage of solutes, including ions and small molecules, across lipid bilayers cannot be easily studied in nanodiscs. For functional studies including transporter assays and ion channel characterization, membrane proteins are usually incorporated into liposomes.[41] However the passive integration of membrane proteins in liposomes again results in a randomly orientated incorporation of these proteins, so only a proportion of the embedded proteins display correct functionality. Cell-free synthesis of membrane proteins is still a challenging task in *E. coli* extracts, although significant progress has been made (reviewed in refs. [45]and [46]). Even a multi-subunit protein complex such as the F_0-F_1-ATP synthetase was successfully assembled by a liposome-containing cell-free system.[47] One of the most notable achievements regarding the high-yield synthesis of cytotoxins in *E. coli* extracts is its technically feasible scalability.[48] In 2011, Murray and co-workers demonstrated CFPS of the multi-disulfide-bonded protein GM-CSF (granulocyte-macrophage colony-stimulating factor) with yields of 700 mg L^{-1} in reaction volumes of up to 100 L.[48] This scalability enables the industrial cell-free production of disulfide-bonded proteins. Moreover, because of the open nature of cell-free systems, transcription and translation factors as well as folding components can be optimized and engineered to result in a highly productive system.

Archaeal extracts

The first archaeal cell extracts were developed to study the effects of different antibiotics on the archaeal translational machinery.[49] New insights into the translation mechanism were gained by investigating the ribosomal subunit assembly in cell extracts based on sulfur-dependent thermophilic Archaea.[50] In 1993, Ruggero et al. reported a linked CFPS system working at high temperatures,[51] as the translationally active extracts were derived from the extreme thermophilic *Sulfolobus solfataricus*. Another coupled cell-free transcription–translation system, based on cell extracts prepared from the hyperthermophilic *Thermococcus kodakaraensis* was introduced by Endoh et al. in 2006.[52] Initial protein yields were comparatively low (1.3 μg mL^{-1}),[52] but after optimization of lysate preparation, reaction mixture composition, and genetic modification of the *T. kodakaraensis* strain, protein yields increased to over 100 μg mL^{-1} in a 30 min batch reaction.[53] Systems based on archaeal cell extracts are of outstanding interest for the synthesis of thermostable proteins, which might need the high translation temperatures (up to 80 °C) for correct protein folding. Additionally, high temperatures in coupled transcription–translation reactions might have a beneficial effect in reducing translation inhibition by mRNA secondary structures.

Eukaryotic Cell-Free Systems

Protozoan extracts

The characteristics of protein synthesis in cell-free extracts of protozoa have been investigated since the end of the 1950s.[54–58] The initial aim of these studies was examination of the translation process. Cell-free systems with ribosomes isolated from various protozoan organisms (*Tetrahymena pyriformis*,[55] *Crithidia oncopelti*,[54,56] *Paramecium aurelia*,[57] *Entamoeba histolytica*[58]) were investigated to characterize the incorporation efficiency of amino acids depending on buffer supplements, pH, energy sources, cation concentrations, and supplementation with spermidine or other compounds.[54] More recently, a cell-free system based on the protozoa *Leishmania tarentolae*[59] was established, in order to understand and characterize parasitic proteins and their influence on parasitic biogenesis.[60] Because transcription and translation initiation mechanisms differ between organisms, parasite protein expression was often inefficient when using conventional in vivo protein synthesis systems. In comparison to other eukaryotic cell-free systems, cultivation, upscaling, and extract preparation of *L. tarentolae* is usually less expensive, and the synthesized proteins show better solubility in comparison to proteins synthesized in *E. coli* lysates.[61] Two noteworthy advantages of *L. tarentolae* extracts are enhanced translation initiation (with a special RNA sequence) and the presence of identical splice leader sequences on all endogenous mRNAs.[62] A special polymeric RNA sequence, SITS (species-independent translation initiation sequence), consists of a long unstructured polymeric extension and three short stem hairpins[63] that support the assembly of the ribosomal subunits as well as the scanning the start codon.[64] Furthermore, the addition during extract preparation of a single antisense oligonucleotide that binds to the splice leader sequence leads to near-complete suppression of endogenous mRNA translation,[65] thereby facilitating translation of exclusively exogenous mRNA encoding the gene of interest. With this optimization, Alexandrov and co-workers synthesized a nearly complete set of Rab GTPases with yields of up to 30 μg mL^{-1} as well as a functionally active heterodimeric farnesyl transferase by using PCR-based DNA templates.[61] Despite these advantages, the system has only been used to study a limited number of proteins. Additionally, it is still an open question as to which types of post-translational modification can be realized in protozoan cell-free systems. With further optimization steps, including for higher production yield and large-scale lysate production, the PCR-based protein synthesis

in *L. tarentolae* might be suitable for high-throughput analysis in the near future.

Fungal Extracts

Yeast extracts

Extracts from *Saccharomyces cerevisiae* ideally combine the characteristics of high-yield protein synthesis and the ability to form correctly folded proteins. In addition, yeast extracts facilitate the formation of post-translational modifications, such as glycosylation.[66] Thus, the significant drawbacks of eukaryotic CFPS systems, such as relatively low protein yield (in comparison to *E. coli*), quite expensive extract preparation, and small reaction volumes might be tolerated. Yeast extracts based on *S. cerevisiae* were developed in the 1970s.[67,68] In the following years, the extracts were used to improve the general understanding of eukaryotic translation initiation,[69,70] thus leading to the identification of cap-dependent translation initiation.[71] In recent years several optimization strategies have increased the protein synthesis rate in yeast extracts: extract preparation,[72] optimization of cultivation conditions,[73] template optimization,[74] substrate replenishment, and byproduct removal[75] during CFPS. Huge efforts have been invested to introduce a novel energy regeneration system into yeast-based CFPS.[76] As for the *E. coli* system, the rapid production of phosphates from high-energy compounds such as phosphoenolpyruvate, creatine phosphate, and acetyl phosphate has an inhibitory effect on CFPS. Moreover, these high-energy compounds are expensive and resulted in limited industrial application.[27] Studies have proven that the use of glucose in combination with phosphate, as alternative energy source, results in more-efficient ATP production in comparison to standard energy sources such as creatine phosphate and phosphoenolpyruvate.[76] Moreover, the accumulation of inhibitory phosphates was avoided. By using this novel energy system, protein yields of up to 3.64 $\mu g\,mL^{-1}$ active luciferase were obtained. Despite the fact that protein yields were low in comparison to the creatine phosphate and creatine kinase system (approximately 8 $\mu g\,mL^{-1}$)[74] the relative costs per microgram of protein was lower by 16%.[76] Further optimization might render the yeast-based cell-free system a potent candidate for industrial protein production, as *S. cerevisiae* is already a highly productive in vivo bio-manufacturing platform.[74]

Plant Extracts

Wheat germ extracts

Wheat germ extraction development started in 1973 when Roberts and Paterson identified an efficient translation mechanism with tobacco mosaic mRNA[77] in wheat germ. After several optimization procedures, including the adjustment of magnesium, potassium, and amino acid concentrations,[78–79] as well as the integration of an adequate energy regeneration system, the wheat germ extract is now a well-established eukaryotic cell-free system. Reaction life-time and productivity of the cell-free wheat germ system was increased by the introduction of a dialysis system to facilitate removal of inhibitory by-products.[7] Nevertheless, in comparison to *E. coli* lysate, the extract preparation procedure is more expensive because of the inhibitory effect of nucleases and proteases in the endosperm: several washing steps of wheat germ embryos are needed for complete removal of inhibitory enzymes.[80] With this setup, Endo and co-workers prepared a highly translationally active and stable lysate; it produced up to 10 mg of green fluorescent protein (GFP) in a 1 mL reaction volume with a steady supply of mRNA over two weeks.[4] A highly productive cell-free wheat germ protein synthesis system was developed.[4,81] For this system, protein yields of the model protein GFP ranged from 1.6 mg mL (batch) to 20 mg mL (dialysis) of wheat germ extract.[20] Despite various high-throughput applications for the wheat germ system, the functionality of synthesized proteins is a key aspect for downstream analysis. Because of the extract preparation method, which includes a gel filtration step, low-molecular-weight components are eliminated from the resulting extracts.[20] These include co-factors that might be relevant for certain enzymatic activities. An analysis of the lipid and metal composition of a commercially available wheat-germ extract was performed by Goren and Fox.[151] They discovered that the iron concentration in the wheat germ extract was approximately 50-fold lower than the amount needed for the cell-free-synthesized desaturase, thus resulting in a limited assembly of the functional complex. In addition, removal of the endoplasmic reticulum (ER) during extract preparation hampers several post-translational modifications. Therefore only restricted modifications are possible (excluding glycosylation). The missing compartment can be mimicked by the addition of microsomes or liposomes: the addition of microsomes from dog pancreas was a common method to supplement wheat-germ extract.[82] Because of the laborious preparation of these microsomes and the required animal material, synthetic microsomes (e.g., liposomes) are currently favored. A problem that often occurs in cell-free systems is the requirement for disulfide bridges in proteins such as antibodies. The presence of the redox agent dithiothreitol (DTT) in the initial extract prevents the formation of disulfide bridges. This limitation was overcome in wheat-germ extracts by exploiting the open nature of cell-free systems. By reducing the DTT concentration and adding protein disulfide isomerase the formation of disulfide bonds was supported, thereby facilitating the production of a functional single-chain antibody variable fragment (scFv).[83] Later on, this approach was enlarged by adding reduced and oxidized glutathione to create an even better environment for the formation of disulfide bonds. With the mentioned modifications, the successful synthesis of disulfide bonded proteins can be applied to other cell-free systems.[48,101] A wheat-germ system was also employed for the production of malaria proteins, in order to discover novel vaccine candidates.[84,85] On-chip protein synthesis has been performed with wheat-germ extracts. The synthesized protein was tagged and thereby directly bound to a detector during CFPS. This method was used to study binding kinetics and enzyme activity.[86] Overall, the wheat-germ system represents one of

the most advanced eukaryotic systems, with applications in protein screening, engineering, and analysis.

Tobacco BY-2 extracts

Another plant-based eukaryotic system that was established during the last 15 years is based on tobacco BY-2 cells.[87–89] In contrast to the four to five days required for the wheat-germ extract preparation procedure, tobacco cells are only treated for four to five hours to obtain translationally active lysate. Only a few evaluations of the tobacco system exist. The initial results suggested a promising system that could compete with the wheat-germ system. A first look at post-translational modifications indicated that glycosylations and disulfide bond formation are possible, thereby resulting in the synthesis of a functionally active full-size antibody.[90] Nevertheless further optimization steps, scale-up, and functional analysis of more complex proteins are essential to obtain a robust protein production system.

Insect Extracts

Spodoptera frugiperda extracts

The baculovirus expression system constitutes one of the most efficient in vivo protein production tools.[91] Most of the proteins synthesized in this system were functional (antigenically and immunogenically similar to their native counterparts). This is mainly attributable to the properties of the insect system, which is able to carry out several post-translational modifications. Based on the promising evidence for protein production in insect cells, a cell-free system derived from cultured *Spodoptera frugiperda* cells was developed.[92,96] In this system, the ER is not fully removed during the gentle lysate preparation procedure. Rather, the remaining structures rearrange themselves into well-defined microsomes. As a result, the insect cell extract contains endogenous microsomes that are translocationally active and provide a nearly natural lipid membrane for protein translocation and embedding[16,93] Moreover, post-translational modifications can be achieved without the requirement for additional enzymes and cofactors. Modifications such as peptide cleavage,[94] lipidation,[95] glycosylation,[96,16] phosphorylation,[97] and disulfide bond formation[98,17] have been reported. These covalent modifications, such glycosylation and disulfide-bond formation, are frequently present in eukaryotic proteins and are often essential for correct protein folding and activity.[99] Translocation of proteins into microsomes was achieved by fusing the signal peptide of honeybee melittin[100] to the target protein.[17] By performing repeated protein synthesis, the same batch of microsomes was used multiple times, and enrichment of the target proteins in the lumen as well as in the membrane of the microsomes was achieved.[17] Microsomes can subsequently be separated from the translation mixture by gentle centrifugation, and the produced protein can be released by treatment with mild detergents such as *n*-dodecyl-β-maltosidase (DDM).[101] Furthermore the microsomes can be converted into giant unilamellar vesicles (GUVs) by applying the "electroswelling process".[95] These GUVs can reach up to 100 μm in diameter,[102,93] and can be considered as a membrane model system to study biological processes in vitro. With these GUVs, a well-defined and cell-independent environment is formed for functional studies of integrated membrane proteins, including microscopic analysis, interaction studies, and ion channel studies.[103]

Using the insect cell-free system, protein synthesis can be carried out in batch format in two different modes: in "linked mode" transcription and translation reactions are separated by a gel filtration step to purify the transcribed mRNA prior to its use in translation; in "coupled mode" transcription and translation take place simultaneously in one reaction chamber. One advantage of the linked method is the option to adapt the reaction temperature and salt concentrations (these differ for transcription and translation), thereby resulting in higher protein yield. The time-saving coupled method is more convenient and user friendly. For a long time, the insect cell-free system was limited to batch reactions that provide fast synthesis of the target protein. However due to the rapid depletion of energy resources and the accumulation of inhibitory byproducts such as free phosphates, only low protein yields (in the $\mu g\,mL^{-1}$ range) range were obtained.[104] In 2014 it was shown that a combination of an insect cell-free translation system with a commercially available CECF device led to a prolonged protein synthesis reaction and a four- to fivefold increase in produced protein (up to $60\,\mu g\,mL^{-1}$).[9] As for the *E. coli* cell-free system, labeling strategies are promising methods to introduce artificial post-translational modifications. For protein interaction studies and single-molecule analysis, functionally active and labeled proteins are required. The commonly used method to label targets with fluorescent proteins (e.g., GFP or YFP) can result in changed or reduced activity of the target due to the relatively large size of the fused proteins.[105] An alternative strategy uses modified tRNAs that are synthetically precharged with a fluorescent non-canonical amino-acid (ncAA). The disadvantage of this strategy is exhaustion of the precharged tRNA pool over time. Therefore an enzymatic tRNA charging procedure was developed, by expanding the cell-free insect system with an orthogonal tRNA/synthetase-pair.[22] The amino-acyl-tRNA-synthetase specifically aminoacylates the matching tRNA with the ncAA. To provide the specific aminoacylation of the orthogonal tRNA, cross-reactivity has to be avoided. Therefore the following conditions have to be fulfilled: 1) the orthogonal synthetase does not aminoacylate endogenous tRNAs with ncAAs; 2) the orthogonal tRNA is not recognized as a substrate for endogenous synthetases; and 3) the orthogonal synthetase does not accept standard amino acids as substrates.[106] Site-directed incorporation can be achieved by introducing a stop codon (usually the amber stop codon UAG) at a defined position in the template. A modified suppressor tRNA that contains the appropriate anticodon (decoding the amber stop codon), the appropriate synthetase, and the desired ncAA are added to the cell-free synthesis reaction mixture, thereby resulting in co-translational incorporation of the supplied amino acid into the target protein. Recently ncAAs were incorporated in a statistical manner as well as in

a site-specific way with subsequent fluorescent labeling of the glycoprotein erythropoietin.[22] The ion channel KcSA was realized in lysates derived from insect cells.[103]

Mammalian Extracts

The successful production of pharmaceutically relevant target proteins is often based on a synthesis setup that is closely related to in vivo conditions; thus, systems have been developed derived from cultured mammalian cells.[107,108] These types of CFPS systems show several characteristics that are beneficial for the production of eukaryotic and especially human proteins. The ability to produce proteins bearing mammalian-like post-translational modifications (thereby exhibiting human-protein-like structures) is the main benefit of these systems.[109] For this reason, mammalian cells have been used for the production of eukaryotic cell-free systems.

Rabbit reticulocytes extracts

CFPS systems based on rabbit reticulocytes have been known since the 1960s. The first protein production was demonstrated by the incorporation of radioactively labeled amino acids into hemoglobin.[110] Reticulocyte lysates were prepared from reticulocytes isolated from rabbits that were made anemic by injection with acetylphenylhydrazine.[111] Initially, rabbit reticulocyte lysate was most frequently used to investigate the molecular steps of protein translation in eukaryotic organisms.[112–114] A development of rabbit reticulocyte lysate was a system for the direct production of target proteins without the background synthesis of endogenous proteins. Removal of endogenous mRNA was achieved by treating the lysate with micrococcal nuclease, an endo-exonuclease that preferentially digests single-stranded nucleic acids.[111] Because the enzyme requires Ca^{2+} ions, its activity can be inhibited by EGTA. Rabbit reticulocyte lysates do not harbor intrinsic microsomal structures. As these structures are often essential for the synthesis of functionally active membrane proteins, the lysate needs to be supplemented with microsomes, which are required for post-translational glycosylation and lipidated proteins as well as for proteins with a cleavable signal peptide. Commonly, heterogeneous canine microsomal membranes are supplemented to reticulocyte lysates[115] for the synthesis of secreted and transmembrane proteins. Alternatively, microsomal membranes from various mammalian systems are added to rabbit reticulocyte lysates.[116,117] The broad range of applications for rabbit reticulocyte lysate includes protein microarray technologies,[118] protein–molecule interaction studies,[119–121] display technologies,[122–124] and screening technologies.[125]

A limitation of protein synthesis systems based on rabbit reticulocyte lysate is the comparatively low protein yield. Interestingly, a recent study has shown that supplementation with viral enhancers of translation, including proteins and mRNA elements like internal ribosomal binding sites, increase protein yield more than tenfold.[126]

Extracts from cultured CHO cells

Chinese hamster ovary (CHO) cells represent the most frequently used mammalian cell-line for the in vivo production of complex therapeutic proteins.[127] Because of the high acceptance and usage of CHO cells for commercial protein production, a cell-free system based on translationally active CHO cell lysates was recently developed.[108,128,129] CHO-lysate-based CFPS systems have enormous potential for the efficient and economic production of a broad range of structurally and functionally diverse proteins. The platform is prepared by mild disruption of cultivated CHO cells, and contains translationally active microsomal structures derived from the ER.[129] Accordingly, CHO cell-free systems promote post-translational modifications including glycosylation of target proteins and co-translational insertion of membrane proteins into biological membranes.[128] Inserted membrane proteins can be directly used for advanced membrane protein characterization. Currently, CHO-lysate CFPS reactions are performed in the coupled transcription–translation mode, thus enabling fast and convenient production of proteins as a basis for convenient high-throughput screening applications.[128] To overcome the key limitation of most eukaryotic cell-free systems (i.e., low protein yield due to limiting translational initiation), internal ribosomal entry sites (IRES) have been used as a strategy to initiate translation. These IRES sequence elements were integrated into the 5′ untranslated region of the DNA template, in order to initiate protein translation in a cap-independent manner. Improved protein production rate for the CHO lysate platform was achieved by using an IRES sequence from the intergenic region (IGR) of the cricket paralysis virus (CrPV).[130] This IRES sequence acts in an initiation-factor-independent manner and consequently bypasses several yield-limiting steps in eukaryotic translational initiation.[131,132] Interestingly, by changing the start codon AUG to GCU, improved IRES sequence efficiency was reached in CHO lysate.[130] The CHO lysate CFPS platform is also a promising system for fast and easy pre-template screening procedures. In this way, DNA constructs can be analyzed in a highly automated and parallel manner in cell-free systems prior to their transfection into CHO cells for subsequent large-scale in vivo production.

Extracts from cultured human cell lines

A wide range of cultured human cell lines is currently available for research and industrial protein production.[133] Originating from almost every kind of human tissue, cell lines have been adapted to in vivo cultivation conditions. Thus, the establishment of several novel cell-free systems offering optimum conditions for human protein production seems feasible. Cell-free systems based on cultured human cells have been derived from HEK293 cells[134] and HeLa cells.[135] A CFPS system based on HeLa cell lysate is commercially available (1-Step Human In Vitro Translation Kit; Thermo Scientific). The characteristics of human cell-free systems are similar to those of CHO cell-free systems. Endogenous microsomes for direct insertion of membrane proteins are available in different human cultivated cell-

lysate-based systems, and different types of post-translational modification can be obtained[107,129] A major benefit of the human CFPS systems is the natural codon usage, thus facilitating the synthesis of high-molecular-weight human proteins.[136] A promising option in extracts derived from cultured human cell lines is the synthesis of entire viruses and virus-like particles.[137] For example, a platform was developed to investigate viral replication mechanisms as a prerequisite for the development of anti-viral drugs.[138] Based on human cell-free systems, picorna viruses,[139,140] the polio virus,[141] and encephalomyocarditis virus[142,143] have been analyzed. Cell-free systems based on cultured human K562 cells displayed protein yields in the range of 20 $\mu g\,mL^{-1}$ in a batch mode for extracts.[129] Various strategies have been applied to increase protein yield, such as focusing on the limited translation initiation in these platforms.[144] It was found that the supplementation with K3L (which binds to eIF2α-kinases) leads to increased protein yield by preventing eIF2α phosphorylation[145,146] Alternatively, addition of GADD34 enhances dephosphorylation of eIF2α, and enhancement of protein synthesis was accomplished by supplementation with translation initiation factors such as eIF2, eIF2B, and eIF4.[144] A further increase in protein yield was achieved in factor-depleted cell lines for lysate production,[147] and by incorporation of IRES sequences into DNA templates.[130]

Outlook and Summary

We have highlighted current CFPS systems and their applications as research tools for understanding translation regulation, and their use as a potential protein production platforms (Table 1).

Beginning with studies on the translational processes in 1960 by Nirenberg and Matthaei,[3] cell-free systems have matured into protein producing, modifying, and analyzing tools. In the long term, well-established systems such as those based on *E. coli* and wheat-germ extracts will compete with the recently developed eukaryotic cell-free systems based on insect and mammalian cell extracts. In these lysates post-translational modifications are feasible, and improved protein folding is usually obtained.[148,149] Therefore, the potential to produce functionally active proteins is improved significantly in the novel eukaryotic cell-free systems. Protein folding and post-translational modifications are facilitated in tobacco-, insect-, and mammalian-based cell-free systems because of the presence of endogenous microsomal structures. These ER-derived structures enable co-translational translocation of de novo synthesized proteins. In *E. coli*, rabbit reticulocyte, and wheat-germ systems exogenous membrane-mimicking structures such as bicelles, liposomes, nanodiscs, and microsomes are usually supplemented (reviewed in ref. [150]). The synthesis of multidomain membrane proteins and their co-translational integration into liposomes is feasible and has been demonstrated in an *E. coli* cell-free system for F_0-F_1-ATP synthetase, and in a wheat germ cell-free system for the human stearoyl-CoA desaturase complex.[47,151] Optimization of this approach facilitated in vitro reconstitution of complex signal transduction pathways. An initial approach to creating a metabolic pathway was presented in 2011 by Guarino and DeLisa.[152] Purified glycosylation components were derived from the bacterium *Campylobacter jejuni* and implemented in *an E. coli* cell-free system. A characteristic of the *C. jejuni* strain is the incorporation an N-linked glycosylation system that is functionally similar to those of eukaryotes and Archaea.[153] The combination of purified glycosylation components with the *E. coli* cell-free system resulted in the glycosylation of the model glycoprotein AcrA (100–150 $\mu g\,mL^{-1}$) as well as the glycoengineered single-chain variable fragment 13-R4-GT (50–100 $\mu g\,mL^{-1}$).[152] The reconstitution of additional components in cell-free systems such as the glycosyltransferase,[154] might result in an expanded glycosylation pathway. Despite the advantage of endogenous membrane structure in eukaryotic cell-free systems, improvements to productivity and scalability are still challenging. The lower protein yields of insect and mammalian cell-free systems are in part attributable to the efficiency of translation initiation. In order to achieve efficient translation initiation, polyadenylation and capping of mRNA are essential.[112] Nevertheless, capping of mRNA is expensive, and unbound caps negatively influence the initiation factor eIF-4E.[155] For this reason, cap-independent translation initiation as described for *L. tarentolae* is desirable. The introduction of a structured IRES mRNA sequence[156] upstream of the target sequence is a promising strategy. By binding directly to ribosomes, the IRES sequence functions as a translation initiator. In 2013, the positive influence of the intergenic region (IGR IRES) of cricket paralysis virus was demonstrated for insect, CHO, and human CFPS systems.[130] Efficient strategies to introduce ncAAs site-specifically into de novo synthesized proteins to improve proteins functionality are highly desirable.[157,158] The increasing number of well-established orthogonal translation systems for site-specific incorporation of ncAAs reflects their impact on this research field.[159] As cell-free systems represent "open" systems, the required additional components, such as modified amino acids and amber suppression tRNA/synthetase pairs, can easily be supplemented. ncAA technology has been applied to mimic the post-translational modifications of eukaryotic proteins, such as in vivo site-specific acetylation of recombinant histones by acetyllysine.[160] Recent applications of ncAA incorporation in *E. coli*-based cell-free systems include the development of novel therapeutics, polymers, and enzymes (reviewed in refs. [37]and [161]). In particular, the cell-free synthesis of homogenous antibody–drug conjugates (an antigen-binding IgG coupled to a chemotherapeutic cytotoxin) is a promising tool to generate highly specific cancer therapeutics.[162] Swartz and co-workers improved the reporter function of *Gaussia princeps* luciferase by incorporation of homopropargylglycine and attaching poly(ethylene glycol).[163] The modified luciferase exhibited a much longer luminescence half-life. Protein engineering in *E. coli* cell-free systems with ncAAs has resulted in human therapeutics,[164] modified enzymes,[163–165] protein polymers,[166] and novel biologics selected by ribosome display methods.[167] As the incorporation of ncAAs into proteins in prokaryotic cell-free systems has resulted in many successful modification of proteins characteristics, this method was applied to eukaryotic cell-free systems. Incorporation of an ncAA into a single-chain

Table 1. Comparison of different CFPS systems.

System	Advantages	Disadvantages	Applications	Refs.
Prokaryotic				
E. coli extract	1. High protein yield 2. Simple cultivation and fast cell growth and lysate preparation 3. Cost-efficient 4. Easy genetic engineering 5. Well-established	1. Limited post-translational modifications 2. No endogenous membrane structures for the synthesis of integral membrane proteins 3. Only native prokaryotic chaperones available: eukaryotic proteins might not be correctly folded	1. Incorporation of non-canonical amino acids, hence protein engineering for the development of human therapeutics, modified enzymes, protein polymers, and ribosome display methods 2. Large-scale synthesis (up to 100 L) reaction volume 3. Industrial production of antibody–drug conjugates	[7, 165] [163] [1] [164] [166]
Archaeal extract	1. Extreme synthesis conditions like high temperature, so reduced inhibition of translation by secondary structures in mRNA 2. Synthesis of correctly folded thermostable proteins	1. Low protein yield	1. Synthesis of thermostable proteins	[49] [51] [52]
Eukaryotic				
Protozoan extract	1. Less expensive cultivation and lysate preparation 2. Good scalability 3. High solubility of synthesized proteins 4. Enhanced initiation of translation by addition of a special RNA sequence	1. Types of post-translational modifications not well known 2. Less used or established 3. Low protein yield	1. High-throughput analysis by PCR-based CFPS	[65] [63] [64] [61]
Yeast extract	1. Ability to perform post-translational modifications like glycosylation 2. Simple and fast cultivation of cells for lysate preparation 3. Well-known in vivo system, so established methods for cell engineering available	1. Low protein yield 2. No mammalian-like post-translational modifications	1. Production of virus like particles for anti-viral drug research 2. Production of bioethanol and (*S*)-L-acetoxyalkan-2-ol in cell-free bioreactors	[66] [177] [28, 72] [73] [74] [174]
Wheat germ extract	1. Highly productive cell-free system, so high yield of complex proteins 2. Systems available for synthesis of disulfide-bridged proteins 3. Correct folding of many protein types, so high solubility of proteins 4. Well-known system	1. Laborious and expensive lysate preparation 2. Limited post-translational modifications possible 3. No endogenous membrane structures 4. Low protein yield compare to prokaryotic and wheat germ systems	1. Production of malaria proteins to characterize novel vaccine candidates 2. On-chip protein synthesis 3. High-throughput applications 4. Production of monoclonal antibodies against GPCRs 5. Investigation of translational processes (conformation analysis of ribosomes)	[7, 80] [4, 83] [86] [81] [20] [175] [176]
Tobacco BY-2 extract	1. Fast and easy lysate preparation procedure 2. Glycosylation and disulfide-bridge formation are possible 3. Yield comparable to wheat germ extracts	1. Limited evaluations of the system are available 2. Contain endogenous amino acids, so difficult to obtain good protein yield	Novel cell-free system High potential for future applications	[87] [88] [89] [90]
Insect cell extract	1. Easy and fast lysate preparation 2. Post-translational modifications are possible (glycosylation, disulfide-bridge formation, lipidation, signal peptide cleavage phosphorylation) 3. Endogenous microsomes are available 4. Direct synthesis and integration of membrane proteins	1. High cultivation costs	1. GUV formation for membrane protein model to study biological processes 2. Engineering of proteins with integration of non-canonical amino acids 3. Automated production of membrane proteins	[92, 104] [95, 97] [17] [16] [103] [21]
Rabbit reticulocyte extract	1. Well-established system 2. Mammalian system 3. Protein synthesis in the presences of supplemented, heterogeneous microsomes possible (e.g., canine pancreas microsomes)	1. Low protein yield 2. Post-translational modifications only possible by supplementation with exogenous microsomes 3. Treatment of living animals required for lysate preparation	1. Protein microarray technologies 2. Protein–molecule interaction studies 3. Display technologies 4. Screening technologies	[110] [111] [115] [117] [125] [126]
CHO cell extract	1. Well-known and characterized cell line (often used for pharmaceutical in vivo protein production) 2. Contain endogenous microsomal structures 3. Mammalian post-translational modifications 4. Direct production of membrane proteins 5. Increase in protein yield by IRES-mediated translation initiation	1. Low protein yield compared to prokaryotic cell-free systems 2. High cultivation cost 3. Robust cell line	1. Novel cell-free system 2. High potential for future applications	[108] [130] [128] [129]

Table 1. (Continued)

System	Advantages	Disadvantages	Applications	Refs.
Extract from cultured human cell lines	1. Optimal environment for correct folding and assembly of human proteins 2. Contain endogenous microsomal structures 3. Human-like post-translational modifications possible 4. Adapted codon usage to facilitate synthesis of high-molecular- weight proteins	1. Low protein yield compared to prokaryotic cell-free systems 2. High cultivation cost 3. Laborious cultivation technologies are necessary (sensitive cells)	1. Investigation of viral replication mechanisms; development of antiviral drugs	[135] [141] [143, 144] [136, 142] [107, 138] [130] [129]

antibody fragment and into the potassium channel KcsA was achieved in an insect-cell free system[168, 101, 103] In addition, structural changes to flavin-mononucleotide-binding protein were achieved by the incorporation of ncAAs into a wheat germ cell-free system.[169] The incorporation of ncAAs bearing a fluorophore has been employed to analyze protein–protein interactions, ligand-binding studies by FRET, and structural conformational changes.[170, 171] In addition to the therapeutical and analytical approaches, a highly investigated field is the industrial large-scale cell-free production of proteins. Scaling up cell-free batch-based reaction systems might be limited in certain cases: decreasing protein yield with increasing reaction volumes caused by an altered surface-to-volume ratio, thus resulting in a reduction in hydrophobic surface area.[172] As a solution to this problem, a thin film was added to the semi-continuous cell-free reaction, in order to provide a hydrophobic surface to facilitate protein synthesis and folding: protein yield did not decrease with increasing reaction volume.[172] Wheat-germ and *E. coli* systems are already used as cell-free production platforms for vaccines and new therapeutics against malaria[85, 84] and human parainfluenza virus type 3,[173] as well as for cytokines and antibodies.[48, 162]

The manifold diversity and increasing number of novel cell-free systems clearly illustrate the high potential of CFPS. The broad range of current applications in research and industrial protein production show the high applicability of CFPS systems. Moreover, the efficient production and characterization of proteins that are difficult to express in living cells (e.g., toxic proteins, several membrane proteins, some post-translationally modified proteins) might provide novel functional and pharmacological insights.

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