Pharmaceutical BIOPROCESSING

Developing cell-free protein synthesis systems: a focus on mammalian cells

Sophisticated cell-free protein synthesis (CFPS) systems have been developed as an alternative to recombinant expression in cultured cells. In this review, we present advances in the field of mammalian-based CFPS by highlighting recently established systems derived from mouse fibroblasts, HeLa, hybridoma, CHO and K562 cells. We further highlight ongoing challenges in the field of mammalian-based CFPS, such as the optimization of already established platforms and the development of novel systems in order to further increase protein yields and reduce manufacturing costs while facilitating the synthesis of a huge number of biologically active target proteins. Advances in mammalian-based CFPS shall expand the number of future applications of CFPS in the area of pharmaceutical research and development.

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Cell-free protein synthesis (CFPS) represents an alternative to cell-based expression as it dramatically improves the development of engineered proteins. CFPS systems utilize the translation machinery preserved in cell extracts in combination with supplemented amino acids, RNA polymerase, salts and nucleoside triphosphates to produce a protein of interest (Figure 1). The advantages of CFPS over recombinant protein production in cultured cells have been described in previous articles [1–3] and will not be discussed here. Instead, we would like to highlight the particular potential of cell-free systems derived from mammalian cells.

Mammalian cell extracts offer a biochemical environment that is closely related to living human cells. By contrast, the protein synthesis and modification machinery present in lysates derived from *Escherichia coli*, insect cells or wheat germ does not necessarily enable the production of human-like recombinant proteins. Thus, the use of mammalian cell extracts is superior for gaining deeper insight into human regulatory networks in particular processes needed for protein synthesis. Furthermore, mammalian-based CFPS offers a better acceptance of human genes as templates for the synthesis and correct folding of complex and multimeric human proteins. These proteins often require the formation of co- and post-translational modifications, such as glycosylation, phosphorylation and lipid modification, in order to achieve their appropriate conformation and thus their functional activity. In this respect, the use of mammalian cell extracts is advantageous, as protein glycosylation and lipid modification are currently limited to CFPS based on mammalian and insect cell lysates [4–7]. Since it can be assumed that co- and post-translational modifications in CFPS depend on the primary cells, mammalian cell extracts favor the generation of more human-like proteins. Last but not least, mammalian-derived CFPS systems can serve as a powerful tool for the time-saving evaluation of plasmid-based expression templates prior to an upscaled protein production in cultured mammalian cells. Some conceivable applications of this method are the evaluation of gene-specific mutations, the analysis of purification tags including their terminal position, the optimization of the encoded gene's codon composition and the investigation of signal peptides.

Advances in mammalian-based CFPS

Rabbit reticulocyte lysates (RRL) have been used for CFPS since the 1950s and are currently the most popular source for eukaryotic CFPS [1,8–10]. Besides the RRL [9,11–12], extracts from Ehrlich ascites cells [13,14],

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Key Terms

Microsomal membranes: Vesicle-like structures re-formed from the endoplasmic reticulum during the process of cell lysate preparation.

Linked system: Transcription and translation reaction is separated by an intermediate mRNA purification step.

HeLa cells [15,16], CHO cells [17,18] and mouse L cells [19] have been prepared as a research tool to study mRNA translation. Transfer of proteins across membranes was investigated by adding canine pancreatic **microsomal membranes** to the translationally active extracts [20–23]. This led to a deeper understanding of basic biological processes, such as protein translocation, the impact of signal recognition particles [22], protein glycosylation and signal peptide cleavage [23]. Meanwhile, translocation-competent microsomes have been derived from a wide variety of additional tissues, such as rat liver [24], sheep pancreas [25], hen oviducts [26] and *Drosophila melanogaster* embryos [27]. Finally, the use of rough microsomes derived from canine pancreas has become the preferred choice for protein translocation studies in RRL [28–30].

Nevertheless, the use of these conventional CFPS systems has several drawbacks. One main limitation is their short lifetime resulting in low yields of only up to a few micrograms target protein per milliliter of reaction mixture. Another drawback is the obvious fact that co- and post-translational processing can only be performed after supplementing microsomal membranes to the cell extracts and preparation of these rough microsomes requires ethically questionable sacrifice of animals [20,21]. Therefore, the use of recently established lysates derived from cultured insect cells and mammalian cells is preferred, as these extracts already contain endogenous microsomes derived from the endoplasmic reticulum of the cultured cells. An emerging number of publications illustrate the application of these cell-free systems for the synthesis of co- and post-translationally modified proteins [5,31–35].

Various CFPS systems based on cultured mammalian cells have been developed in the past decade in order to address drawbacks such as high manufacturing costs, variations in translational activity depending on lysate batches and the ethically questionable preparation of RRL as it requires the sacrifice of rabbits (Table 1).

Zeenko and coworkers have established a **linked CFPS system** based on cultured mouse embryonic fibroblasts exhibiting a mutation in the eIF2α gene [6]. Phosphor-

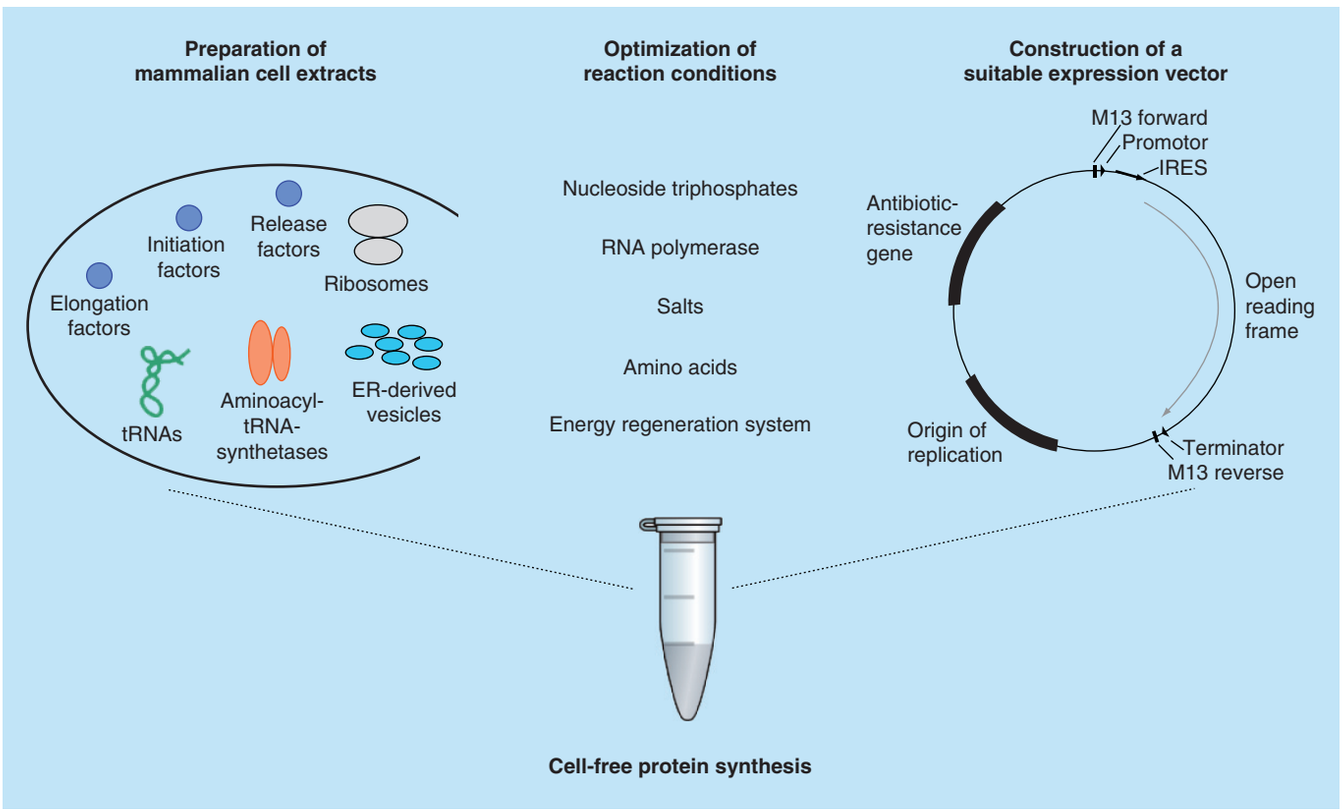

Figure 1. Development of cell-free protein synthesis systems based on extracts from cultured mammalian cells. The development includes the preparation of mammalian cell extracts, the optimization of reaction conditions and the construction of a suitable expression construct.

Table 1. Cell-free protein synthesis systems derived from mammalian cells. Protein yield was determined based on cell-free synthesized active firefly luciferase.

Cell extract	Mode of operation	Yield (μg/ml)	N-glycosylation	Ref.
CHO cells	Coupled	49	Yes	[32]
HeLa cells	Linked	NA	NA	[15]
HeLa cells	Linked/coupled	NA	Inefficient	[36,37]
Hybridoma cells	Linked	NA	Yes	[5]
K562 cells	Coupled	21	Yes	[7]
Mouse fibroblasts	Linked	20	Yes	[6]
Rabbit reticulocytes	Linked/coupled	1–5	No	[9]

NA: Not available.

ylation of the initiation factor eIF2α during translation reaction has been reported as a limiting step in the achievement of higher protein yields [5]. In this context, lysates derived from mutated fibroblasts avoid the insufficient translation initiation caused by phosphorylation of the initiation factor eIF2α on Ser51, resulting in a 30-fold increased translational efficiency compared with wild-type extracts. Protein synthesis based on these fibroblast extracts is active for at least 2 h, generating up to 20 μg/ml firefly luciferase [6]. Furthermore, the established CFPS platform has the potential to perform signal sequence processing and N-linked glycosylation.

Mikami and coworkers have developed a cell-free translation system based on HeLa cells that is configured in a linked mode of operation, meaning that transcription and translation reactions are separated by an intermediate mRNA purification step [36]. For enhanced protein synthesis levels, the HeLa cell extract is supplemented with translation initiation factors (eIF2, eIF2B, eIF4) and translational regulators, such as the nuclear protein import factor p97. The linked mode of operation was later transferred to a coupled reaction mode [37]. **Coupled systems** are often favored as they save user's time and effort, require reduced manufacturing costs due to the avoidance of gel filtration columns for mRNA purification and are easily applicable in automated devices. In the coupled system derived from HeLa extracts, cost-intensive capping of mRNA was replaced by cap-independent translation based on the encephalomyocarditis virus **internal ribosomal entry site** (IRES) or the hepatitis C virus IRES. As glycosylation of target proteins was very inefficient using HeLa cell extracts, a hybridoma-based *in vitro* translation system that enables N-linked glycosylation has been additionally developed by Mikami and coworkers [5]. Hybridoma cell extracts have been described to be superior to HeLa cell lysates in terms of glycosylation efficiency, the synthesis of membrane-associated proteins as well as the cleavage of signal peptides. The authors have reported an increased protein synthesis in both, HeLa and hybridoma cell extracts by adding the recombinant factors GADD34 and/or K3L that diminish phosphorylation of eIF2α [5]. Additionally, the batch-based CFPS system derived from HeLa cells was transferred to a continuous mode of operation for obtaining higher yields of the desired protein.

A novel high-yield CFPS system based on translationally active lysates from CHO cells has been recently reported [32]. The coupled CFPS platform uses an IRES from the intergenic region (IGR) of the cricket paralysis virus (CrPV) that functions without the need for any limiting initiation factors, such as eIF2α. IGR IRES elements have been previously reported to even increase translational efficiency if the amount of functional initiation factors is limited [38,39]. The cap-dependent decrease of protein synthesis by eIF2α phosphorylation under stress conditions, for instance, enhances IGR IRES-mediated translation owing to the increased availability of 40S ribosomal subunits [39,40]. The combination of the CrPV IGR IRES-based expression vector and the CHO cell extract enables the production of up to 50 μg/ml active firefly luciferase within 4 h [32]. In addition, the CHO cell extract contains translocationally active microsomes that are capable of performing glycosylation, as has been demonstrated by the analysis of human erythropoietin. These microsomes can further be used for the embedment of membrane-spanning proteins into the lipid bilayers for advanced functional studies. As the CrPV IGR IRES has been shown to function efficiently in a broad range of eukaryotic cell lysates, a human K562-derived CFPS

Key Terms

Coupled system: Combination of transcription and translation in one reaction compartment.

Internal ribosomal entry site: A highly structured RNA sequence found within the 5′ untranslated region of viral genomes or cellular mRNAs that functions to recruit ribosomes for the initiation of translation.

Key Term

Bio-orthogonal: Refers to any chemical reaction that occurs without interfering with native biochemical processes in cell-based and cell-free systems.

system has been developed based on CrPV IGR IRES-mediated translation [7]. The coupled CFPS platform enables the synthesis of up to 21 µg/ml active firefly luciferase, the formation of N-glycosylation and the embedment of membrane proteins for downstream functional characterization [7].

In the last decade, CFPS systems derived from primary mouse keratinocytes [41,42], HeLa cells [15,43], Krebs-2 ascites cells [44] and HEK293F cells [45] have been developed to provide biochemical tools for the investigation of basic biological processes in specific types of cell lysates. For instance, Bergamini and coworkers have established an *in vitro* translation system derived from HeLa cells in order to study translation of mammalian and viral mRNAs in a mammalian-like environment [15]. The linked HeLa-based CFPS system has been successfully applied for investigating the effect of the poly(A) tail on the translation of picornaviral RNAs from three different classes of IRES [46]. Subsequently, the utilized *in vitro* translation system has been modified to an efficient factor-depleted platform providing a powerful tool for the investigation of translational control [47]. This CFPS system is based on RNA interference-mediated knockdown of the factor of interest prior to cell extract preparation and thus overcomes limitations regarding the availability of specific antibodies as well as the co-depletion of proteins associated with the factor under study.

Ongoing challenges

In the past decade, substantial progress has been made in the development of novel mammalian-based CFPS systems. However, there are still limitations that are currently in the focus of research and developmental efforts. Ongoing challenges in the field of mammalian-based CFPS are the optimization of already established platforms and the development of novel systems in order to enable even higher protein yields and lower manufacturing costs while facilitating the synthesis of a huge number of biologically active target proteins.

A better understanding of the biological processes taking place in the individual CFPS reaction might enable the improvement of the technology. For instance, expensive energy regenerating agents, RNase and protease inhibitors as well as polymerases currently supplied to eukaryotic lysates could be replaced by cheaper ones in order to lower manufacturing costs. Increased protein yields might be achieved by optimizing reaction conditions and transferring batch-based CFPS reactions to continuous dialysis systems [3,36,37,48]. The challenge of synthesizing correctly folded proteins, in particular proteins with co- and post-translational modifications, might be addressed by advanced cell extract preparation procedures, optimized reaction conditions as well as the supplementation of the lysates with the appropriate chaperones. Furthermore, mammalian cell extracts holding an appropriate oxidizing environment enable the formation of disulfide bonds in cell-free-produced proteins, as has been demonstrated for *Sf*21-based CFPS [49,50]. Target proteins could be engineered in a desired manner by making use of the open accessible nature of the CFPS reaction. In this context, **bio-orthogonal** systems provide a promising tool for the incorporation of chemoselective reactive amino acids into synthesized proteins at defined positions by expanding the genetic code. These amino acids can subsequently be modified in a desired manner by the addition of a corresponding reaction partner, resulting in novel characteristics of the target protein. Several bioorthogonal and chemoselective reactions have been identified and applied in cell-based [51,52] as well as cell-free systems [31,53–54] in recent years. The copper(I)-catalyzed azide alkyne cycloaddition, based on the click chemistry concept, is one prominent example of such a chemoselective reaction [54–56]. In general, characterization of cell-free synthesized proteins by mass spectrometry and additional analytical methods will assist in achieving the above-mentioned goals.

Future perspective

Technical advances in mammalian-based CFPS will obviously expand the number of applications of CFPS in the field of pharmaceutical research and development (Figure 2). In this review, we provide an insight into some prospective applications.

Just-in-time protein production using high-throughput technologies

High-throughput protein synthesis platforms for drug screening are becoming increasingly important. To date, mostly conventional expression systems based on intact cells are applied for protein-based drug discovery. However, the use of CFPS platforms may be advantageous to cell-based methods as it enables the parallel synthesis of different target proteins directly from PCR-based templates without time-consuming cloning procedures [57–59]. In this way, ligation of the DNA template to a linearized plasmid, transformation, selection of colonies harboring the desired plasmid, cultivation of positive colonies, plasmid isolation, sequencing, cell transfection and cell cultivation

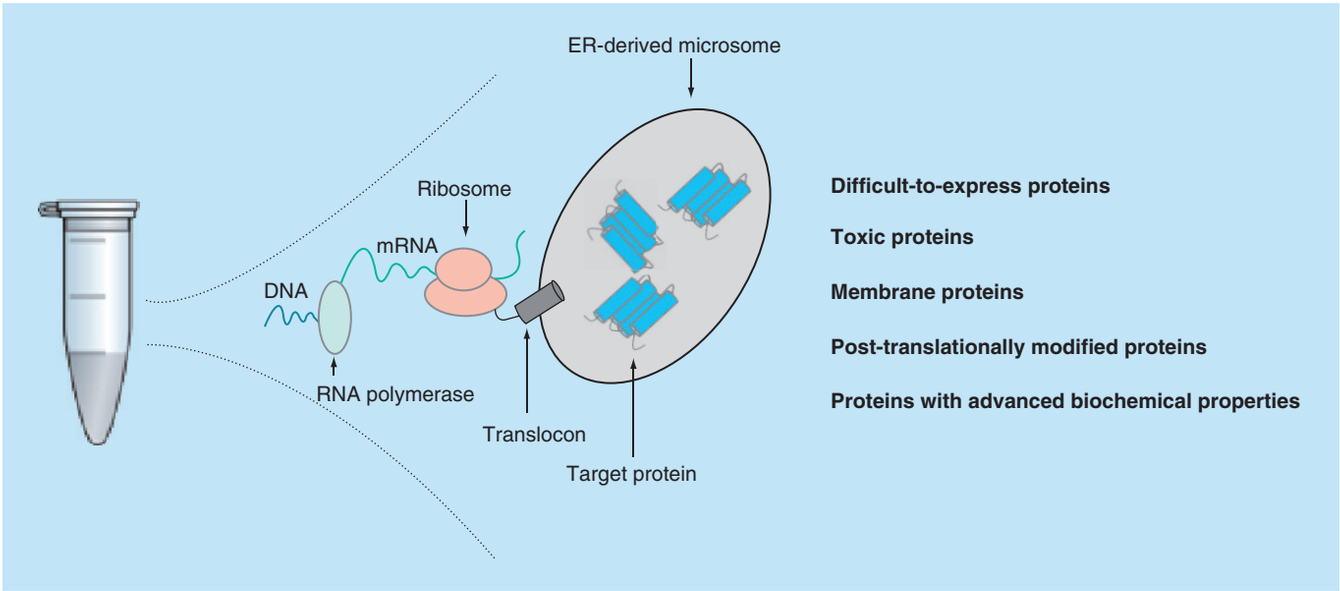

Figure 2. Cell-free synthesis of pharmaceutically relevant proteins. Advanced mammalian-based cell-free protein synthesis systems already contain endogenous microsomes derived from the endoplasmic reticulum of the cultured cells. These systems can be used for the synthesis of pharmaceutically relevant proteins, such as difficult-to-express proteins (e.g., toxic proteins, membrane proteins), post-translationally modified proteins (e.g., disulfide-bonded proteins, glycoproteins) and proteins with advanced biochemical properties.
Modified with permission from [33].

for protein production can be avoided. High-throughput CFPS methods are further favored by short reaction times and their potential for miniaturization and automation [60,61]. These benefits turn CFPS into a valuable tool to speed up the entire process and product development timeline. Thus, mammalian-based CFPS is a promising alternative to conventional cell-based expression methods as it dramatically accelerates the development and manufacture of proteinogenic pharmaceuticals, for example, antibodies, as well as the production of novel targets, for example, viral surface proteins, for early-stage pharmacological validation.

Difficult-to-express proteins

Despite many promising aspects of cell-based recombinant protein production, CFPS platforms are the system of choice for the synthesis of pharmaceutically relevant proteins that are not physiologically tolerated by an intact cell, such as toxic proteins [62], certain membrane proteins [63–66], proteolytically sensitive proteins and unstable proteins [1]. In particular, membrane-spanning proteins are of pharmaceutical interest since approximately one-third of all human genes encode membrane proteins and these proteins often possess essential relevance for cell viability [67,68]. Accordingly, many diseases and more than half of all drug targets are attributed to membrane proteins [69–72]. Despite their physiological significance, biophysical and structural analysis of membrane-spanning proteins lags far behind the characterization of cytosolic proteins due to low abundance in membranes and their low yields in cell-based expression systems [69,73,74]. These low yields can mainly be attributed to toxic effects of the synthesized membrane protein, for example, particular ion channels, transporters and receptors on host cells [66,69]. CFPS offers the potential to bypass these toxic effects and thus facilitates the expression, optimization and production of recombinant membrane-spanning proteins in the presence of microsomes or synthetic lipids to embed the target protein into a native-like environment [34,75–78].

Structural proteomics

Labeling of recombinant proteins with stable isotopes is an essential prerequisite for a variety of techniques, such as NMR spectroscopy and x-ray crystallography [79]. Protein labeling in intact cells is often hindered by interfering amino acid metabolic processes, which may be bypassed by CFPS [80,81]. Amino acid-selective and site-directed stable-isotope labeling of cell-free synthesized proteins is often accomplished in prokaryotic *in vitro* translation systems [79,81]. Nevertheless, preparation of specifically labeled NMR samples is still challenging owing to isotope scrambling problems. In this context, improved mammalian-based CFPS platforms are a promising tool in the field of structural proteomics, as in general isotopic labeling in CFPS sys-

tems can be easily accomplished compared with *in vivo* methods [2,81].

Synthesis of proteins with advanced biochemical properties

Novel methods for engineering proteins by the introduction of noncanonical amino acids in either a residue-specific or site-specific manner have advanced rapidly in the past few years. In this context, CFPS systems provide a versatile platform for protein engineering by directed evolution [82–86]. CFPS platforms enable the incorporation of chemically modified amino acids, for example, fluorescence-detectable amino acids and modified amino acids that can undergo click-chemistry reactions, into *de novo* synthesized target proteins at defined positions in order to introduce novel characteristics for advanced biochemical properties [87–91]. In this respect, CFPS systems derived from mammalian cells offer important advantages over their corresponding recombinant expression platforms based on intact cells as toxic side effects of noncanonical amino acids can be circumvented and novel eukaryotic cell-free orthogonal translation systems have been successfully implemented [31]. In addition, CFPS platforms can be easily modified to incorporate unusual side chain structures into target proteins by adjusting the components of the translation machinery [53,89,92]. Thus, the use of mammalian-based CFPS systems opens a wide variety of new opportunities for protein engineering, structural analysis and protein interaction studies in the field of pharmaceutical research and development [3].

Conclusion

CFPS constitutes a versatile alternative to recombinant protein production in cultured mammalian cells since major limitations, such as high manufacturing costs and low protein yields, have been successfully addressed in prokaryotic and eukaryotic CFPS in recent years [61,93]. Eukaryotic CFPS platforms are currently applied for basic science as well as for novel technological applications, for example, antibody production [49,50]. As we assume that a growing number of the remaining challenges will be solved within the next few years, the number of applications of CFPS derived from mammalian cells will steadily increase as well. In conclusion, mammalian-based CFPS has a great potential to establish itself as a powerful bioproduction system in the postgenomic era.

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Executive summary

Background

- Mammalian-based cell-free protein synthesis (CFPS) systems are superior to *in vitro* production platforms derived from other cell sources for several reasons:
- The contribution of eukaryotic translational control mechanisms to the regulation of gene expression can be monitored and optimized in mammalian CFPS systems.
- The linked correlation of the codon usage in human genes of interest with their translation efficiency in mammalian CFPS systems ensures high-yield protein production.
- The synthesis of complex human proteins has been demonstrated in mammalian cell extracts.
- Time-saving evaluation of expression templates in cell-free mammalian systems prior to protein production in mammalian cells ensures maximum yield of functional protein.
- Eukaryotic chaperones present in mammalian-based CFPS systems favor optimum protein folding as a prerequisite for maximum functionality.

Advances in mammalian-based CFPS

- Several CFPS systems derived from mammalian cells have been developed in the last decade in order to address the drawbacks of conventional CFPS platforms. Advantages of these mammalian-based CFPS systems include:
- Option to maintain low manufacturing costs while reaching high protein yields.
- Endotoxin-free production of cytosolic, secreted and transmembraneous proteins.
- Formation of co- and post-translational modifications, for example, glycosylation.
- Cotranslational embedment of membrane-spanning proteins into microsomal membranes present in mammalian cell lysates supports maximum functional activity of the synthesized membrane proteins.

Ongoing challenges & future perspective

- Ongoing challenges in the field of mammalian-based CFPS are the optimization of already established platforms and the development of novel systems in order to further increase protein yields and reduce manufacturing costs while facilitating the synthesis of a huge number of biologically active target proteins. Advances in mammalian-based CFPS will further expand the number of future applications of CFPS in the area of pharmaceutical research and development.

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