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Overview of Cell-Free Protein Synthesis: Historic Landmarks, Commercial Systems, and Expanding Applications

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Abstract

During early days of molecular biology, cell-free protein synthesis played an essential role in deciphering the genetic code and contributed to our understanding of translation of protein from messenger RNA. Owning to several decades of major and incremental improvements, modern cell-free systems have achieved higher protein synthesis yields at lower production costs. Commercial cell-free systems are now available from a variety of material sources, ranging from "traditional" *E. coli*, rabbit reticulocyte lysate and wheat germ extracts to recent insect and human cell extracts to defined systems reconstituted from purified recombinant components. Though each cell-free system has certain advantages and disadvantages, the diversity of the cell-free systems allows *in vitro* synthesis of a wide range of proteins for a variety of downstream applications. In the post-genomic era, cell-free protein synthesis has rapidly become the preferred approach for high throughput functional and structural studies of proteins and a versatile tool for *in vitro* protein evolution and synthetic biology. This article provides a brief history of cell-free protein synthesis and describes key advances in modern cell-free systems, practical differences between widely used commercial cell-free systems, and applications of this important technology.

Keywords

Cell-free protein synthesis; high throughput; unnatural amino acid; isotope labeling; *in vitro* protein evolution; artificial cell

INTRODUCTION

Cell-free protein synthesis is one of widely used methods in molecular biology. Production of proteins using cell-free protein synthesis usually takes a few hours, in contrast to production of proteins in cells, which typically takes days if not weeks. In fact, even first-time users can often obtain newly synthesized proteins on the same day he or she begins to use a commercial system. But, even though many users will use commercial systems rather than making them in their own laboratories, users should understand the processes and mechanisms underlying cell-free protein synthesis. These have recently been reviewed in (Spirin and Schwartz, 2008). Here, I briefly review early history of cell-free protein synthesis, then describe key advances that have led to modern cell-free systems. I review

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practical differences between currently available commercial cell-free systems and the increasing number of applications of cell-free protein synthesis.

EARLY HISTORY OF CELL-FREE PROTEIN SYNTHESIS

At the dawn of molecular biology, many laboratories used cell-free protein synthesis to address the question of how amino acids are incorporated into proteins. Researchers conducted cell-free protein synthesis using the extracts of rat liver cells (Zamecnik et al., 1948), bacteria (Gale and Folkes, 1954; Lamborg and Zamecnik, 1960), human and rabbit reticulocytes (Bank and Marks, 1966; Schweet et al., 1958), ascites cells (Keller and Littlefield, 1957) and wheat germ (Marcus and Feeley, 1966). They discovered protein synthesis occurred in the microsome fraction that requires ATP and GTP (Hoagland et al., 1956; Littlefield et al., 1955). In 1961, Nirenberg and Matthaei found the first correspondence between nucleotide triplets and the amino acids they encoded, demonstrating cell-free synthesis of polyphenylalanine from synthetic polyuridylic acid in the *E. coli* extract (Nirenberg and Matthaei, 1961). In the years that followed, cell-free synthesis continued to play an important part in identifying the amino acids encoded by the remaining triplets, that is, in breaking the remaining characters in the genetic code (Nirenberg, 2004), and establishing the “central dogma of molecular biology” (Crick, 1970; Strasser, 2006).

LANDMARK ADVANCES IN THE DEVELOPMENT OF CELL-FREE PROTEIN SYNTHESIS SYSTEMS

Since the preparation processes did not remove messenger RNA (mRNA), the cell extracts that comprised early cell-free systems synthesized mostly native proteins from endogenous mRNA. These extracts were essentially supernatants obtained by high speed centrifugation of lysates from disrupted cells *e.g.*, at 30,000*g* (the so-called S30 extract) (Lamborg and Zamecnik, 1960; Matthaei and Nirenberg, 1961; Schweet et al., 1958; Zamecnik et al., 1948). To direct the protein synthesis machinery to translate exogenous mRNA, Nirenberg and Matthaei incubated the *E. coli* S30 extract in the presence of 20 amino acids and ATP regeneration components (Nirenberg and Matthaei, 1961). Such “incubated S30” improved the amount of the protein synthesized from exogenous mRNA, possibly by freeing ribosomes from attaching to endogenous mRNA (ribosome run-off) and allowing endogenous mRNA to be degraded by ribonucleases in the extract. Similarly, Pelham and Jackson added a Ca^{2+}-dependent micrococcal ribonuclease during the preparation of the rabbit reticulocyte extract (lysate) to degrade endogenous mRNA (Pelham and Jackson, 1976). The micrococcal ribonuclease can be subsequently inactivated by EGTA to prevent its digestion of exogenous mRNA (Pelham and Jackson, 1976). Interestingly, the wheat germ extract does not translate a significant amount of endogenous proteins, therefore, there is no need for incubation or nuclease-treatment (Roberts and Paterson, 1973).

The next landmark improvement in cell-free protein synthesis was the introduction of coupled transcription-translation systems that used DNA as the template instead of mRNA (Chen and Zubay, 1983; Zubay, 1973). Zubay and colleagues used the *E. coli* RNA polymerase in the S30 extracts to transcribe DNA into mRNA for protein synthesis, mainly

for the purpose of studying gene regulation *in vitro* (Zubay, 1973). To obtain higher amounts of mRNA, other researchers later used stronger phage promoters and the more efficient phage RNA polymerases (T7 or SP6) for the coupled transcription-translation from the DNA template (Craig et al., 1992; Krieg and Melton, 1987; Nevin and Pratt, 1991). The coupled transcription-translation based on the phage promoters and RNA polymerases has since been adopted by most cell-free systems (Table 1). In addition to using the more stable DNA template and avoiding a separate *in vitro* transcription step, coupled transcription and translation in the cell-free system often result in a higher protein synthesis yield, probably due to newly synthesized mRNA is immediately translated into protein, which may minimize the adverse effects of mRNA degradation or inhibitory structures.

Cell-free protein synthesis is commonly conducted in batch format. However, the protein synthesis reactions often stop after a short period of time, in many cases in only 1 hr or so, due to depletion of ATP and GTP, degradation of mRNA and accumulation of by-products to inhibiting concentrations. Consequently, the batch cell-free protein synthesis normally yields μg ml^{-1} of protein. Spirin and colleagues introduced the concept of continuous cell-free protein synthesis in both *E. coli* and eukaryotic cell-free systems (Spirin et al., 1988) (Ryabova et al., 1998; Shirokov et al., 2007). The continuous cell-free protein synthesis circumvents some of the issues of the batch format by using a flow chamber or dialysis membrane to maintain the levels of small molecules energy sources and amino acids, and at the same time removing inhibitory by-products (e.g., phosphate). As a result, a continuous cell-free protein synthesis reaction can often last more than 10 hours and yield mg ml^{-1} of protein.

Due to continuous improvement by academic and industrial researchers, the cell extracts in cell free systems have improved significantly. From the “incubated S30” first described by Nirenberg and Matthaei (Nirenberg and Matthaei, 1961), Zubay, Pratt and colleagues developed the robust protocols for making the *E. coli* extract that have become the standards (Pratt, 1984; Zubay, 1973). Other important improvements included the use of *E. coli* strains that lacked RNase I (*rna*) (MRE600) and Exonuclease V (A19) to stabilize RNA and linear DNA templates, respectively (Gesteland, 1966) (Yang et al., 1980). Recently, Swartz and colleagues pioneered the use of genome engineered *E. coli* strains to stabilize amino acids during *in vitro* reactions (Michel-Reydellet et al., 2004), *in vitro* activation of endogenous metabolic pathways to sustain high-yield protein synthesis (Jewett et al., 2008; Jewett and Swartz, 2004a), and systematic optimization of *E. coli* growth conditions and extraction procedures to reduce the production cost (Kim et al., 2006; Liu et al., 2005; Zawada and Swartz, 2005; Zawada and Swartz, 2006).

Since their initial development, the protocols for making the “classic” eukaryotic extracts, rabbit reticulocyte lysate (Jackson and Hunt, 1983) and wheat germ (Anderson et al., 1983; Erickson and Blobel, 1983), remain largely unchanged, and the protein synthesis yields from these eukaryotic extracts are generally lower than that from the *E. coli* extract. Recently, researchers developed improved protocols for making highly active extracts from wheat germ and mammalian cells. Endo and colleagues discovered that the remnants of endosperm on the surface of wheat germ were the sources of ribosome-inactivating proteins and other translation inhibitors, responsible for the poor stability and low yield of the conventional

wheat germ cell-free system (Madin et al., 2000). By extensively washing isolated wheat germ, the Endo group developed a high-yield long-lasting wheat germ cell-free system (Madin et al., 2000; Sawasaki et al., 2007). Until recently, with the exception of the rabbit reticulocyte lysate, the extracts of mammalian cells have not been widely used for cell-free protein synthesis mainly due to low yields. Imataka and colleagues at RIKEN found that the lack of functional translation initiation factors in the HeLa cell extract was a major limiting factor (Mikami et al., 2006b). By supplementing with exogenous translation initiation factors, the Imataka group developed significantly improved mammalian cell-free systems (Mikami et al., 2010; Mikami et al., 2006a; Mikami et al., 2006b), effectively taking over the rabbit reticulocyte lysate system.

Finally, the Ueda group reconstituted the minimal protein translational machinery from *E. coli*, thereby completely circumventing the issues of inhibitory factors in cell extracts (Shimizu et al., 2001). The PURE system (Protein synthesis Using Recombinant Elements), created by the Ueda group, consists of entirely purified components with minimal nucleases, proteases, and other enzymatic activities detrimental or unrelated to protein translation. Though other laboratories had attempted to reconstitute the protein synthesis with purified components (Ganoza et al., 1985; Kung et al., 1977), the PURE system was the first robust cell-free system containing a complete set of purified translation factors and aminoacyl-tRNA synthetases (Shimizu et al., 2001; Shimizu et al., 2005). Taking the same approach, my laboratory reconstituted the minimal protein translational machinery from *Thermus thermophilus* and demonstrated cell-free protein synthesis at temperatures up to 65°C (Zhou et al., 2012).

To make cell-free protein synthesis a protein factory rather than just an analytic tool, Swartz and colleagues pioneered chemical engineering approaches to making the *E. coli* extract. Their goal was to manufacture therapeutic proteins in the cell-free system at liter or any scale (Carlson et al., 2012). The entire processes of cell growth, extract preparation, protein synthesis and downstream purification were subject to cost analyses and process engineering. The “black-box” nature of the *E. coli* extract became sets of biochemical reactions amenable to pathway engineering. One of key advances was the creation of the “Cytomim” system that can activate central metabolism and oxidative phosphorylation in the *E. coli* extract, allowing not only the use of inexpensive energy substrates, such as glutamate or glucose, but also continuous protein synthesis for more than 6 hr in the batch format (Jewett et al., 2008; Jewett and Swartz, 2004a; Jewett and Swartz, 2004b). The Cytomim system was the basis for further improvements that led to the cell-free synthesis of recombinant human granulocyte-macrophage colony-stimulating factor in a 100-liter industrial stir tank (Jewett et al., 2008; Zawada et al., 2011).

USE OF COMMERCIALLY AVAILABLE CELL-FREE PROTEIN SYNTHESIS SYSTEMS

The quick and simple way to synthesize proteins *in vitro* is to use a commercially available cell-free system (Table 1). Most commercial cell-free systems provide DNA expression vectors into which the researcher clones his or her gene and allow protein production from the cloned gene. These expression vectors often contain promoter and other sequences

optimized for cell-free protein synthesis. Almost all commercial cell-free systems have adopted coupled transcription and translation, which also allows PCR-generated linear DNA templates to be used for protein synthesis.

To choose an appropriate commercial cell-free system, one should consider protein size and origin, protein folding, post-translational modification, downstream assays, applications and costs. In general, the prokaryotic systems (e.g., *E. coli* extracts) yield more proteins (especially small proteins, <50 kDa) than the eukaryotic systems, whereas the latter are more suited for expressing large multi-domain eukaryotic proteins. The continuous cell-free synthesis format generally produces more proteins (often >1 mg ml^{-1}) than the batch format. Systems based on cell extracts tend to provide better folding environments and post-translational modifications, whereas the reconstituted systems afford mRNA and protein stability. The reconstituted systems have minimal enzymatic activities and macromolecules other than those involved in protein translation, therefore, it is possible to carry out downstream characterization immediately after protein synthesis without purification. Table 1 provides a comparison of current commercial cell-free systems.

APPLICATIONS OF CELL-FREE PROTEIN SYNTHESIS

High-throughput cell-free protein synthesis for functional and structural analyses

The use of cell-free protein synthesis has made the most impact on functional and structural genomics. For the first time, researchers have been able to express and purify a large number of proteins in a short period of time for subsequent high throughput functional and structural analyses. For instance, a number of laboratories have used low-cost *E. coli* extract and wheat germ cell-free systems in high-throughput automated format to produce stable isotope-labeled proteins for nuclear magnetic resonance analyses (Aoki et al., 2009; Sawasaki et al., 2005; Vinarov and Markley, 2005). The advantage is that only newly synthesized proteins are labeled during cell-free protein synthesis and can be analyzed without extensive purification.

Like DNA microarrays for gene expression, protein arrays have been used for high throughput analyses of protein functions. Initially researchers had to individually purify proteins and spot each protein on solid surfaces (MacBeath and Schreiber, 2000; Zhu et al., 2001). These protein arrays are laborious to make and surface-bound proteins can lose functions during storage. Cell-free protein synthesis circumvents these problems by allowing proteins to be synthesized and captured in the vicinity of immobilized DNA templates, effectively generating just-in-time protein arrays from more stable DNA arrays. Two such protein array technologies, protein *in situ* array (PISA) (He et al., 2008) and nucleic acid programmable protein array (NAPPA) (Miersch and LaBaer, 2011) have been used for functional proteomics studies of protein-protein interactions.

Incorporation of unnatural amino acids into proteins

The translational machinery, including aminoacyl-tRNA synthetases, can tolerate a wide range of unnatural amino acids, allowing the genetic code to be expanded or even reassigned (Forster et al., 2003; Hartman et al., 2006; Josephson et al., 2005). One of the earliest applications of cell-free protein synthesis was the incorporation of unnatural amino acids

into a specific position of an *in vitro* synthesized protein. Schultz and colleagues first demonstrated the incorporation of unnatural amino acids into an amber stop codon that substituted a sense codon in a protein in an *E. coli* extract (Noren et al., 1989). It took more than a decade for the Schultz laboratory to engineer living cells to allow such expansion of the genetic code *in vivo* (Wang et al., 2001). And still, compared to cell-based approaches (Young and Schultz, 2010), cell-free systems are easier to manipulate for incorporation of unnatural amino acids. The reconstituted cell-free systems are especially suited for these applications (Forster et al., 2004; Hipolito and Suga, 2012; Shimizu et al., 2001). Using the reconstituted cell-free system, researchers can simply remove the competing factors, such as release factors, tRNAs or aminoacyl-tRNA synthetases, or replace them with an tRNA-synthetase pair from another organism, or an engineered aminoacyl-tRNA synthetase (Forster et al., 2004; Singh-Blom et al., 2013). Moreover, tRNAs can be pre-acylated with unnatural amino acids using natural enzymes (Forster et al., 2003) or engineered ribozymes (so called “Flexizymes”) (Murakami et al., 2006). The combined use of pre-charged tRNAs and the reconstituted cell-free system has allowed unnatural peptides or peptidomimetics containing mostly unnatural amino acids to be synthesized for potential applications in therapeutics (Hipolito and Suga, 2012; Josephson et al., 2005).

Directed evolution of proteins

Protein functions, such as binding affinity, thermostability and catalytic activity, can often be improved by iterative rounds of mutation and selection – an approach called directed protein evolution (Dougherty and Arnold, 2009; Romero and Arnold, 2009). Directed protein evolution is critically dependent on a stable physical linkage between protein (phenotype) and nucleic acid (genotype) throughout the selection process. The cell-based approaches (Farinas et al., 2001; Hibbert and Dalby, 2005), *e.g.,* phage display (Sidhu and Koide, 2007; Smith, 1985) and yeast surface display (Boder and Wittrup, 1997; Gai and Wittrup, 2007), maintain such genotype-phenotype linkage because the different nucleic acid sequences are confined to different phage particles or cells. The use of these cell-based approaches can potentially be limited by phage packaging or cellular transformation efficiencies and growth biases as phages expressing different members of a protein library may amplify at different rates (Derda et al., 2011).

In comparison, *in vitro* directed evolution based on cell-free protein synthesis can potentially incorporate protein libraries with higher complexities (more genes to select from) and less amplification bias (Golynskiy et al., 2013). These *in vitro* approaches include ribosome display (Hanes and Pluckthun, 1997; Pluckthun, 2012) and mRNA display (Roberts and Szostak, 1997; Takahashi and Roberts, 2009), both of which create a physical linkage between mRNA and protein, and *in vitro* compartmentalization (Griffiths and Tawfik, 2000; Griffiths and Tawfik, 2006; Lu and Ellington, 2013), which encapsulates DNA, mRNA and protein in water-in-oil emulsion droplets. Although researchers have successfully used extract-based cell-free systems for ribosome display and mRNA display, they have recently shown the advantages of the reconstituted cell-free systems. Since all of their components are purified, the reconstituted cell-free systems contain minimal nuclease and protease activities, which lead to significant stabilization of the mRNA-protein linkage (Ueda et al., 2010; Villemagne et al., 2006). A reconstituted cell-free system without the release factor 1

has been used in ribosome display, resulting in increased efficiency of ribosome stalling at the stop codon and displaying the full-length protein (Ueda et al., 2010). When incorporation of multiple unnatural amino acids is required for *in vitro* evolution and selection of drug-like molecules, the reconstituted cell-free systems become the only choice as only components necessary for *in vitro* synthesis are included in the systems (Hipolito and Suga, 2012; Josephson et al., 2013; Watts and Forster, 2012).

Cell-free synthetic biology towards artificial cell

Cell-free protein synthesis is likely a key starting point for any attempt to create an artificial cell either for understanding origin of life or for practical applications (Noireaux et al., 2011). Of the 200 or so essential genes believed to the minimal set for a free-living cell, a large portion are directly involved in protein synthesis (Gil et al., 2004; Koonin et al., 1996). Protein synthesis has been coupled to cell-free transcription regulation and DNA/RNA replication (Asahara and Chong, 2010; Forster and Church, 2006; Oberholzer et al., 1995; Shin and Noireaux, 2012). In parallel, cell-free protein synthesis has been performed in liposomes (Pereira de Souza et al., 2009), phospholipid vesicles (Noireaux and Libchaber, 2004; Shin and Noireaux, 2012) and water-in-oil droplets (Courtois et al., 2008; Dittrich et al., 2005; Fallah-Araghi et al., 2012). By such means, the reconstituted cell-free systems could be an important tool for bottom-up reconstruction of the minimal cell (Forster and Church, 2006; Jewett and Forster, 2010).

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Table 1

Comparison of commercial cell-free systems from major suppliers.

Source of cell-free systems	*E. coli* extract	Human cell extract	Wheat germ extract	Rabbit reticulocyte lysate	Insect cell extract	Reconstituted purified *E. coli* components
Commercial kits (suppliers)	RTS (5 PRIME); Expressway™ (Life Technologies); S30 T7 high yield (Promega);	One-step human IVT (Thermo Scientific)	WEPRO® (CellFree Sciences) TNT® coupled (Promega) RTS CECF (5 PRIME)	TNT® Coupled (Promega); Retic lysate IVT™ (Life Technologies);	TNT® T7 (Promega) EasyXpress Insect kit(Qiagen/RiN A)	PURExpress® (New England Biolabs) PURESYSTEM® (BioComber)
Typical yield range	100 Mg ml^{-1} to 1 mg ml^{-1}	Up to 750 μg ml^{-1}	Up to 10 mg ml^{-1}	<100 μg ml^{-1}	<100 μg ml^{-1}	50-200 μg ml^{-1}
DNA template	Circular and linear	Circular and linear	Circular and linear except WEPRO®	Circular and linear	Circular	Circular and linear
mRNA template	Not recommended	yes	WEPRO® mRNA only	yes	yes	yes
In vitro reaction format	Batch and continuous	Batch and continuous	Batch and continuous	batch	batch	batch
Detection of newly synthesized protein	[^{35}S]methionine; FluoroTect™Green$_{Lys}$-tRNA; Transcend™tRNA.	[^{35}S]methionine	[^{35}S]methionine	[^{35}S] methionine; FluoroTect™ GreenLys-tRNA	[^{35}S] methionine; FluoroTect™Green$_{Lys}$-tRNA; Transcend™tRNA.	[^{35}S] methionine; FluoroTect™Green$_{Lys}$-tRNA
Protein folding capability	Prokaryotic endogenous chaperones	Mammalian endogenous chaperones	Plant endogenous chaperones	Mammalian endogenous chaperones	Insect endogenous chaperones	No chaperones
Membrane protein folding	Add exogenous detergents or lipids, or use MembraneMax™ (Life Technologies)	Possible	Add exogenous detergents or liposomes	Add exogenous microsomal membranes	Possible	Add inverted membrane vesicles
Disulfide bond formation	Use disulfide bond enhancer (New England Biolabs) or RTS disulfide kit (5 PRIME)	Possible	Possible	Possible	Possible	Use disulfide bond enhancer (New England Biolabs)
Post-translational modification	No eukaryotic modification	N-glycosylation possible	N-myristoylation or N-glycosylation in the presence of microsomal membranes	N-glycosylation in the presence of microsomal membranes	N-glycosylation, N-myristoylation, prenylation	No modification
Applications	Unnatural amino acid incorporation; ribosome display; PISA	Express active human proteins; NAPPA	Stable isotope incorporation; ribosome display; mRNA display;	NAPPA; ribosome display; mRNA display	Express soluble active eukaryotic proteins	Unnatural amino acid incorporation; ribosome display; mRNA display
References	(Hanes et al., 1999; He and Taussig, 2008; Pratt, 1984; Schneider et al., 2010; Swartz et al., 2004; Zubay, 1973)	(Festa et al., 2013; Mikami et al., 2006a; Mikami et al., 2006b)	(Kawasaki et al., 2003; Nozawa et al., 2011; Nozawa and Tozawa, 2014; Takahashi et al., 2002; Yamauchi et al., 2010)	(Craig et al., 1992; He and Taussig, 1997; Jackson and Hunt, 1983; Miersch and LaBaer, 2011; Takahashi and Roberts, 2009)	(Ezure et al., 2006; Ezure et al., 2010; Suzuki et al., 2007; Suzuki et al., 2010; Tarui et al., 2000; Tarui et al., 2001)	(Kuruma et al., 2005; Shimizu and Ueda, 2010)