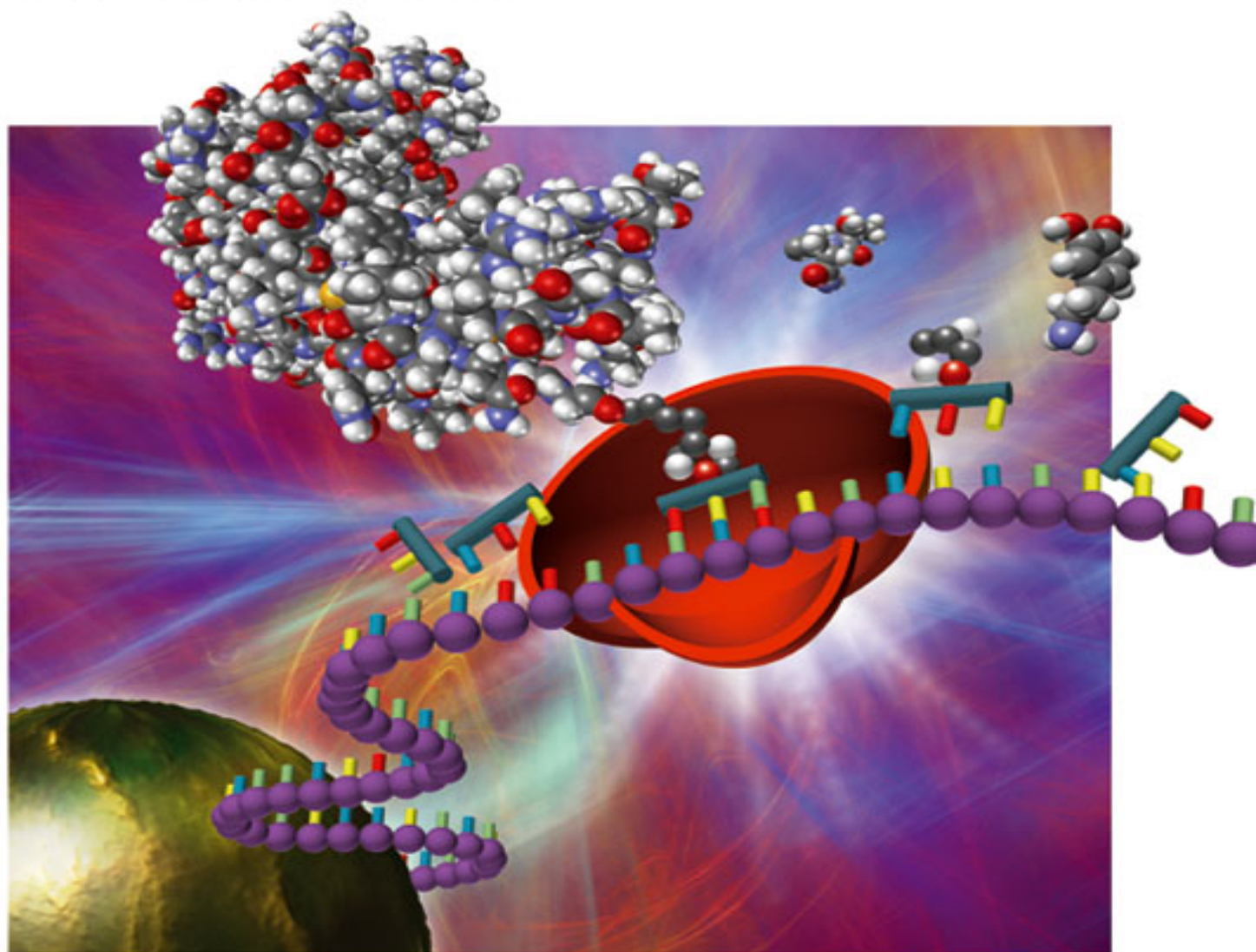


Edited by Alexander S. Spirin
and James R. Swartz

 WILEY-VCH

Cell-free Protein Synthesis

Methods and Protocols



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Preface

We are pleased to offer the following chapters as an update on the rapidly developing field of cell-free protein synthesis (CFPS), a subset of a potentially larger field termed cell-free biology. Although this collection is far from comprehensive, it is intended to provide an overview of the field by offering examples of some of the more exciting developments.

CFPS is a unique embodiment of our fascination with biology and of our desire to harness biology for societal benefit. Early on, agriculture began to harness biology and dramatically changed our social structures by providing plentiful and local food supplies. In more recent times, we industrialized biology. For example, acetone/butanol fermentations helped supply munitions for World War I, and the large scale production of antibiotics saved millions of lives during and after World War II.

But the most dramatic development came with the deciphering of the genetic code and the subsequent ability to reprogram living organisms. While CFPS was instrumental as a research tool in breaking the code, it languished relative to its potential as a protein production tool. Even the breakthrough developments of continuous-flow (CFCF) and continuous-exchange (CECF) operational modes failed to rapidly launch cell-free synthesis as a wide-spread research and production technology. However, over time these developments have combined with the analysis and activation of cell-free metabolism and with a variety of other advances to open exciting new opportunities for the production of complex proteins, for convenient protein evolution, for expanding our knowledge of basic biology, and even for producing complex proteins such as membrane proteins that are very difficult to produce *in vivo*.

In this collection, we first set the stage in Chapter 1 by describing the history of cell-free protein synthesis with emphasis on providing an overview of many of the recent advances. Chapter 2 describes a particularly interesting and powerful development, the use of purified components to catalyze robust protein synthesis. Chapter 3 describes a series of developments that enable CFPS to analyze genomes for those sequences encoding gene products that impact a broad variety of central metabolic processes. Both Chapters 2 and 3 have the potential to provide important new knowledge about protein synthesis and microbial metabolism. They also both provide unique platforms for general protein synthesis.

With Chapter 4, the focus is shifted to a particular application: patient-specific medicine. CFPS offers the speed and economy required when a new protein vaccine is needed for each patient, as is the case for Non-Hodgkins Lymphoma. This chapter describes the development of complex fusion protein vaccines that require the formation of multiple disulfide bonds, but that nevertheless can be rapidly produced by CFPS. Data are also presented to show that these vaccines elicit protective immune responses in mouse tumor models.

Chapter 5 provides new advances in improving the efficiency of CFPS in CECF format using bacterial cell extracts. Chapter 6 then extends that description by showing how these advances provide significant advantages in producing the proteins required for exploring structural biology and structural proteomics.

Although Chapters 2 through 6 describe work with bacterial cell extracts, this is definitely not the only viable approach. Chapter 7 describes the considerable progress made with the use of wheat germ extracts in the CECF format. This eukaryotic system has been used by a variety of laboratories to produce proteins for structural

determination as well as for other research applications. An impressive recent achievement of Roche Applied Science group (Penzberg, Germany) in collaboration with GeneCopoeia (Germantown, USA) and Fulengen (Guangzhou, China) is the expression of 12,000 genes encoding for human proteins using a wheat germ CECF system.

One of the most significant challenges for CFPS has been the production of membrane proteins. Chapters 8 and 9 describe the significant progress made in this pursuit using cell-free methods to avoid the product toxicity that limits *in vivo* production. Chapter 10 then provides impressive examples showing how complex proteins requiring disulfide bonds, special chaperones, and cofactors can be evolved to provide new catalytic function. This is all the more significant since the evolution depends upon single molecule PCR to isolate individual genes and their products in a process termed SIMPLEX (single-molecule PCR-linked *in vitro* expression). With a very interesting alternative approach, Chapter 11 describes the separation, amplification, and cell-free expression of individual DNA molecules within a gel matrix. In this way, the entire process of cloning, expression, and screening of genes and gene libraries can be accomplished without living cells, and, more importantly, in hours instead of days and, potentially, with more complete evaluation of the gene library.

Finally, Chapter 12 addresses technology to enable the cell-free production of proteins at industrial scales and economies. Advances are discussed that allow the relatively efficient use of inexpensive raw materials to dramatically reduce costs. Efficient energy in the form of ATP is supplied by oxidative phosphorylation, but this requires oxygen supply. Thus, methods are also described that allow the use of conventional bioreactors for the large scale, economical cell-free production of proteins.

Taken together, these chapters illustrate the power and versatility of CFPS. Biological evolution has provided an incredible catalog of protein structures and functions. With CFPS, we have the opportunity to directly control the environment in which these polypeptides are produced, folded, and, when appropriate, combined with other polypeptides. We are freed from the need to maintain cell viability, and we can also channel most, if not all, of the metabolic resources to the production only of our product. We can also design and produce a system of catalysts that will remain relatively invariant over the period of protein production. These considerations suggest that CFPS will be able to produce many proteins that are difficult to produce *in vivo* (as is the case with membrane proteins, for example) and may also be able to produce many proteins more economically than with *in vivo* approaches. For the same reasons, CFPS is developing into an even more powerful research tool. The exciting advances and applications described in this book suggest that CFPS will play an expanding role in modern biotechnology.

July 2007

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1

Cell-free Protein Synthesis Systems: Historical Landmarks, Classification, and General Methods

Alexander S. Spirin and James R. Swartz

1.1 Introduction: Historical Landmarks

1.1.1 Discovery of Protein Synthesis in Cell Extracts

The demonstration of the capability of disintegrated cells and cell extracts to continue protein synthesis was among the great discoveries of the early 1950s that led to the birth of molecular biology. The original observations were made independently in several laboratories working with homogenates and homogenate fractions of animal tissues [16, 132, 154, 155, 174, 175]. Shortly afterwards, disrupted bacterial cells were also shown to be capable of synthesizing proteins [42] and the fraction of ribonucleoprotein particles called ribosomes was identified as the heart of the protein-synthesizing machinery of the

cell [138]. Cell extracts freed from heavy components by centrifugation at 30000g (the so-called S30 extracts) and supplemented with amino acids, ATP and GTP, were the first cell-free protein-synthesizing systems [65, 95, 99, 100, 114, 137, 148, 168].

In those systems, however, the ribosomes just continued to translate endogenous mRNAs and elongate the polypeptides for which synthesis had already been started. Nevertheless, a high level of globin synthesis from endogenous mRNA templates could be achieved using rabbit reticulocyte lysates, and several general molecular mechanisms of protein-synthesizing machinery were studied (see, e.g., Refs. [54, 135]).

1.1.2 Translation of Exogenous Messages

The principal breakthrough in the development of cell-free protein-synthesizing systems was made in 1961 when Nirenberg and Matthaei managed to destroy endogenous mRNA in the bacterial (*E. coli*) extract without damaging ribosomes and the rest of the protein-synthesizing machinery [124, 125]. The ribosome run-off and the selective destruction of endogenous mRNAs was accomplished by a simple procedure of pre-incubation of the extract at 30–37 °C. The introduction of polyribonucleotides in such mRNA-depleted extracts resulted in effective *translation of exogenous messages*. The experiments with translation of synthetic polyribonucleotides, such as poly(U), poly(A), poly(C) and random copolymers poly(U,C), poly(U,C,A), etc., deciphered the genetic code. Furthermore, natural alien messages, including eukaryotic mRNAs, could be successfully expressed in such bacterial extracts.

Later, addition of a Ca²⁺-dependent micrococcal ribonuclease with subsequent inactivation of the enzyme by

removal of Ca^{2+} with EGTA was used for destruction of endogenous globin mRNA in the reticulocyte lysate [130]. Such mRNA-depleted animal extracts became the basis of the most efficient eukaryotic cell-free systems [55, 57, 115].

Another type of eukaryotic cell-free system was based on wheat germ extracts [106, 136]. In this case the content of active endogenous mRNA in the extract was found to be so low that there was no need for the pre-treatment procedures described above [5, 105].

1.1.3 Coupled Transcription-translation in Bacterial Extracts

In the case of bacterial cell-free translation systems, the addition of pre-synthesized mRNA to a cell extract violates the principle of natural prokaryotic translation. In prokaryotic cells, translation of a mRNA by ribosomes is initiated soon after the beginning of its synthesis on the DNA template. The ribosomes move along mRNA chain not far behind the RNA polymerase, and both processes proceed with synchronized rates (*coupled transcription-translation*) [102, 181]. When pre-synthesized, complete mRNAs are used in cell-free translation systems, the initiation of translation sometimes may be hindered by mRNA folding and tertiary structure formation, especially if ribosome-binding sites are involved.

The first demonstration of DNA-dependent incorporation of amino acids into synthesized proteins as well as the first evidence for the coupled transcription-translation process in bacterial cell-free systems were also made by Nirenberg's group in the beginning of the 1960s [21, 110]. In 1967, Zubay and colleagues made significant improvements [36, 97] and introduced an efficient bacterial coupled transcription-translation system for expression of exogenous DNA [27, 189]. Their system was based on crude cell-free *E.*

coli extract containing endogenous RNA polymerase, but devoid of endogenous DNA and mRNA due to exhaustive nuclease degradation. This improved method was broadly adopted, although somewhat modified protocols were reported by others (see, e.g., Ref. [134]).

Another practical version of the bacterial coupled transcription-translation system for exogenous gene expression was proposed by Gold and Schweiger [49, 50, 149], who used the mixture of isolated *E. coli* ribosomes, tRNA and ribosome-free supernatant (the so-called S100 extract) purified by ion-exchange chromatography from all nucleic acids, instead of the crude extract with degraded nucleic acids.

1.1.4 Combined Transcription-translation Systems

The next important step in the development of cell-free gene expression was the combination of a cell extract with a specific bacteriophage RNA polymerase that used a phage-specific promoter for transcription. Either SP6 polymerase [161] or T7 polymerase [30, 123] were suggested for such cell-free systems. These polymerases direct the exclusive synthesis of the proteins encoded by genes preceded by the corresponding phage promoters. Such systems possess several advantages: (a) the phage polymerases provide a higher level of transcripts than endogenous bacterial RNA polymerase; (b) the addition of rifampicin selectively inhibits the endogenous RNA polymerase, and thus there is no need to self-digest or treat cell extract for removal of endogenous DNA; (c) due to the promoter specificity of the phage RNA polymerase, only the gene of interest is expressed; (d) the systems are convenient for expression from any plasmid constructs and PCR products where the simple phage promoters are inserted; and (e) the phage RNA polymerases

and DNA templates with phage promoters can be combined both with prokaryotic [123, 161] and eukaryotic [30, 161] extracts.

Strictly speaking, these systems cannot be referred to as “coupled transcription-translation” systems: both spatial and temporal coupling is absent in this case because the T7 and SP6 RNA polymerases work much faster than the endogenous bacterial polymerase and translation machinery, the transcripts quickly accumulate in excess over translating ribosomes, and thus mRNA is synthesized mainly in advance in such systems. The term “combined transcription-translation” is a more appropriate designation. It should be mentioned that both purified *E. coli* RNA polymerase [18, 28, 98, 137, 164] and animal virus-associated RNA polymerases [7, 29, 131] were also used in the combined cell-free transcription-translation systems based on eukaryotic extracts, but the use of SP6 and T7 phage polymerases proved to be the most successful.

1.1.5 Continuous Flow/Continuous Exchange Principle

In cell-free translation and transcription-translation systems performed in a fixed volume of a test-tube (batch format) the reaction conditions change during incubation as a result of the consumption of substrates and the accumulation of products. Translation stops as soon as any essential substrate is exhausted or any product or by-product reaches an inhibiting concentration, usually after 20–60 minutes of incubation. The limited lifetimes and, as a consequence, low yields of protein products made the early batch systems useful mainly for analytical purposes and inappropriate for preparative synthesis of polypeptides and proteins.

A principal breakthrough was the invention of the so-called *continuous-action* or *continuous-duty* translation [4, 160] and transcription-translation systems [8, 10] (see also Refs. [14, 15, 153, 161, 162]). Instead of incubating the reaction mixture in a fixed volume in a test-tube, the reaction was performed under conditions of *persistent supply* of the consumable substrates (amino acids, nucleoside triphosphates and energy-regenerating compounds) and with *removal* of the reaction products (mainly inorganic phosphates and nucleoside monophosphates, as well as polypeptide products and by-products in some reactor versions). To achieve that, a porous (ultrafiltration or dialysis) membrane was used to retain the high-molecular-weight components of the protein-synthesizing machinery (ribosomes, mRNA, ARSases, etc.) within a defined reaction compartment. The membrane separated the reaction compartment from another compartment containing a feeding solution with a reservoir of low-molecular-weight components (substrates) for the reaction. This technique was reproduced in a number of laboratories [37, 38, 66, 90, 126, 127, 171, 172, 177].

In so-called continuous-flow cell-free (CFCF) systems [8, 10, 160] the feeding solution with substrates is continuously pumped into the chamber containing the reaction mixture, and the products are continuously removed through the ultrafiltration membrane by the outgoing flow. Reactors working in pulsating [161] (see also Ref. [162]) or “discontinuous” (see Chapter 7 of this book) modes, with alternating flow-in and flow-out instead of the direct continuous flow, can also be used.

In the dialysis format of the continuous systems, designated also as continuous-exchange cell-free (CECF) systems, the passive (diffusional) exchange of substrates and low-molecular-weight products through a porous barrier takes place [4, 26, 107, 153, 162]. The dialysis (CECF)