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Review

Engine out of the chassis: Cell-free protein synthesis and its uses

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ABSTRACT

The translation machinery is the engine of life. Extracting the cytoplasmic milieu from a cell affords a lysate capable of producing proteins in concentrations reaching to tens of micromolar. Such lysates, derivable from a variety of cells, allow the facile addition and subtraction of components that are directly or indirectly related to the translation machinery and/or the over-expressed protein. The flexible nature of such cell-free expression systems, when coupled with high throughput monitoring, can be especially suitable for protein engineering studies, allowing one to bypass multiple steps typically required using conventional *in vivo* protein expression.

1. Introduction

The ability to produce a functional protein in the test tube, rather than in cells, is the essence of cell-free protein synthesis (CFPS) [1,2]. The preparation of a CFPS kit requires the separation of the cytoplasmic milieu from the cell wall, and has been applied to a variety of cell types, spanning bacteria, protozoa, plants, insects and mammals [1–8]. The cell lysate is a crowded environment of active biomolecules, capable of supporting many cellular functions. These functions include, but are not limited to, many metabolic pathways, as well as transcription and translation. The preparation of lysates for CFPS was significantly improved over the years in terms of buffer composition [3], energy recycling [9], the utilization of various mutated cell strains [10,11], the supplementation of small molecules [12,13], the addition of proteins such as T7 RNA polymerase (RNAP) to generate a transcription/translation coupled system [1,3] and chaperones to improve the yields of properly folded target proteins.

In *Escherichia coli* CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [14]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [15]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation.

This paper will outline progress in the field of CFPS that applies this approach in ways that would be challenging, if not impossible, to implement using standard *in vivo* expression systems (Fig. 1). The use of improved fluorescent proteins, such as Emerald GFP [16], and of fluorescence detection technologies using a plate reader platform, allow real time monitoring of protein expression in a high-throughput format [17]. These advances allow the straightforward screening of the effects on translation rate of various procedural modifications. These include the introduction of exogenous materials, (chemical reagents, proteins, and nucleic acids) and the substitution of mutated/modified components of the translation machinery (ribosomes, mRNAs, tRNAs) for their endogenous counterparts.

2. Methods of cell-free protein synthesis

The two basic types of CFPS are optimized cell extracts (often termed lysate-based CFPS), an approach that has been in use for more than five decades, and the more recently developed PURE system, which employs a mixture of a minimal set of purified components (e.g., ribosome, tRNAs, tRNA synthetases, factors, amino acids, energy sources) required for full-length protein synthesis. Below we present a brief description of each approach and discuss various factors that can influence protein yield and function, before considering some specific examples.

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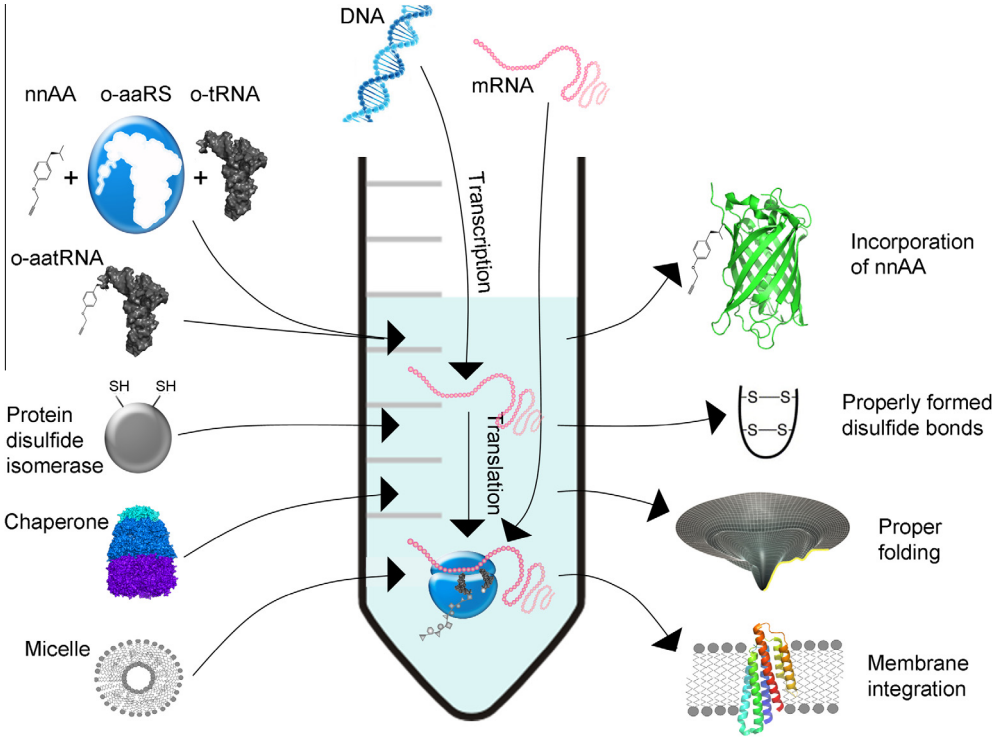

Fig. 1. Cell-free protein synthesis and its functionalities. Translation commences upon the addition of DNA (PCR product or plasmid) in a coupled system or by adding separately transcribed mRNA. Modified CFPS may exhibit various functionalities, some of which are depicted.

2.1. Lysate-based CFPS, coupled transcription/translation

Some commercially available prokaryotic and eukaryotic CFPS kits produce transcribed mRNA and translated protein in a coupled fashion. Adding a DNA encoding the protein of interest along with T7 or SP6 RNA polymerase (RNAP) generally produces transcribed mRNA at a faster rate than protein synthesis, with the result that protein expression is not limited by mRNA availability. There also exists a protocol for the generation of an *E. coli* CFPS that utilizes endogenous RNAP [18]. In general, coupled CFPS expresses proteins in higher yields, and eliminates the separate *in vitro* transcription step required for mRNA-dependent CFPS (see below).

Coupled CFPS utilizes DNA in three forms: linear PCR product, linearized plasmid and circular plasmid. Circular DNA plasmid has typically been preferred to linearized plasmid or PCR products, due to the greater susceptibility of linear DNAs to nucleolytic cleavage [19]. On the other hand, use of the linear PCR product has the distinct advantage of simplicity, since it eliminates the need for time-consuming cloning steps required when generating an expression plasmid, that include: ligation of the DNA template to a linearized plasmid, transformation of the plasmid to compatible cells, selection of colonies harboring the foreign plasmid, growth of positive colonies in culture for plasmid production, plasmid isolation, sequencing, transformation of the plasmid to compatible cells, culture growth for protein overexpression, harvesting and lysis (Fig. 2). In contrast, addition of an amplified linear DNA fragment to a CFPS affords single step protein expression via transcription/translation coupled CFPS, either in analytic amounts amenable to a high throughput format or in preparative amounts [20,21]. Utilization of a linear PCR product on an analytical scale allows facile optimization of translation of the amplified DNA fragment, coupled with any desirable extension, as, for example, in the case of N-terminal extensions of the adiponectin hypervariable domain [21]. Moreover, the yield of expressed protein can be raised utilizing procedures that increase the stability of the linear PCR-amplified DNA via both the removal of nucleases [10,20] and the utilization of overhang extensions to cyclize PCR products, exploiting the endogenous ligase activity of lysates [22]. In the case of dengue virus NS2B/NS3 protease, the latter approach gave protein yields comparable to those obtained using plasmid-based CFPS [22].

In general, these improvements make PCR product an attractive alternative to circular plasmid for applications of protein expression ranging from protein engineering to NMR structural characterization of proteins. Commercial CFPS kits, optimized for testing the coupled transcription/translation of PCR products, are available from suppliers such as: 5-prime, Promega, Jena Bioscience, New England Biolabs, Life technologies, Pierce, and Cell-Free Sciences.

2.2. PURE CFPS

The PURExpress kit was developed by Ueda and co-workers [23] and further optimized by New England Biolabs [24,25], which also distributes it commercially. The PURExpress kit has several advantages over lysate-based kits. Because it is devoid of any biomolecules and metabolites that do not directly participate in protein synthesis, it lacks both nucleases and proteases that decrease the lifetime of DNA, mRNA and proteins, and metabolic enzymes that can convert some nucleotides and amino acids necessary for transcription and translation to non-functional products not participating in these processes. In addition, it allows straightforward elimination of specific components of the translational machinery or substitution of exogenous for endogenous components. Thus, for example, by omitting the tRNA synthetase PheRS and all traces of the amino acid Phe, we could make EmGFP synthesis, measured at the single molecule level, totally dependent on the addition of fluorescently labeled Phe-tRNA^{Phe} as the sole source of Phe incorporated into protein. An alternative approach, based on manipulation of a lysate-based kit [17], was successful for parallel