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The past, present and future of cell-free protein synthesis

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Recent technical advances have revitalized cell-free expression systems to meet the increasing demands for protein synthesis. Cell-free systems offer several advantages over traditional cell-based expression methods, including the easy modification of reaction conditions to favor protein folding, decreased sensitivity to product toxicity and suitability for high-throughput strategies because of reduced reaction volumes and process time. Moreover, improvements in translation efficiency have resulted in yields that exceed a milligram of protein per milliliter of reaction mix. We review the advances on this expanding technology and highlight the growing list of associated applications.

Introduction

Since the pioneering studies conducted by Nirenberg and Matthaei more than four decades ago [1] cell-free protein synthesis has been a valuable tool for understanding how mRNAs are translated into functional polypeptides. In addition, it has been used for antibiotic drug discovery and has been a means for small-scale expression of toxic products. With the advent of the proteomics era, the field has experienced a technical renaissance expanding into a myriad of applications covering both functional and structural proteomics. Significant improvements made to the configuration, energetics and robustness of reactions have led to productivities that far surpass the milligram levels of product per milliliter of reaction. Also, important progress has been made on key reaction components so that it is now possible to obtain a polypeptide with its corresponding post-translational modifications or, in the case of a membrane protein, properly imbedded into a lipid bilayer. Finally, the ability to easily manipulate the reaction components and conditions makes *in vitro* protein synthesis particularly amenable to automation and miniaturization, enabling application to the fields of protein arrays, *in vitro* evolution and multiplexed real-time labeling, among others. In this article we review the key aspects of this field, emphasizing the most recently developed applications for both basic and applied research. A review of the entire field is beyond the scope of this article and further details in this regard can be found in previously published literature [2–6].

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Configurations and history

In vitro translation systems are based on the early demonstration that cell integrity is not required for protein synthesis to occur. In its simplest form, translation can be accomplished using a crude lysate from any given organism (that provides the translational machinery, accessory enzymes, tRNA and factors) in combination with exogenously added RNA template, amino acids and an energy supply. This classical *in vitro* translation scheme is called 'uncoupled' as opposed to the 'coupled' or 'combined' transcription and translation configuration in which the mRNA is transcribed *in situ* from a DNA template added to the reaction (see [2] for details). Usually coupled systems exhibit higher protein yields and are easier and faster to operate than systems that are not coupled although they require supplementing the reaction with additional NTPs and a highly processive RNA polymerase, such as those encoded by T7, T3 or SP6 bacteriophages. The use of plasmid or PCR templates rather than purified mRNAs resulted in the emergence of a variety of new applications.

Sources of lysates

Although any organism could potentially be used as a source for the preparation of a cell-free protein expression system, the most popular are those based on *Escherichia coli*, wheat germ and rabbit reticulocytes (for recent reviews see [2,6,7]). The choice of the system should be determined by the origin and biochemical nature of the protein and the specifics of the downstream application. In general, *E. coli*-based systems provide higher yields and more-homogeneous samples suitable for structural studies. The protein yields of *E. coli*-based systems range from a few micrograms up to several milligrams per milliliter of reaction, depending on the protein and the reaction format [8]. Eukaryotic-based systems, although less productive, provide a better platform for functional studies particularly for post-translationally modified proteins. Reported protein yields for the rabbit reticulocyte lysate are in the microgram or in the fraction of a microgram per milliliter of reaction range whereas protein yields of the wheat germ extract are typically two orders of magnitude higher [2,6,9].

Crude cell lysates are not the only source of the enzymatic machinery. Despite being one of the most complicated basic cellular processes, the whole translational mechanism from *E. coli* was recently reconstituted

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in vitro starting with >100 individually purified components [10]. The system exhibits high translational efficiency with the added advantage of simpler manipulation of the reaction conditions and easy purification of untagged protein products.

Productivity and automation

The principal limitation of the first generation batch-formatted reactions (Figure 1a) is their short lifetimes (<1 hr) and consequently their low yield. This is primarily because of the rapid depletion of the high-energy phosphate pool, which occurs even in the absence of protein synthesis [11]. In turn, this leads to the accumulation of free phosphates, which can apparently complex with magnesium, and further inhibit protein synthesis. This problem was first overcome by Spirin and coworkers with the introduction of the continuous-flow cell-free (CFCF) translation system, which relies on the continuous supply of energy and substrates and the continuous removal of the reaction by-products (Figure 1b) [12]. The reaction time can then be extended for 20 h and increases the product yield by two orders of magnitude. Despite the improvement in yield, the operational complexities make this system extremely impractical. The technology was later simplified by the development of a semi-continuous or continuous-exchange cell-free (CECF) method, in which a passive rather than active exchange of substrates and by-products extended the reaction lifetime [13,14] (Figure 1c). However, semi continuous systems are not easily applicable to high-throughput processes, which require miniaturization and automation.

To this end several laboratories have focused either on developing high-throughput-friendly systems or on maximizing the energetics of the batch reaction. Endo and coworkers have devised a highly efficient bilayer diffusion system devoid of membranes that is compatible with high-throughput formats [9,15] (Figure 1d). Swartz's group has consistently sought more efficient and economical alternatives to the traditional ATP and/or GTP regeneration systems with promising results. For example, they have recently demonstrated that is possible to produce close to a milligram of protein per milliliter of reaction using a batch format (see [16] and references therein). The same group has recently shown that a higher efficiency cell-free system can be obtained simply by mutating genes involved in the catabolism of certain amino acids [17].

Folding and post-translational modifications

A key goal for cell-free translation systems is to synthesize biologically active proteins. Currently, the primary issues are protein folding and post-translational modifications. A clear advantage that these systems have over *in vivo* protein synthesis is that the environmental conditions can be adjusted easily. Strategies to improve protein folding and post-translational processing include the addition of a variety of reagents and folding catalysts to the reaction.

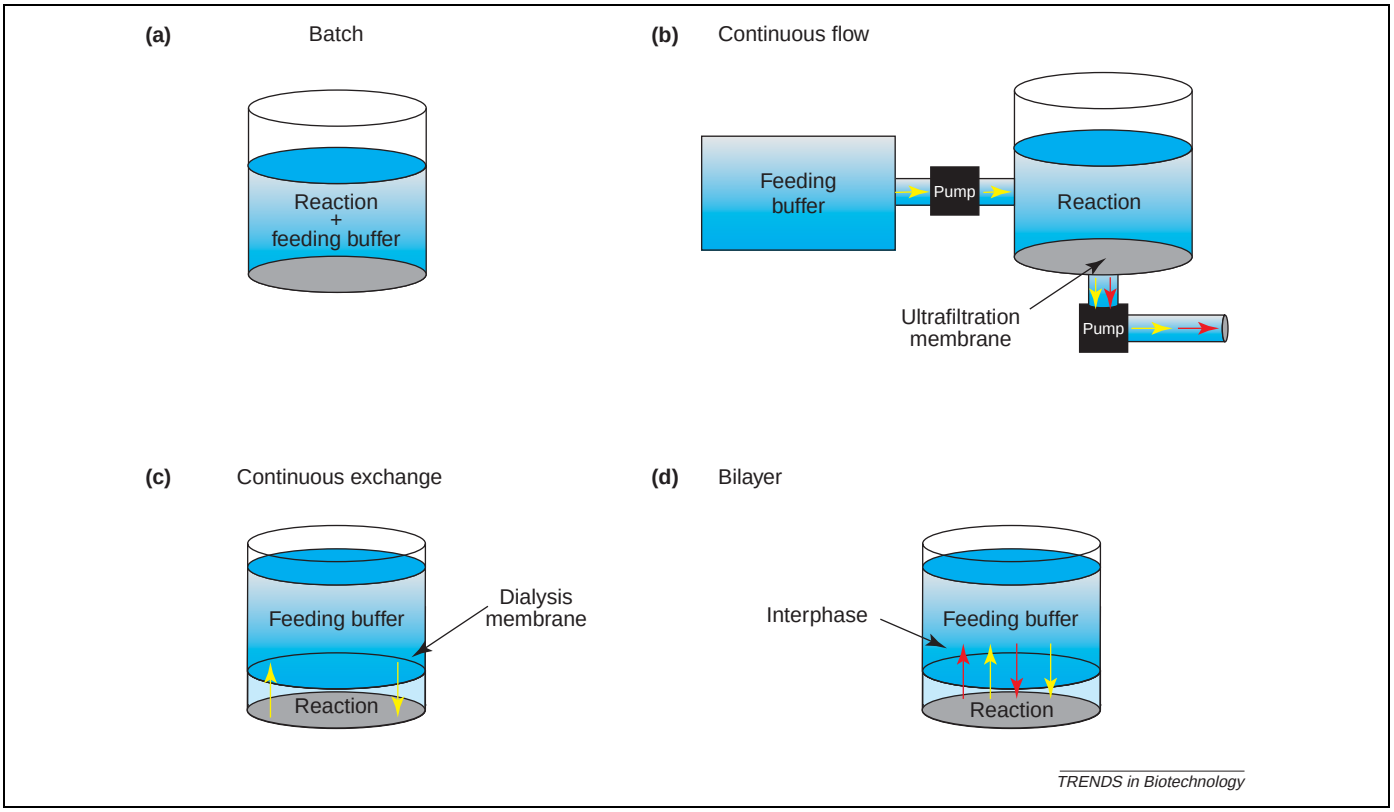

Figure 1. Current formats of cell-free protein expression systems. Formats are classified according to how the reaction is fed: **(a)** batch **(b)** continuous-flow cell-free **(c)** continuous exchange cell-free and **(d)** bilayer. Reaction components include ribosome, translation factors, tRNA, aminoacyl tRNA synthetases, template (RNA or DNA), and RNA polymerase (when necessary). The feeding buffer includes amino acids, energy components, NTPs (when necessary), co-factors and other accessory reagents. Yellow arrows indicate the flow of buffer components and red arrows represent the flow of protein product.

Chaperones

Reports on the exogenous supply of chaperones to cell-free protein synthesis reactions suggest that the effect that these catalysts have is protein-dependant. For example addition of purified DnaK, DnaJ, GroEL and GroES has been reported to be beneficial for the synthesis of single chain and fragment antigen binding (Fab) antibodies [18,19] but it appears to have no effect on the folding or activity of luciferase [20].

Membrane proteins

One of the greatest and potentially most far-reaching impacts of *in vitro* translation could be in the area of membrane protein production. Membrane proteins account for nearly a third of the genes encoded by most fully sequenced genomes. However, only a handful of integral membrane protein structures (<80) have been solved to high resolution. The paucity of membrane structures reflects the hurdles that must be overcome when expressing and preparing these proteins. This difficulty is particularly daunting when over-producing large quantities of eukaryotic or mammalian integral membrane proteins. Over-expression of membrane proteins *in vivo* frequently results in cell toxicity largely owing to their hydrophobicity, protein aggregation, misfolding and low yield. In some cases, the over-expression of integral membrane proteins, such as ion channel proteins, transporters and receptors can disrupt the integrity of the cell membrane and lead to cell lysis. What is exciting is that nearly all of these obstacles can be overcome by cell-free expression. *In vitro* translation offers a unique opportunity to use the highly efficient bacterial transcription and translational machinery while introducing natural mammalian or other synthetic lipids. In addition, *in vitro* expression can circumvent the problem of post-translational modification that is essential *in vivo* and can impair crystallization. For example, milligram amounts of active transmembrane multi-drug transporters have recently been synthesized *in vitro* [21,22]. The presence of mild detergents or lipid mixtures during the reaction notably eased aggregation and insolubility issues and apparently did not interfere with the translation activity. Moreover, membrane proteins that require their cognate partner protein in the proper molar ratios for folding and solubility can easily be co-expressed by adjusting the relative amounts of template encoding the two different subunits [21].

Disulfide bond formation

Disulfide-bonded proteins are ordinarily formed in extracytoplasmic compartments, such as the periplasm of prokaryotes and the lumen of the endoplasmic reticulum (ER) of eukaryotes in which conditions are more oxidizing. Although cell-free protein systems have two intrinsic features that might prevent the formation of disulfide bonds (reducing agents that stabilize the protein synthesis machinery and the lack of compartments with oxidizing redox potential) these hurdles can easily be overcome. One method eliminates dithiothreitol from the cell-free extract before the translation reaction, which results in the high production of single-chain antibodies with dual disulfide bonds [23]. Also, the combination of alkylation of the extract with iodoacetamide, a suitable glutathione redox buffer and a disulfide bond isomerase added to the *in vitro* reaction can have a profound positive effect on the production of active proteins with multiple disulfide bonds [18,24].

Glycosylation and other post-translational modifications

Glycosylation is the most widespread and complex form of post-translational modification in eukaryotes (for a review see [25]). A major problem for the production of glycoproteins is that they are ordinarily produced as a mixture of glycoforms. Only the glycosidic core remains relatively uniform while the protein is in transit within the ER. In cell-free systems, core glycosylation can be achieved by supplementing extracts with microsomal fractions [26]. Proteins are translocated to the lumen of the vesicles where their leader peptide is cleaved and they acquire the oligosaccharide chain. Given that intracellular transport in cell-free protein synthesis systems is disrupted, further processing of the oligosaccharides is being prevented. However, some variation in the glycosylation pattern can still be observed in cell-free protein synthesis systems owing to inhomogeneous folding that apparently restricts the access of the glycosylating enzymes [27]. Recently, the generation of a *Spodoptera frugiperda* 21 cell-based lysate has been reported [28]. The system provides core protein glycosylation enzymes without the need for supplementing the reaction with membrane vesicles. The newest approach for the *in vivo* synthesis of homogeneous samples of glycoproteins exploits the use of a non-natural amino acid linked to a monosaccharide moiety [29]. This strategy could easily be adapted to cover *in vitro* protein synthesis. When incorporated into a protein, the monoglycosylated amino acid can be further modified by glycosyltransferases added to the *in vitro* reaction resulting in more-complex glycoforms.

Other post-translational modifications, such as phosphorylation, myristylation, farnsylation, isoprenylation and adenylation have been observed in lysates from higher eukaryotes (for further details see [2,6,30] and references therein). With all these modifications, the dynamic complexity of post-translational modifications makes it difficult to produce homogeneous protein samples. Methods for creating artificial post-translational modification mimics (artificial modifications that imitate the structure of the natural ones) have been proposed as a solution for this problem (for a review see [31]). Cell-free systems seem to be the most favorable platform for this novel strategy.

Applications

Until several years ago, the use of cell-free protein systems was restricted to low-throughput applications with low protein concentration requirements. Today, thanks to significant progress made to the miniaturization, automation and optimization of fed-batch and semicontinous reactions, *in vitro* synthesis of proteins has found a large variety of low- and high-throughput applications suitable for functional and structural proteomics.

Structural proteomics

Proteins that pose obstacles for expression and analysis are under-represented in the protein structure databases. Toxic proteins, membrane proteins, complex assemblies and proteins that exhibit insolubility issues are examples of under-characterized proteins. The flexibility that cell-free protein synthesis offers for manipulating reaction conditions and directing all metabolic resources to protein production has already proven to be effective in filling this void. First, the productivity of *in vitro* systems based on *Esherichia. coli* and wheat germ extracts has surpassed the milligram levels per milliliter of reaction and the products can be directly used for structural studies [8,32]. Second, several cytotoxic proteins, which are difficult or impossible to overexpress in cellular environments can be readily produced *in vitro* (for an example see [33]). Selenomethionine labeling, which is often used by protein crystallographers to facilitate protein phasing by multi-wavelength dispersion, frequently leads to poorly substituted proteins *in vivo* owing to its cytotoxicity, but it is efficiently incorporated *in vitro* [34]. The cell-free technique can also overcome the problem of large-scale cell growth using expensive media and materials. And finally, integral membrane proteins that are notoriously difficult to express *in vivo* can be produced *in vitro* at more than a milligram per milliliter of reaction and are suitable for biophysical analysis by nuclear magenetic resonance (NMR) [22].

Today, NMR spectroscopy techniques enable researchers to gather information from relatively large and supramolecular structures [35]. However, the application of this technology on proteins expressed from cell-based systems presents two major shortcomings. First, it requires extensive isotope labeling, which involves costly expression procedures *in vivo*. Second, the dual amino selective labeling technique [36], which helps to identify the signals, can only be used with certain amino acids owing to limited uptake and amino acid scrambling. With the absence of bilayer barriers, reduced amino acid metabolism and small reaction volumes, an *in vitro* protein expression system is a more convenient and economically feasible option for heavy isotope labeling. Several successful examples of amino-selective or uniform stable-isotope labeling using cell-free systems have been reported (for a recent example see [37] and for a review see [38]). More importantly, heteronuclear single quantum coherence spectra can be recorded directly from the reaction without further purification of the products facilitating the simultaneous handling of multiple samples [39].

High throughput and functional proteomics

With the vast and growing amount of genetic information available today, it is becoming increasingly difficult to use the traditional cell-based expression systems to rapidly screen protein activities or interactions using a multiplexed approach. By contrast, batch-fed cell-free protein synthesis offers tremendous flexibility for fast and parallel analysis of the products. One of the earliest strategies was the so-called '*in vitro* expression cloning', in which small plasmid pools are used to express desired protein products and screen for the presence of a desired biochemical activity. The subsequent positive pools are then progressively subdivided until a single plasmid coding for the active protein is isolated [40]. This strategy can be further improved by the use of the Gateway technology (http://www.invitrogen.com/gateway) which has recently proven to be convenient for the rapid screening of large libraries using protein cell-free synthesis [41]. Using this technology a large collection of genes can be tagged in tandem and screened for expression and solubility in a few hours (Figure 2).

With the finding that PCR products can be used as templates for the reaction, the procedure could be expanded to the construction and screening of random mutagenesis libraries (for a recent example see [42]). Other strategies are based on tandem PCR reactions that add specific 5′ and 3′ elements to the amplified product [43]. However, in this case the use of extremely long primers increases the chances of introduction of unwanted mutations. In our laboratory we have improved this approach by combining the power of PCR with the robustness of directional topoisomerase I-mediated joining to create DNA fragments optimized for *in vitro* expression (http://www.invitrogen.com/Content/Focus/320_022127_Focus25_3.pdf)

Protein evolution

Directed evolution is a powerful tool for accelerating the improvement of enzymes and interacting proteins. The key principle behind this strategy is the creation of a physical link between the nucleic acid and the protein that permits multiple rounds of amplification and selection. Cell-free translation-based display technologies offer faster procedures, lower costs and wider dynamic range when compared with traditional cell-based strategies. The most established methods are ribosome display, *in vitro* virus (also known as RNA-peptide fusion or mRNA display) and *in vitro* compartmentalization (for a review see [44]). Ribosome display uses cell-free extracts to translate mRNAs devoid of a stop codon, resulting in the RNA–ribosome–protein ternary complex remaining intact. For *in vitro* virus, a puromycin-tagged DNA is ligated to the 3′-end of the template messenger. The translating ribosome stalls at the RNA–DNA junction and the attached puromycin serves as a covalent acceptor of the polypeptide chain. In the third method a DNA library of enzyme mutants together with the transcription/translation mix are partitioned in water-in-oil-emulsion at an average of one DNA molecule per droplet. The synthesized product either modifies or attaches to the DNA from which it was produced (e.g. by the use of biotinylated DNA and a streptavidin tag). Then the emulsion is collapsed and the corresponding selection and amplification steps are performed.

Unnatural amino acids and in vitro protein labeling

The incorporation of non-natural amino acids, particularly those with chemically or physically reactive side chains, has the potential to be one of the most useful tools for functional and structural proteomics. A variety of labels, including fluorescent dyes for functional studies,

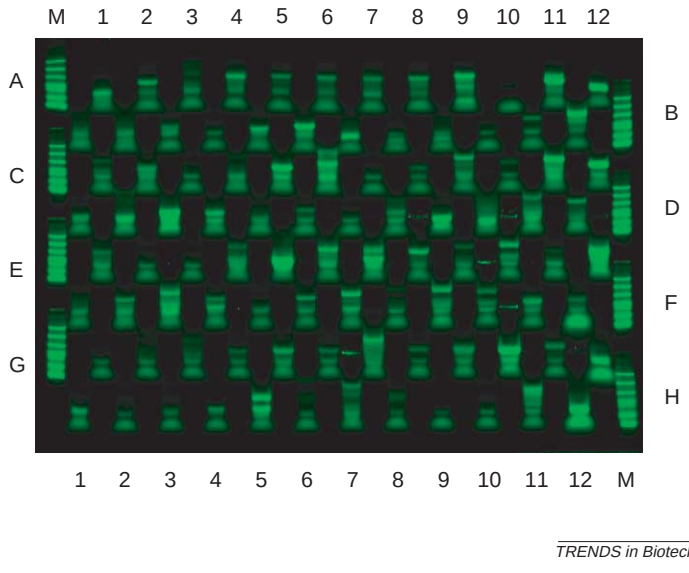

Figure 2. Rapid high-throughput protein expression screening method. A collection of human open reading frames were recombined in tandem into a vector that encodes a tetracysteine motif N-terminal fusion tag (pEXP3, Invitrogen, http://www.invitrogen.com). Proteins were synthesized in parallel using a commercial *E. coli* cell-free lysate (Expressway HTP Cell-Free *E. coli* Expression Kit, Invitrogen) and loaded in a 96-well SDS–PAGE (E-PAGE 96 Protein Electrophoresis System, Invitrogen) in the presence of a biarsenical fluorescein derivative [48] (Lumio Green Detection Kit, Invitrogen). Products were visualized in-gel with no further staining under a laser scanner (Typhoon 8600 Variable Mode Imager, Amersham Biosciences, http://www.amershambiosciences.com). Abbreviations: M, Molecular weight marker.

biotinylated moieties to facilitate purification and many others, including those for structural studies and for post-translational modifications, can be sequence-specifically incorporated into proteins. An efficient way to incorporate artificial amino acids into polypeptides is to supplement the cell-free extracts with chemically aminoacylated suppressor tRNAs that recognize a particular stop codon [45]. Genes containing the desired nonsense mutation at the appropriate site are used as templates. For example the incorporation of a single label at the N-terminal position, highly desirable for the preparation of protein micro arrays, can be readily accomplished by using an amber initiator suppressor tRNA and a DNA template with an amber codon instead of the normal initiation codon [46].

In vitro co-translational labeling is not limited to the use of unnatural amino acids. For example, puromycin derivatives can be used in cell-free expression systems to specifically label proteins at the C-terminus [47]. Recently, a novel tetracysteine motif was shown to specifically bind biarsenical ligands that become fluorescent only after binding [48]. Using a fluorometer, these compounds have been directly added to cell-free transcription–translation systems to monitor real-time protein synthesis in high-throughput expression format (Figure 3). This approach is particularly useful for high-throughput screening of pharmacological agents with translation-inhibiting activity. Although some of these labeling techniques can be applied to cell-based systems, problems such as cytotoxicity of the compounds, reduced protein yields, low label incorporation, or transport across membranes are issues largely reduced or eliminated when using a cell-free expression systems.

Finally, protein interaction validation studies should be greatly facilitated in cell-free expression systems, for instance by combining dual labeling with fluorescence cross-correlation spectroscopy [49]. This method allows monitoring whether two molecules labeled with different fluorophores bind to each other or not by the identification of fluctuations that occur simultaneously in two channels of a fluorescence correlation microscope.

Miniaturization and protein arrays

A clear application of cell-free protein expression reactions is in the area of miniaturization and protein arrays. For example, protein 'macro' arrays can be generated by small 25 μl reactions to synthesize tagged products that are *in situ* immobilized in separate wells coated with tag-binding beads [50].

Reactions can be downscaled to levels (nanoliter scale) that are unimaginable for cell-based approaches [51] and yet still synthesize enough products to perform individual enzymatic assays in 96-well glass microplates. Finally, cell-free protein synthesis offers tremendous advantages to the construction of protein micro arrays. Ramachandran and coworkers developed a self-assembling protein chip starting with DNA gene microarrays, which are transcribed and translated by a cell-free system. The resulting proteins, fused to glutathione-S-transferase (GST), are immediately captured *in situ* because of an antibody, anti-GST, printed simultaneously with the expression plasmid [52]. This technique saves considerable labor, time and costs by eliminating the need to express, purify and print proteins separately.

Diagnostics and therapeutics

In the field of genetic diagnostics, cell-free expression has attracted considerable interest with the protein truncation test as a method for detection of nonsense or frame-shift mutations in marker genes. Briefly, gene specific RT–PCR or PCR amplified products are added to transcription and translation reactions and truncated

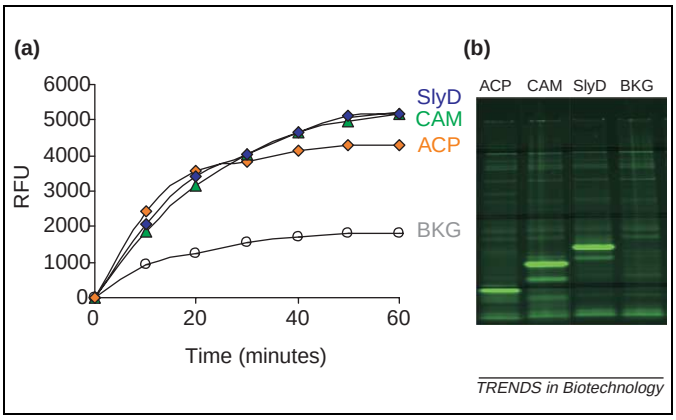

Figure 3. Real-time protein synthesis. Proteins were expressed and labeled simultaneously using a commercial cell-free expression kit (Expressway Plus Expression System with Lumio Technology, Invitrogen, http://www.invitrogen.com). Proteins were tagged with the tetracysteine motif Cys-Cys-X-X-Cys-Cys (where X is any non-cysteine amino acid). A biarsenical fluorescein derivative [48] was added directly to the reaction becoming fluorescent upon covalently binding to the tetracysteine tag. Products were followed by fluorometry as they emerge from the ribosome (SpectraMax Gemini XS, Molecular Devices, http://www.moleculardevices.com) **(a)** or visualized in gel with no further staining **(b)**. Abbreviations: ACP, acyl carrier protein (Accession # NP_455689); BKG, background signal (no DNA added to the reaction); CAM, calmodulin like protein (Accession # AAH31889); SlyD, FKBP-type peptidyl-prolyl cis-trans isomerase, (Accession # NP_417808, with naturally occurring Lumio binding site); RFU, relative fluorescence units.

products are detected using SDS–PAGE or ELISA in a high-throughput format [53]. Finally, the use of cell-free protein expression has been useful on the study of synthesis and assembly of macromolecular protein complexes [2]. Among them, *de novo* synthesis of infectious viral particles by cell-free systems has been the focus of considerable attention during the past years [54]. Aside from renewed concerns about biological weapons and new threats posed by genetic engineering, *in vitro* production of virus has enabled the development of multiple antiviral agents (for a recent example see [55]).

Future perspectives

Most of the advantages that cell-free expression systems offer can only be attained by high productive batch-fed configurations. Although protein concentrations up to a milligram per milliliter of reaction can be achieved now, this is still not enough for certain applications. But there is plenty of room for improvement. For example the incorporation of membrane vesicles loaded with the oxidative phosphorylation enzymes might have a positive effect by recycling ADP and lowering the free phosphate contents [56]. Finding a highly efficient energy regeneration system is also a key issue for lowering the costs of this expensive technology.

Another area that cell-free technology can make a significant impact on is protein folding. A relatively high fraction of proteins obtained by *in vitro* and *in vivo* systems are usually insoluble or misfolded. The addition of detergents or chaperones to the reaction sometimes has a productive effect but there might also be complementary approaches. For instance, hybrid systems composed of lysates from different sources, including those from archaea, might provide a more robust folding context.

The number and type of cell-free expression applications continues to grow. As an illustration, RNA silencing is a rapidly growing field in which cell-free expression systems can be extensively applied because they offer a less-restrictive biochemical framework for studying the RNAi machinery or validating siRNAs. Indeed, many of the key-findings of this area were achieved using wheat germ and *Drosophila* embryo cell-free extracts [57].

Cell-free expression is a powerful, flexible and ever expanding technology. The ability to manipulate the reaction conditions and to generate novel applications will probably only be limited by our creativity.

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