Bacterial cell-free expression technology to in vitro systems engineering and optimization

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ABSTRACT

Cell-free expression system is a technology for the synthesis of proteins in vitro. The system is a platform for several bioengineering projects, e.g. cell-free metabolic engineering, evolutionary design of experiments, and synthetic minimal cell construction. Bacterial cell-free protein synthesis system (CFPS) is a robust tool for synthetic biology. The bacteria lysate, the DNA, and the energy module, which are the three optimized sub-systems for in vitro protein synthesis, compose the integrated system. Currently, an optimized E. coli cell-free expression system can produce up to ~2.3 mg/mL of a fluorescent reporter protein. Herein, I will describe the features of ATP-regeneration systems for in vitro protein synthesis, and I will present a machine-learning experiment for optimizing the protein yield of E. coli cell-free protein synthesis systems. Moreover, I will introduce experiments on the synthesis of a minimal cell using liposomes as dynamic containers, and E. coli cell-free expression system as biochemical platform for metabolism and gene expression. CFPS can be further integrated with other technologies for novel applications in environmental, medical, and material science.

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1. Introduction

The conception and design of novel biological systems using bio-molecular components and modules [1–3] is a constructive approach to solve problems, and provide useful solutions for the development of the future bio-economy [4–6]. This is an evolving context where cell-free synthetic biology has emerged as a new bioengineering discipline that aims to reconstitute complex biological functions outside of the living cells seeking applications in medical, environmental, and material science [7–10]. One of the main enabling technologies of this new scientific domain is cell-free protein synthesis [11,12] (CFPS). CFPS is a methodology to synthesize polypeptides and reconstruct molecular machineries (ribosomes) in vitro from synthetic DNA recombinant molecules [13], as well as re-activate complex metabolic pathways in cytoplasmic extracts [14]. Cell-free expression systems can be of prokaryotic and eukaryotic cell origin. For instance, depending on the application eukaryotic cell-free expression systems can be prepared from tobacco BY-2, yeast, wheat germ, insect, rabbit reticulocyte, and Chinese hamster ovary (CHO) cell lines [13]. Herein, I will discuss the features and engineering capabilities of the E. coli cell-free expression system, which is the most widely used in...
synthetic biology. Cell-free expression technology can be more advantageous than in vivo expression of recombinant proteins [15]. At a glance two major advantages should be mentioned: (i) the expression of cytotoxic products, and (ii) rapid prototyping through the design-built-test (DBT) cycle [16]. Nonetheless, CFPS is only one of the possible developments of an emerging scientific field that aims to engineer new complex behaviors and systems in vitro. Some examples of such systems are minimal cells [17], microfluidic based artificial cells [18], in vitro DNA, RNA circuits, and molecular programs [19–22], DNA nanostructure self-assembly [23,24], and cell-free enzyme technology [25].

Initially, E. coli cell-free expression systems were developed as platforms for in vitro protein synthesis [26,27], and as tools for understanding DNA replication [28]. Since then, several improvements have been demonstrated and bacterial cell-free expression systems have become a robust, cost-effective, versatile and scalable technology for production of proteins [29,30] such as: vaccines [31], antibodies [32], cytokines [33], and it has also paved the way to the new exciting field of cell-free metabolic engineering (CFME) [34–36]. In the recent years, E. coli cell-free expression systems have been decoupled and standardized [37], and the preparation streamlined and democratized [38]. In particular, modularity has been demonstrated useful to prototype newly in vitro designed genetic circuits [39], develop new transcriptional factors [40], incorporate non-standard amino acids [41], produce human proteins [42], synthesize a bacteriophage from its genomic DNA [43], conceive low-cost portable devices for diagnostics [44], and develop bio-sensors [45]. Furthermore, due to its robustness and engineering capabilities, cell-free expression technology provides a useful framework for developing new directed evolution platforms using microfluidic and high-throughput technologies [46–49].

Table 1, describes some important results achieved with CFPS. In addition to the aforementioned applications, cell-free expression system was envisioned as the main platform for building a minimal cell using liposomes as model cellular container [61,62]. Firstly, the synthesis of a minimal cell was a project mainly related to the origin of life research field [63]. However, demonstration of a genetic circuit within liposomes [64], a DNA programmed bioreactor [51], the biosynthesis of phospholipidin liposomes [65], and the original design of a cell-free expression system for the construction of synthetic ribosomes [55], emphasized the minimal cell construction as a synthetic biology project. The ribosome is the central molecular machinery for sustaining a protein-based self-replicating minimal cell [66]. Overall, these results laid the foundations of cell-free expression systems applied to the field of synthetic biology to the synthesis of a minimal cell.

The main advantage of conceiving new ideas using cell-free expression systems is the lack of cell viability, which gives the possibility to achieve different designs assembling biological parts and modules adjusting the experimental parameters [9]. Moreover, the open nature of the system allows performing quantitatively studies based on mathematical modeling [67–69]. Herein, I will review the energetic and preparation of bacterial cell-free expression systems. In particular, I will present (i) the most powerful E. coli cell-free expression system capable of producing >2 mg/mL of GFP fluorescent reporter in a test tube, which has been recently upgraded for extending the performance of an all E. coli cell-free platform for in vitro gene circuitry [70] (ii) the optimization of complex systems in vitro using machine learning and liquid handling robotics (iii), the design of minimal cells based on in vitro gene expression within liposome compartments.

2. Bacterial cell-free expression and polysaccharide system energetic

Cell-free protein synthesis is an in vitro technology for many bioengineering projects [9]. The technology is well established, and exploited in many laboratories worldwide. Cell-free expression systems can be abstracted in three different modules, as shown in Fig. 1: (i) the bacterial lysate (crude extract) is the extracted, and clarified cytoplasm containing all the necessary molecular machineries for in vitro transcription, translation and metabolism (ii)

| Reference           | Original Work                                                                 | Year       | Relevance                                                                 |
|---------------------|------------------------------------------------------------------------------|------------|---------------------------------------------------------------------------|
| Noireaux, V. et al. | Gene circuits in vitro using a cell-free expression system                    | 2003       | Definition of the fundamental principles for in vitro gene circuitry      |
| Noireaux, V. et al. | Bioreactor controlled by DNA program                                          | 2004       | Compartmentalization of gene expression for functional protein pore in    |
| Calhoun, K. A. et al.| Cell-free protein synthesis (CFPS) using glucose and nucleotide monophosphate| 2005       | Cell-free activation of central metabolism for energy components regeneration |
| Jewett, M.C. et al. | Co-activation of multiple biochemical reactions in a cell-free platform        | 2005       | Identification and re-activation of oxidative phosphorylation in cell-free expression systems |
| Jawada, J. F. et al.| Cell-free protein synthesis system for biopharmaceutical products            | 2011       | Milestone production of active disulfide-bonded therapeutics at 100 L scale |
| Caschera, F. et al.| Optimization of cell-free protein synthesis using machine learning           | 2011       | Automatic, evolutionary, iterative design of experiments applied to cell-free protein synthesis |
| Shin, J. et al.     | Reconstruction of the transcriptional repertoire of E. coli cells              | 2012       | Parts standardization for in vitro circuits with E. coli cell-free expression systems |
| Jewett, M.C. et al. | Integrated system for re-construction of functional ribosome                 | 2013       | First platform for building synthetic ribosome from DNA program           |
| Chappell, J. et al. | Utilization of CFPS to characterize DNA regulatory elements                   | 2013       | Rapid prototyping of DNA libraries, and in vitro — in vivo correlation    |
| Caschera, F. et al.| Polysaccharides to re-cycle inorganic phosphate using endogenous catalysts   | 2014       | Highest protein yield (2.3 mg/mL) achieved in batch mode                  |
| Hong, S.H. et al.  | Site specific incorporation of non standard amino acids using a cell-free expression system | 2014       | Cell-free multi site incorporation of non standard amino acids with a genetically recoded strain |
| Karbroun, E. et al. | DNA compartments on a silicon chip as artificial cells                       | 2014       | Oscillating protein expression as function of chip geometry               |
| Caschera, F. et al.| Inexpensive polyposephate molecule activates glycolysis, important for scale-up projects | 2015       | High yielding cost-effective formulation for cell-free protein synthesis |
| Caschera, F. et al.| First detailed amino acid preparation at high concentration for CFPS          | 2015       | Standardization of a complex molecular mixture for the cell-free expression research community |
| Niederholtmeyer, H. et al. | Implementation of in vitro biological networks in cells                   | 2015       | Rapid prototyping of bacterial oscillatory networks                      |
| Karim, A. K. et al.| Cell-free metabolic engineering from DNA programs                            | 2016       | Design, built, test cycle (DBT) of in vitro metabolic networks           |
the recombinant DNA module is the information unit with the sequences codifying and directing protein/s synthesis and molecular networks, (iii) the energy module is the mixture of small molecules necessary to trigger an in vitro metabolism for ATP-regeneration and fuel the system.

Importantly, for generating efficient cell-free protein synthesis systems, the three modules described above must be optimized and interlocked. The transcription of DNA molecules can be either under the control of the T7 promoter [71] or endogenous bacterial sigma factors [57]. The design and assembly of the gene regulatory parts is an example of DNA module optimization [72]. Cell-free protein synthesis and gene circuitry from recombinant DNA can be executed either from optimized plasmids amplification [79] or PCR generated linear templates [73,74]. Generally, protein synthesis from plasmid DNA gives higher protein yields than synthesis from linear DNA templates. Nevertheless, working with linear constructs is convenient to bypass extensive and tedious cloning procedure necessary for plasmid preparation and amplification. However, performing cell-free protein synthesis from PCR generated linear templates requires molecular strategies for protecting the sequences from cytoplasmic exonucleases. The protection of the gene of interest can be achieved by addition of extra base pairs, by supplementing DNA binding proteins, e.g. GamS, by chemical modification with PTO (phosphorothioate bonds modifications), or with short oligos blocking the RecBCD complex [74,75]. Besides, stabilization is also possible by DNA circularization strategies [76], crosslinking into hydrogels [77], or even by E. coli genome engineering [78]. Overall, the expression using linear templates is preferred because of the reduce number of cloning steps, in fact cell-free protein synthesis can be initiated by linear sequences amplified by PCR where regulatory elements and genes are joined using multistep overlap PCR [79] or Gibson assembly [80].

The design and optimization of the energy module is also important. Indeed, an efficient metabolism able to re-generate ATP is critical for in vitro protein synthesis [81]. The construction of this element implies the preparation of stable molecular mixtures containing, potassium, magnesium, glutamate, enzyme co-factors, high-energy phosphate donor molecules, tRNA, and nucleotides triphosphate (NTP), among other components [57,59]. The function of this chemical mixture is to reanimate the bacteria central metabolism for energy production using the enzymes already present in the crude lysate. Diverse metabolic modules based on different high-energy phosphate molecule donors, such as: 3-PGA (3-phosphoglycerate), PEP (phosphoenolpyruvate) and CP (creatine phosphate) have been designed to sustain in vitro protein synthesis [72,82]. However, in some cases, the overall cost of phosphate donor molecules and NTP can be limiting for high-throughput screening and large-scale applications. Therefore, cost-effective formulations to bypass substrate level phosphorylation have been developed using glucose [83], pyruvate [84], or fructose 1,6-biphosphate [85] as energy sources for ATP-regeneration, as well as nucleotides mono-phosphate (NMP) as molecular precursors [86]. In addition, it has been proven that cell-free protein synthesis can proceeds efficiently with glutamate, a metabolic intermediate of the tricarboxylic acid (TCA) cycle that generates NADH. The reducing molecule is then used in the oxidative phosphorylation to produce ATP through inverted membrane vesicles present in the lysate [53]. Nevertheless, the cell-free reaction is less efficient if the byproduct of protein synthesis, inorganic phosphate (iP), is accumulated upon ATP hydrolysis. In fact, iP chelates magnesium ions thereby depriving the reaction of an essential salt necessary for the stability and functionally of enzymes and ribosomes [84,87]. Taking into consideration the inhibiting mechanism, long-lasting high-yielding cell-free protein synthesis has been designed with maltose and maltodextrin to efficiently recycle the inorganic phosphate and re-generate ATP through the glycolysis [57]. This novel in vitro metabolism utilizes only endogenous catalysts already present in the cellular lysate. The recycling scheme was inspired by previous results showing efficient protein synthesis with maltodextrin and exogenous enzymes [88,89]. Further improvement was possible with higher amino acids concentrations, and coupling maltose or maltodextrin to 3-PGA. As a result, production of 2.3 mg/mL of a fluorescent reporter protein was demonstrated in batch mode reaction [57]. Besides, a cost-effective formulation of this energy module was implemented using hexamethaphosphate (HMP). The polynucleotide molecule HMP releases iP and triggers production of glucose-1-phosphate upon polysaccharide phosphorylation. This cost-effective cell-free expression system could synthesize up to 1.6 mg/mL of protein in batch mode bypassing high-energy substrate level phosphorylation [58]. Importantly, these polysaccharide based systems are fueled by a custom made amino acid mixture, which allows precise control on important parameters for in vitro protein synthesis such as: amino acid pH, relative and total concentration [59].

For making cell-free expression technology easy accessible to many laboratories worldwide, different methods of crude lysate preparation have been developed. Indeed, french-press or high-pressure homogenization [53,57], used for disrupting bacteria cells, can be substituted by cost-effective methods, such as: beads beating [37], sonication [38], and chemical lysis [90]. All these methods can generate lysates for high yielding cell-free expression systems synthesizing proteins in the concentration range between 0.5 and 2 mg/mL. Moreover, further protocol simplifications have decreased the requirements for expensive laboratory equipment. Indeed, high-speed centrifugation (30000 g) can be replaced by lower speed centrifugation (12000 g), which is reachable by less costly benchtop centrifuges [91,92].

Bacterial cell-free expression systems are generally prepared using E. coli A19, D10 and BL21 [93]. However, more recently new cell-free expression systems from diverse bacterial strains have been introduced. These new in vitro protein synthesis systems have been developed using Bacillus Subtilis [94] and Streptomyces [95]. The alternative systems have been conceived for prototyping DNA regulatory elements such as endogenous promoters, as well as for synthesizing soluble proteins from high GC content genes, such as
non-ribosomal peptides [96]. These novel platforms demonstrate the potential of cell-free expression technology as bioengineering tool for prototyping gene circuits from bacteria strains important for industrial applications, and also to synthesize secondary metabolites for medical applications.

Cell-free expression systems technology has provided the test bed to conceive an ambitious synthetic biology project, i.e. the total synthesis of ribosome in vitro [55,97]. The synthesis of ribosomes is important to build and sustain a protein based minimal cell, i.e. capable of self-maintenance and self-replication [66], and moreover, to engineer ribosomes with extended capabilities [98]. The cell-free expression system designed for building ribosomes in vitro from DNA encoding sequences is named iSAT: integrated ribosomal RNA synthesis, self-assembly, and translation [55]. The platform has been optimized for using ribosomes self-assembled upon in vitro transcription of the E. coli natural rRNA operon encoding the 16s, 23s, and 5s ribosomal rRNAs [89]. In addition, further system’s improvements have been achieved implementing molecular crowding and reducing conditions [100], as well as alleviating substrate limitation using a reaction set-up for continuous buffer exchange [101]. The iSAT system is an advanced and unique platform for in vitro protein synthesis from in situ self-assembled ribosomes. The system is highly complex with many interdependent components interacting non-linearly, which direct the correct self-assembly of active ribosomes for in vitro protein synthesis. The possibility of intelligently design such complex process from the bottom-up, will pave the way towards the engineering of new synthetic ribosome with extended capabilities. Remarkably, the system constitutes a central module for building a minimal cell that can regenerate its components during cycles of self-replication.

Overall, cell-free expression systems are platforms with important bioengineering capabilities. In fact, the lack of cell viability provides direct access to system’s parameters, and open the possibility to re-configure the biochemical variables into new layers with optimal out-puts. Therefore, parallelized experimentation and data driven automatic optimization will be a future field of application for this technology.

3. In vitro cell-free experiments and automatic driven optimization

One of the endeavors of synthetic biology is the implementation of automatic approaches to design and optimize complex biological systems. For instance, computer algorithms have been recently applied to design bacterial synthetic gene circuits [102], and to improve the performance of CRISPR-Cas9 gene-editing tool [103]. Furthermore, a robotic workstation programmed for on demand production of proteins has been recently presented [104]. Automation may represent a breakthrough technology in modern science. For instance, a robotic crew system could be applied to improve the workflow of biological experimental research [105]. However, the conception of a robot scientist [106] able of making and testing hypothesis experimentally without human intervention, i.e. using laws derived from large data-set already available [107], dates back to ~10 years ago. More recently, an approach based on an iterative cycle of experiments grounded on high-throughput screening protocols and statistical modeling of the fitness landscape was applied for discovering a new Amphoteracin B liposomal drug formulation [108]. Such approach is different because is based on an initial human in-put that defines the experimental space, and is followed by a machine learning algorithm that predicts the best interactions in the fitness landscape over each iterated generation of experiments. This intelligent exploration has efficiently optimized the cargo capacity of an insoluble drug into liposome bilayers of different compositions. The result was possible by screening a library of buffers, salts, phospholipids, and fatty acids underlying an experimental space of ~90^10^3 possible molecular combinations. The high-throughput exploration was conducted using liquid handling robotics guided by a machine-learning algorithm. As a result, by sampling only a small subset (0.5%) of the possible experimental space, a new formulation with 2.5-fold increase cargo capacity was discovered in only 1 week of experiments [108]. This optimization has demonstrated the possibility of optimizing a biochemical system in vitro, through a non-exhaustive exploration of a complex multidimensional experimental space exploiting a laboratory robotic workstation, and an intelligent computer algorithm, i.e. an artificial neural network. This method could be further extended to the optimization of amphiphile systems for cell-free synthesis of membrane proteins [109]. In vitro cell-free expression systems are complex biochemical systems, therefore, they can be optimized using the state of the art of machine learning algorithms [110], and liquid handling robotics. Indeed, the iterative automated approach discussed above was applied to the optimization of an E. coli cell-free expression system [54].

A cell-free expression system is designed for in vitro protein synthesis from synthetic DNA in a test tube; see modular organization in Fig. 1. The open nature of the system allows both theoretical optimization with liquid handling robotics and machine learning. In this experiment [54], in vitro protein synthesis was optimized using a commercial kit reconstituted in sub-optimal conditions (diluted). In particular, a library composed of various salts, co-factors, and amino acids at different concentrations was tested at three time variables (T = 0, T = 30 or T = 120 min) during the course of cell-free protein synthesis. Moreover, a library of 6 DNA constructs (plasmids) with variations in the distances between the T7 promoter, the ribosome binding site, and the start codon, as well as the stop codon and the terminator was designed around two common core sequences of 5 bp and evaluated to generate GFP. Overall, the library’s components generated a possible space of 1.5*10^8 combinations in a 16-dimensional space. Using iterative high-throughput screening and a neural network type algorithm, the yield of protein synthesized in vitro was progressively increased up to 300% trying only 0.014% of the total experimental combinations over 8 generations of experiments (8 days of experiments). In this iterative experimentation was introduced a trade-off mechanism to choose between model based and random combinations. This novel mechanism was used to cope with an experimental space too large to be efficiently optimized. However, the number of random combinations was progressively decreased over the evolutionary exploration towards generations of experiments entirely based on model predicted combinations. As a result, a better system configuration was discovered by a robotic workstation guided through the fitness landscape by an artificial neural network.

These experiments represent the state of the art of cell-free expression technology optimization using machine learning, and can be extended to the optimization of different bacterial cell-free protein synthesis systems [94,95], to the synthesis of human proteins [42], synthetic ribosomes [97], proteins incorporating non-standard amino acids [41], and even to the optimization of non-ribosomal peptides synthesized through cell-free expression technology [96]. In addition to many other complex bio-chemical systems. Besides, machine learning could be applied to optimize and scale-up the production of valuable products synthesized through cell-free metabolic engineering [34]. Interesting would be also the development of more powerful and precise machine learning methods for designing DNA libraries encoding for better biocatalysts [111]. In conclusion, robotic workstations are fundamental for big data generation, and consistent execution of the
experiments, which overall are essential premises for complex systems optimization driven by computers algorithms. Looking forward, automation and machine learning could be also useful to implement the integration of the modules necessary for the synthesis of artificial cells, either using a top-down or a bottom up approach [112].

4. Synthesis of minimal cells: compartmentalization, liposomes dynamics and molecular machineries

One of the grand challenges of synthetic biology is to construct a minimal synthetic cell [113,114]. The synthesis of such complex system can be achieved following the top-down or the bottom-up approach [62,112]. These approaches are based on two specular designs: the bottom-up approach pursues a gradual increase of complexity through the integration of three subsystems, i.e. compartment, metabolism and information [17,115], conversely, the top-down aim to reduce the complexity of modern cells by implanting a computer designed DNA program into the cellular hardware, and as a result, direct minimal life functions [116].

Bacterial cell-free expression technology is relevant to design minimal cells, and is the platform used to build minimal cells through the bottom-up approach [117]. Several laboratories worldwide have contributed to the development of this research project. For instance, long lasting protein synthesis inside phospholipid vesicles has been achieved through the synthesis of a membrane pore (protein) facilitating nutrients up-take from an external feeding solution [51]. Furthermore, in order to control minimal cell division, the MreB cytoskeletal bacterial structures were recently characterized using micrograph image analysis. In particular, genetic expression of one, two proteins simultaneously, as well as, a simple genetic circuit based on the melibiose operator have been investigated inside liposomes [118]. This preliminary study shown that liposome gene expression is highly variable and difficult to predict accurately within a population. The observations suggested that fluctuations resulting in a high level of correlated noise [119], could be most likely due to heterogeneous encapsulation of DNA molecules (plasmids), which in turn could be due to the inverted emulsion transfer method utilized to generate liposomes [120]. Therefore, methods to precisely control the encapsulation of DNA constructs during compartmentalization [121] are necessary for precise system out-put control, and decrease high level of unnessary correlated noise (protein yields) in a simple expression system. Importantly, through the construction of multifunctional minimal cells, is the design of systems capable of reaction spatial control [122] and chemical communication [123]. For instance, mechanisms based on riboswitches regulatory elements and two-way chemical communication strategies based on quorum sensing, have been implemented to establish interaction between artificial cell and bacterial cell [123,124]. These results are important as they show progression towards the development of technologies based on life principles [125].

The stepwise increase of minimal cell complexity was envisioned as a programmed vesicle-vesicle fusion process similar to the symbiogenesis [126]. Moreover, continuous liposome fusion is necessary to generate a life-cycle supporting Darwinian evolution of proliferating minimal cell systems [127]. The strategy implemented to induce fusion is based on the interaction of oppositely charged membranes, lipid mixing and internal mixing contents [128,129]. This vesicle fusion strategy was further characterized using flow-cytometry [130], which is a tool that has been well established for studying gene expression within liposomes [131]. Importantly, to develop experimental methods for increasing minimal cell complexity, the vesicle fusion strategy was integrated with activated compartmentalized gene expression. In particular, compartmentalized gene expression was activated upon internal mixing contents of two vesicle populations encapsulating complementary components necessary for in vitro protein synthesis, i.e. DNA and RNA polymerase [132]. Recently, different vesicle fusion strategies, e.g. liposome fusion induced by repeated freeze and dry cycles or mediated by the SNARE complex were demonstrated to be compatible with internal RNA replication, and genetic circuits respectively [133,134]. Also, compartmentalized amplification of DNA molecules has been linked to liposome self-reproduction, triggered by chemical transformation of a bolaamphiphilic precursor intercalated in the membrane [135], and liposome fusion induced by a change in pH [136]. This model suggested an experimental route for studying minimal cell evolution through a life-cycle.

Towards the establishment of a minimal cell cycle supporting Darwinian evolution, liposome growth by internal membrane components generation was also studied. In particular, a bacterial metabolic pathway for synthesizing phospholipids was reconstituted in liposomes using complex enzymatic membrane proteins, which were internally synthesize by their DNA encoding sequences [65]. Finally, for building a self-replicable protein based minimal cell [66], ribosome reconstitution was demonstrated within liposomes [137]. The iSAT system [55] was compartmentalized within liposomes of different phospholipid formulations, and protein synthesis from self-assembled ribosomes, was assessed as function of the membrane composition. It was shown bacterial rRNA operon transcription, and reconstitution of functional ribosomes from purified ribosomal proteins inside liposomes. In addition, it was proved that protein synthesis depends on phospholipid bilayer composition, which by passive diffusion regulates the permeability of small molecule nutrients fueling in vitro protein synthesis [137].

Overall, these results demonstrate that the synthesis of a minimal cell, through the bottom-up approach using liposomes as dynamic containers mimicking the modern cellular organization, is an active field of research. However, it is still not clear which is the road map towards the synthesis of a minimal cells, and how the sub-systems integration of such complex system can be efficiently completed [17,138]. Then, would be important to specify or simulate how a minimal genome [139], assembled from scratch and compartmentalized, could support autonomy and open ended evolution [140].

5. Conclusions and perspectives

Cell-free protein synthesis is an enabling technology, an important platform for many synthetic biology projects, such as: cell-free metabolic engineering [34], machine learning optimization [54], and directed evolution [47]. Herein was given an overview of the numerous applications and growing interest for cell-free expression technology, which has become an important tool for cell-free synthetic biology [9]. However, other in vitro technologies such as: molecular programs [22], and DNA supra-molecular structures [141] may provide a new level of complexity for developing original, integrated cell-free biotechnologies. In this review were presented three main topics: (i) cell-free expression system modular organization and energetic, (ii) machine learning optimization, (iii) and minimal cell synthesis. The three subjects are important for different reasons. In particular, to develop a robust bacterial cell-free expression system is essential to design accurately the energy module, which provides the main framework for high-yielding and reproducible in vitro protein synthesis. The open nature of cell-free expression technology allowed automation with
robotic workstations [137]. Therefore, large amount of data can be generated by parallelized experimentation for machine learning optimization to synthesize, for example, difficult to fold proteins and small molecules for industrial and medical applications. The synthesis of a minimal cell is an important project to understand the fundamental design principles of life. This understanding will unveil novel design rules to the development of new life-like technologies that could be useful for environmental remediation, material, and medical science. The increasing interest for cell-free expression systems will generate new results, and will continue to advance cell-free synthetic biology research. As mentioned, CFPS was developed many years ago, and nowadays has become a robust tool for engineering and prototyping new systems, such as: assembly of DNA regulatory parts for gene circuit design, and on-demand production of recombinant proteins. Cell-free expression technology will be more and more a playground for engineers, microbiologists, molecular and computer scientists. The next challenges for cell-free expression systems technology could be the design of a self-replicating, out-of-equilibrium system for enzymatic DNA synthesis [142], and continuous in vitro evolution [143], thus contributing to further decrease the cost of DNA synthesis and improving protein design for biotechnological applications.

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