Synthetic and Systems Biotechnology 2 (2017) 97–104

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology/

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

Filippo Caschera

École Supérieure de Physique et de Chimie Industrielles de la Ville de Paris (ESPCI Paris), CNRS UMR 7083, 10 Rue Vauquelin, 75231 Paris Cedex 05, France

ARTICLE INFO

Article history:
Received 13 June 2017
Received in revised form
25 July 2017
Accepted 25 July 2017

Keywords:
Cell-free protein synthesis
Engineering
Optimization
Polysaccharide
ATP-regeneration
Machine-learning
Synthetic minimal cell

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.

Contents

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

E-mail address: filippocaschera@gmail.com.
Peer review under responsibility of KeAi Communications Co., Ltd.

http://dx.doi.org/10.1016/j.synbio.2017.07.004

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 system has 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 phospholipidsin 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 $\geq$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)

Table 1
Recent landmarks of *E. coli* cell-free expression systems to synthetic biology.

Reference	Original Work	Year	Relevance
Noireaux, V. et al. [50]	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. [51]	Bioreactor controlled by DNA program	2004	Compartmentalization of gene expression for functional protein pore in phospholipid bilayer
Calhoun, K. A. et al. [52]	Cell-free protein synthesis (CFPS) using glucose and nucleotide monophosphate precursors	2005	Cell-free activation of central metabolism for energy components regeneration
Jewett, M.C. et al. [53]	Co-activation of multiple biochemical reactions in a cell-free platform	2008	Identification and re-activation of oxidative phosphorylation in cell-free expression systems
Zawada, J. F. et al. [33]	Cell-free protein synthesis system for biopharmaceutical products	2011	Milestone production of active disulfide-bonded therapeutics at 100 L scale
Caschera, F. et al. [54]	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. [39]	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. [55]	Integrated system for re-construction of functional ribosome	2013	First platform for building synthetic ribosome from DNA program
Chappell, J. et al. [56]	Utilization of CFPS to characterize DNA regulatory elements	2013	Rapid prototyping of DNA libraries, and *in vitro* − *in vivo* correlation
Caschera, F. et al. [57]	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. [41]	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
Karzbrun, E. et al. [18]	DNA compartments on a silicon chip as artificial cells	2014	Oscillating protein expression as function of chip geometry
Caschera, F. et al. [58]	Inexpensive polyphosphate molecule activates glycolysis, important for scale-up projects	2015	High yielding cost-effective formulation for cell-free protein synthesis
Caschera, F. et al. [59]	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. [60]	Implementation of *in vitro* biological networks in cells	2015	Rapid prototyping of bacterial oscillatory networks
Karim, A. K. et al. [16]	Cell-free metabolic engineering from DNA programs	2016	Design, built, test cycle (DBT) of *in vitro* metabolic networks

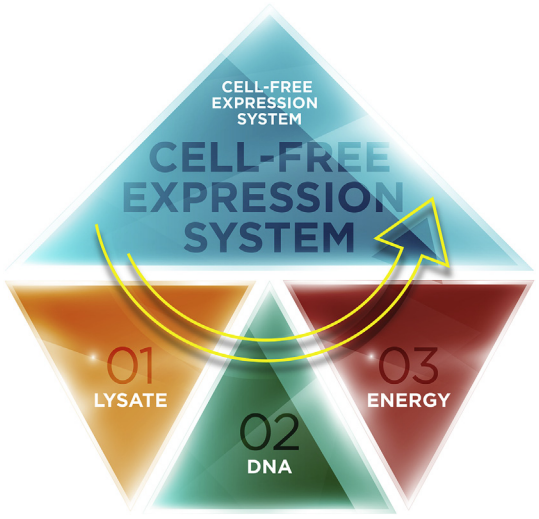

Fig. 1. Cell-free expression system modular organization. In the schematic illustration three modules, i.e. 0.1 the Lysate, 0.2 the DNA, 0.3 the Energy are interlocked to constitute a system for *in vitro* protein synthesis.

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 amplified in *E. coli* 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 reactivate 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 this inhibiting mechanism, long-last 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 concentration, 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 polyphosphate molecule HMP releases iP and triggers production of glucose-1-phospahte 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 (12000g), 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 syntheses 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 [99]. 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 crowd 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 Amphotericin 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 allowed combinatorial 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 $1.5^{*}10^{6}$ 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 [17]. 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 synthesized from encapsulated DNA [117]. Besides, gene expression inside liposome populations has been 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 unnecessary 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 symbiogensis [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 complexes 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.

References

[1] Kahl LJ, Endy D. A survey of enabling technologies in synthetic biology. J Biol Eng 2013;7(1):13.
[2] Schwille P. Bottom-up synthetic biology: engineering in a tinkerer's world. Science 2011;333(6047):1252–4.
[3] Heinemann M, Panke S. Synthetic biology putting engineering into biology. Bioinformatics 2006;22:2790–9.
[4] Endy D. Foundations for engineering biology. Nature 2005;438(7067):449–53.
[5] Auslander S, Auslander D, Fussenegger M. Synthetic biology-the synthesis of biology. Angew Chem Int Ed Engl 2017;56(23):6396–419.
[6] de Lorenzo V, Schmidt M. Biological standards for the knowledge-based bioeconomy: what is at stake. New Biotechnol 2017. http://dx.doi.org/10.1016/j.nbt.2017.05.001.
[7] Smith MT, Wilding KM, Hunt JM, Bennett AM, Bundy BC. The emerging age of cell-free synthetic biology. FEBS Lett 2014;588(17):2755–61.
[8] Hodgman CE, Jewett MC. Cell-free synthetic biology: thinking outside the cell. Metab Eng 2012;14(3):261–9.
[9] Perez JG, Stark JC, Jewett MC. Cell-free synthetic biology: engineering beyond the cell. Cold Spring Harb Perspect Biol 2016;8(12).
[10] Karig DK. Cell-free synthetic biology for environmental sensing and remediation. Curr Opin Biotechnol 2017;45:69–75.
[11] Lee KH, Kim DM. Applications of cell-free protein synthesis in synthetic biology: interfacing bio-machinery with synthetic environments. Biotechnol J 2013;8(11):1292–300.
[12] Rosenblum G, Cooperman BS. Engine out of the chassis: cell-free protein synthesis and its uses. FEBS Lett 2014;588(2):261–8.
[13] Zemella A, Thoring L, Hoffmeister C, Kubick S. Cell-free protein synthesis: pros and cons of prokaryotic and eukaryotic systems. ChemBioChem 2015;16:2420–31.
[14] Swartz JR. Transforming biochemical engineering with cell-free biology. AIChE J 2011;58:5–13.
[15] Lu Y. Cell-free synthetic biology: engineering in an open world. Synth Syst Biotechnol 2017;2:23–7.
[16] Karim AS, Jewett MC. A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery. Metab Eng 2016;36:116–26.
[17] Caschera F, Noireaux V. Integration of biological parts toward the synthesis of a minimal cell. Curr Opin Chem Biol 2014;22:85–91.
[18] Karzbrun E, Tayar AM, Noireaux V, Bar-Ziv RH. Programmable on-chip DNA compartments as artificial cells. Science 2014;345:829–32.
[19] Chen Y-J, Dalchau N, Srinivas N, Phillips A, Cardelli L, Soloveichik D, et al. Programmable chemical controllers made from DNA. Nat Nanotechnol 2013;8:755–61.
[20] Qian L, Winfree E. Scaling up digital circuit computation with DNA strand displacement cascades. Science 2011;332(6034):1196–201.
[21] Weitz M, Simmel FC. Synthetic *in vitro* transcription circuits. Transcription 2012;3(2):87–91.
[22] Montagne K, Plasson R, Sakai Y, Fujii T, Rondlez Y. Programming an *in vitro* DNA oscillator using a molecular networking strategy. Mol Syst Biol 2011;7:466.
[23] Andersen ES, et al. Self-assembly of a nanoscale DNA box with a controllable lid. Nature 2009;459(7243):73–6.
[24] Czogalla A, Franquelim HG, Schwille P. DNA nanostructures on membranes as tools for synthetic biology. Biophys J 2016;110:1698–707.
[25] Hold C, Billerbeck S, Panke S. Forward design of a complex enzyme cascade reaction. Nat Commun 2016;7:12971.
[26] Nirenberg MW, Matthaei JH. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. Proc Natl Acad Sci U S A 1961;47:1588–602.
[27] Zubay G. *In vitro* synthesis of protein in microbial systems. Annu Rev Genet 1973;7:267–87.
[28] Kornberg T, Gefter ML. Purification and DNA synthesis in cell-free extracts: properties of DNA polymerase II. Proc Natl Acad Sci U S A 1971;68(4):761–4.
[29] Spirin AS, Swartz JR. Cell-free protein synthesis systems: historical landmarks, classification, and general methods. In: Cell-free protein synthesis: methods and protocols. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2008. p. 1–34.
[30] Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB. A continuous cell-free translation system capable of producing polypeptides in high yield. Science 1988;242(4882):1162–4.
[31] Lu Y, Chan W, Ko BY, VanLang CC, Swartz JR. Assessing sequence plasticity of a virus-like nanoparticle by evolution toward a versatile scaffold for vaccines and drug delivery. Proc Natl Acad Sci U S A 2015;112(40):12360–5.
[32] Xu Y, Lee J, Tran C, Heibeck TH, Wang WD, Yang J, et al. Production of bispecific antibodies in "knobs-into holes" using a cell-free expression system. mAbs 2015;7:231–42.
[33] Zawada JF, Yin G, Steiner AR, Yang J, Neresh A, Roy SM, et al. Microscale to manufacturing scale-up of cell-free cytokine production–a new approach for shortening protein production development timelines. Biotechnol Bioeng 2011;108(7):1570–8.
[34] Dudley QM, Karim AS, Jewett MC. Cell-free metabolic engineering: biomanufacturing beyond the cell. Biotechnol J 2015;10(1):69–82.
[35] Dudley QM, Anderson KC, Jewett MC. Cell-free mixing of escherichia coli crude extracts to prototype and rationally engineer high-titer mevalonate synthesis. ACS Synth Biol 2016;5(12):1578–88.
[36] Guo W, Sheng J, Feng X. Mini-review: *in vitro* metabolic engineering for biomanufacturing of high-value products. Comput Struct Biotechnol J 2017;15:161–7.
[37] Sun ZZ, Hayes CA, Shin J, Caschera F, Murray RM, Noireaux V. Protocols for implementing an Escherichia coli based TX-TL cell-free expression system for synthetic biology. J Vis Exp 2013;79:e50762.
[38] Kwon YC, Jewett MC. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. Sci Rep 2015;5:8663.
[39] Shin J, Noireaux V. An *E. coli* cell-free expression toolbox: application to synthetic gene circuits and artificial cells. ACS Synth Biol 2012;1(1):29–41.
[40] de los Santos ELC, Meyerowitz JT, Mayo SL, Murray RM. Engineering transcriptional regulator effector specificity using computational design and *in vitro* rapid prototyping: developing a vanillin sensor. ACS Synth Biol 2016;5:287–95.
[41] Hong SH, Ntai I, Haimovich AD, Kelleher NL, Isaacs FJ, Jewett MC. Cell-free protein synthesis from a release factor 1 deficient Escherichia coli activates efficient and multiple site-specific nonstandard amino acid incorporation. ACS Synth Biol 2014;3(6):398–409.
[42] Oza JP, Aerni HR, Pirman NL, Barber KW, ter Har CM, et al. Robust production of recombinant phosphoproteins using cell-free protein synthesis. Nat Commun 2015;6:8168.
[43] Shin J, Jardine P, Noireaux V. Genome replication, synthesis, and assembly of the bacteriophage T7 in a single cell-free reaction. ACS Synth Biol 2012;1(9):408–13.
[44] Pardee K, Green AA, Ferrante T, Cameron DE, Daleykeyser A, Yin P, et al. Paper-based synthetic gene networks. Cell 2014;159(4):940–54.
[45] Salehi AS, Shakalli Tang MJ, Smith MT, Hunt JM, Law RA, Wood DW, et al. A cell-free protein synthesis approach to biosensing htrβ-specific endocrine disruptors. Anal Chem 2017;89:3395–401.
[46] Stapleton JA, Swartz JR. Development of an *in vitro* compartmentalization screen for high-throughput directed evolution of [FeFe] hydrogenases. PLoS One 2010;5(12):e15275.
[47] Dodevski I, Markou GC, Sarkar CA. Conceptual and methodological advances in cell-free directed evolution. Curr Opin Struct Biol 2015;33:1–7.
[48] Mastrobattista E, Taly V, Chanudet E, Treacy P, Kelly BT, Griffiths AD. High-throughput screening of enzyme libraries: *in vitro* evolution of a beta-galactosidase by fluorescence-activated sorting of double emulsions. Chem Biol 2005;12(12):1291–300.
[49] Kim TW, Chockhawala HA, Hess M, Dana CM, Baer Z, Sczyrba A, et al. High-throughput *in vitro* glycoside hydrolase (HIGH) screening for enzyme discovery. Angew Chem Int Ed Engl 2011;50(47):11215–8.
[50] Noireaux V, Bar-Ziv R, Libchaber A. Principles of cell-free genetic circuit assembly. Proc Natl Acad Sci U S A 2003;100(22):12672–7.
[51] Noireaux V, Libchaber A. A vesicle bioreactor as a step toward an artificial cell assembly. Proc Natl Acad Sci U S A 2004;101:17669–74.
[52] Calhoun KA, Swartz JR. An economical method for cell-free protein synthesis using glucose and nucleoside monophosphates. Biotechnol Prog 2005;21:1146–53.
[53] Jewett MC, Calhoun KA, Voloshin A, Wuu JJ, Swartz JR. An integrated cell-free metabolic platform for protein production and synthetic biology. Mol Syst Biol 2008;4:220.
[54] Caschera F, Bedau MA, Buchanan A, Cawse J, de Lucrezia D, Gazzola G, et al. Coping with complexity: machine learning optimization of cell-free protein

synthesis. Biotechnol Bioeng 2011;108(9):2218–28.
[55] Jewett MC, Fritz BR, Timmerman LE, Church GM. *In vitro* integration of ribosomal RNA synthesis, ribosome assembly, and translation. Mol Syst Biol 2013;9:678.
[56] Chappell J, Jensen K, Freemont PS. Validation of an entirely *in vitro* approach for rapid prototyping of DNA regulatory elements for synthetic biology. Nucleic Acids Res 2013;41(5):3471–81.
[57] Caschera F, Noireaux V. Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-free transcription-translation system. Biochimie 2014;99:162–8.
[58] Caschera F, Noireaux V. A cost-effective polyphosphate-based metabolism fuels an all *E. coli* cell-free expression system. Metab Eng 2015;27:29–37.
[59] Caschera F, Noireaux V. Preparation of amino acid mixtures for cell-free expression systems. Biotechniques 2015;58(1):40–3.
[60] Niederholtmeyer H, Sun ZZ, Hori Y, Yeung E, Verpoorte A, Murray RM, et al. Rapid cell-free forward engineering of novel genetic ring oscillators. Elife 2015;4:e09771.
[61] Noireaux V, Bar-Ziv R, Godefroy J, Salman H, Libchaber A. Toward an artificial cell based on gene expression in vesicles. Phys Biol 2005;2(3):P1–8.
[62] Luisi PL, Ferri F, Stano P. Approaches to semi-synthetic minimal cells: a review. Naturwissenschaften 2006;93(1):1–13.
[63] Szathmary E. Life: in search of the simplest cell. Nature 2005;433(7025):469–70.
[64] Ishikawa K, Sato K, Shima Y, Urabe I, Yomo T. Expression of a cascading genetic network within liposomes. FEBS Lett 2004;576:387–90.
[65] Kuruma Y, Stano P, Ueda T, Luisi PL. A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. Biochim Biophys Acta 2009;1788(2):567–74.
[66] Jewett MC, Forster AC. Update on designing and building minimal cells. Curr Opin Biotechnol 2010;21(5):697–703.
[67] Karzbrun E, Shin J, Bar-Ziv RH, Noireaux V. Coarse-grained dynamics of protein synthesis in a cell-free system. Phys Rev Lett 2011;106(4):048104.
[68] Nagaraj VH, Greene JM, Sengupta AM, Sontag ED. Translation inhibition and resource balance in the cell-free gene expression system. bioRxiv 2017. http://dx.doi.org/10.1101/142869.
[69] Vilkhovoy M, Horvath N, Shih C-H, Wayman J, Calhoun K, Swartz J, et al. Sequence specific modeling of *E. coli* cell-free protein synthesis. bioRxiv 2017. http://dx.doi.org/10.1101/139774.
[70] Garamella J, Marshall R, Rustad M, Noireaux V. The all *E. coli* TX-TL toolbox 2.0: a platform for cell-free synthetic biology. ACS Synth Biol 2016;5:344–55.
[71] Hoffmann M, Nemetz C, Madin K, Buchberger B. Rapid translation system: a novel cell-free way from gene to protein. Biotechnol Annu Rev 2004;10:1–30.
[72] Shin J, Noireaux V. Efficient cell-free expression with the endogenous *E. coli* RNA polymerase and sigma factor 70. J Biol Eng 2010;4:8.
[73] Schinn SM, Broadbent A, Bradley WT, Bundy BC. Protein synthesis directly from PCR: progress and applications of cell-free protein synthesis with linear DNA. N Biotechnol 2016;33(4):480–7.
[74] Sun ZZ, Yeung E, Hayes CA, Noireaux V, Murray RM. Linear DNA for rapid prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free system. ACS Synth Biol 2014;3(6):387–97.
[75] Marshall R, Maxwell CS, Collins SP, Beisel CL, Noireaux V. Short DNA containing chi sites enhances DNA stability and gene expression in *E. coli* cell-free transcription-translation systems. Biotechnol Bioeng 2017;114(9):2131–41.
[76] Wu PS, Ozawa K, Lim SP, Vasudevan SG, Dixon NE, Otting G. Cell-free transcription/translation from PCR-amplified DNA for high-throughput NMR studies. Angew Chem Int Ed Engl 2007;46(18):3356–8.
[77] Park N, Um SH, Funabashi H, Xu J, Luo D. A cell-free protein-producing gel. Nat Mater 2009;8(5):432–7.
[78] Michel-Reydellet N, Woodrow K, Swartz J. Increasing PCR fragment stability and protein yields in a cell-free system with genetically modified Escherichia coli extracts. J Mol Microbiol Biotechnol 2005;9(1):26–34.
[79] Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2007;2(4):924–32.
[80] Gibson DG, Young L, Chuang RY, Venter JC, Hutchinson III CA, Hamilton OS. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 2009;6:343–5.
[81] Calhoun KA, Swartz JR. Energy systems for ATP regeneration in cell-free protein synthesis reactions. Methods Mol Biol 2007;375:3–17.
[82] Jewett MC, Swartz J. Rapid expression and purification of 100 nmol quantities of active protein using cell-free protein synthesis. Biotechnol Prog 2004;20:102–9.
[83] Calhoun KA, Swartz JR. Energizing cell-free protein synthesis with glucose metabolism. Biotechnol Bioeng 2005;90(5):606–13.
[84] Kim DM, Swartz JR. Prolonging cell-free protein synthesis with a novel ATP regeneration system. Biotechnol Bioeng 1999;66(3):180–8.
[85] Kim TW, Keum JW, Oh IS, Choi CY, Kim HC, Kim DM. An economical and highly productive cell-free protein synthesis system utilizing fructose-1,6-bisphosphate as an energy source. J Biotechnol 2011;130:389–93.
[86] Kim DM, Swartz JR. Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis. Biotechnol Bioeng 2001;74(4):309–16.
[87] Failmezger J, Nitschel R, Sanchez-Kopper A, Kraml M, Siemann-Herzberg M. Site-Specific cleavage of ribosomal RNA in Escherichia coli-Based cell-free protein synthesis systems. PLoS One 2016;11(12):e0168764.
[88] Wang Y, Zhang YH. Cell-free protein synthesis energized by slowly-metabolized maltodextrin. BMC Biotechnol 2009;9:58.
[89] Kim HC, Kim TW, Kim DM. Prolonged production of proteins in a cell-free protein synthesis using polymeric carbohydrates as an energy source. Process Biochem 2011;46:1366–9.
[90] Fujiwara K, Doi N. Biochemical preparation of cell extract for cell-free protein synthesis without physical disruption. PLoS One 2016;11(4):e0154614.
[91] Kim TW, Keum JW, Oh IS, Choi CY, Park CG, Kim DM. Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. J Biotechnol 2006;126(4):554–61.
[92] Krinsky N, Kaduri M, Shainsky-Roitman J, Goldfeder M, Ivanir E, Benhar I, et al. A simple and rapid method for preparing a cell-free bacterial lysate for protein synthesis. PLoS One 2016;11(10):e0165137.
[93] Schwarz D, Junge F, Durst F, Frolich N, Schneider B, Reckel S, et al. Preparative scale expression of membrane proteins in Escherichia coli-based continuous exchange cell-free systems. Nat Protoc 2007;2(11):2945–57.
[94] Kelwick R, Webb AJ, MacDonald JT, Freemont PS. Development of a Bacillus subtilis cell-free transcription-translation system for prototyping regulatory elements. Metab Eng 2016;38:370–81.
[95] Li J, Wang H, Kwon YC, Jewett MC. Establishing a high yielding streptomyces-based cell-free protein synthesis system. Biotechnol Bioeng 2017;114(6):1343–53.
[96] Goering AW, Li J, McClure RA, Thomson RJ, Jewett MC, Kelleher NL. *In vitro* reconstruction of nonribosomal peptide biosynthesis directly from dna using cell-free protein synthesis. ACS Synth Biol 2017;6:39–44.
[97] Li J, Haas W, Jackson K, Kuru E, Jewett MC, Fan ZH, et al. Cogenerating synthetic parts toward a self-replicating system. ACS Synth Biol 2017;6(7):1327–36.
[98] Des Soye BJ, Patel JR, Isaacs FJ, Jewett MC. Repurposing the translation apparatus for synthetic biology. Curr Opin Chem Biol 2015;28:83–90.
[99] Fritz BR, Jewett MC. The impact of transcriptional tuning on *in vitro* integrated rRNA transcription and ribosome construction. Nucleic Acids Res 2014;42(10):6774–85.
[100] Fritz BR, Jamil OK, Jewett MC. Implications of macromolecular crowding and reducing conditions for *in vitro* ribosome construction. Nucleic Acids Res 2015;43(9):4774–84.
[101] Liu Y, Fritz BR, Anderson MJ, Schoborg JA, Jewett MC. Characterizing and alleviating substrate limitations for improved *in vitro* ribosome construction. ACS Synth Biol 2015;4(4):454–62.
[102] Nielsen AA, Der BS, Shin J, Vaidyanathan P, Paralanov V, Strychalski EA, et al. Genetic circuit design automation. Science 2016;352(6281):aac7341.
[103] Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol 2016;34(2):184–91.
[104] Boles KS, Kannan K, Gill J, Felderman M, Gouvis H, Hubby B, et al. Digital-to-biological converter for on-demand production of biologics. Nat Biotechnol 2017;35(7):672–5.
[105] Yachie N, Robotic Biology C, Natsume T. Robotic crowd biology with maholo LabDroids. Nat Biotechnol 2017;35(4):310–2.
[106] King RD, Rowland J, Oliver SG, Young M, Aubrey W, Byrne E, et al. The automation of science. Science 2009;324(5923):85–9.
[107] King RD, Whelan KE, Jones FM, Reiser PG, Bryant CH, Muggleton SH, et al. Functional genomic hypothesis generation and experimentation by a robot scientist. Nature 2004;427(6971):247–52.
[108] Caschera F, Gazzola G, Bedau MA, Bosh Moreno C, Buchanan A, Cawse J, et al. Automated discovery of novel drug formulations using predictive iterated high throughput experimentation. PLoS One 2010;5(1):e8546.
[109] Sachse R, Dondapati SK, Fenz SF, Schmidt T, Kubick S. Membrane protein synthesis in cell-free systems: from bio-mimetic systems to bio-membranes. FEBS Lett 2014;588(17):2774–81.
[110] Ghahramani Z. Probabilistic machine learning and artificial intelligence. Nature 2015;521(7553):452–9.
[111] Carlin DA, Caster RW, Wang X, Betzenderfer SA, Chen CX, Duong VM, et al. Kinetic characterization of 100 glycoside hydrolase mutants enables the discovery of structural features correlated with kinetic constants. PLoS One 2016;11(1):e0147596.
[112] Rasmussen S, Chen L, Nilsson M, Abe S. Bridging nonliving and living matter. Artif Life 2003;9(3):269–316.
[113] Porcar M, Danchin A, de Lorenzo V, Dos Santos VA, Krasnogor N, Rasmussen S, et al. The ten grand challenges of synthetic life. Syst Synth Biol 2011;5(1–2):1–9.
[114] Rasmussen S, Bedau MA, Chen L, Deamer D, Krakaver DC, Packard NH, et al. Protocells: bridging nonliving and living matter. The MIT Press; 2008.
[115] Noireaux V, Maeda YT, Libchaber A. Development of an artificial cell, from self-organization to computation and self-reproduction. Proc Natl Acad Sci U S A 2011;108(9):3473–80.
[116] Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, et al. Creation of a bacterial cell controlled by a chemically synthesized genome. Science 2010;329(5987):52–6.
[117] Maeda YT, Nakadai T, Shin J, Uryu K, Noireaux V, Libchaber A, et al. Assembly of MreB filaments on vesicular membranes: a synthetic biology approach. ACS Synth Biol 2012;1:53–9.
[118] Caschera F, Noireaux V. Compartmentalization of an all-*E. coli* cell-free

expression system for the construction of a minimal cell. Artif Life 2016;22(2):185–95.
[119] Nishimura K, Tsuru S, Suzuki H, Yomo T. Stochasticity in gene expression in a cell-sized compartment. ACS Synth Biol 2015;4(5):566–76.
[120] Chiba M, Miyazaki M, Ishiwata S. Quantitative analysis of the lamellarity of giant liposomes prepared by the inverted emulsion method. Biophys J 2014;107(2):346–54.
[121] Kamiya K, Takeuchi S. Giant liposome formation toward the synthesis of well-defined artificial cells. J Mater Chem B 2017. http://dx.doi.org/10.1039/C7TB01322A.
[122] Elani Y. Construction of membrane-bound artificial cells using microfluidics: a new frontier in bottom-up synthetic biology. Biochem Soc Trans 2016;44:723–30.
[123] Lentini R, Perez Santero S, Chizzolini F, Cecchi D, Fontana J, Marchioretto M, et al. Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. Nat Commun 2014;5:4012.
[124] Lentini R, Martini NY, Forlin M, Belmonte L, Fontana J, Cornella M, et al. Two-way chemical communication between artificial and natural cells. ACS Cent Sci 2017;3:117–23.
[125] Bedau MA, McCaskill JS, Packard NH, Rasmussen S. Living technology: exploiting life's principles in technology. Artif Life 2010;16(1):89–97.
[126] Luisi PL. The emergence of life: from chemical origins to synthetic biology. Cambridge University Press; 2016.
[127] Szostak JW, Bartel DP, Luisi PL. Synthesizing life. Nature 2001;409(6818):387–90.
[128] Marchi-Artzner V, Jullien L, Belloni L, Raison D, Lacombe L, Lehn JM. Interaction, lipid exchange, and effect of vesicle size in systems of oppositely charged vesicles. J Phys Chem 1996;100:13844–56.
[129] Caschera F, Stano P, Luisi PL. Reactivity and fusion between cationic vesicles and fatty acid anionic vesicles. J Colloid Interface Sci 2010;345(2):561–5.
[130] Sunami T, Caschera F, Morita Y, Toyota T, Nishimura K, Matsuura T, et al. Detection of association and fusion of giant vesicles using a fluorescence-activated cell sorter. Langmuir 2010;26:15098–103.
[131] Nishimura K, Matsuura T, Nishimura K, Sunami T, Suzuki H, Yomo T. Cell-free protein synthesis inside giant unilamellar vesicles analyzed by flow cytometry. Langmuir 2012;28(22):8426–32.
[132] Caschera F, Sunami T, Matsuura T, Suzuki H, Hanczyc MM, Yomo T. Programmed vesicle fusion triggers gene expression. Langmuir 2011;27(21):13082–90.
[133] Tsuji G, Fujii S, Sunami T, Yomo T. Sustainable proliferation of liposomes compatible with inner RNA replication. Proc Natl Acad Sci U S A 2016;113(3):590–5.
[134] Adamala KP, Martin-Alarcon DA, Guthrie-Honea KR, Boyden ES. Engineering genetic circuit interactions within and between synthetic minimal cells. Nat Chem 2016;9:431–9.
[135] Kurihara K, Tamura M, Shohda K, Toyota T, Suzuki K, Sugawara T. Self-reproduction of supramolecular giant vesicles combined with the amplification of encapsulated DNA. Nat Chem 2011;3(10):775–81.
[136] Kurihara K, Okura Y, Matsuo M, Toyota T, Suzuki K, Sugawara T. A recursive vesicle-based model protocell with a primitive model cell cycle. Nat Commun 2015;6:8352.
[137] Caschera F, Lee JW, Ho KK, Liu AP, Jewett MC. Cell-free compartmentalized protein synthesis inside double emulsion templated liposomes with *in vitro* synthesized and assembled ribosomes. Chem Commun (Camb) 2016;52(31):5467–9.
[138] Jia H, Heymann M, Bernhard F, Schwille P, Kai L. Cell-free protein synthesis in micro compartments: building a minimal cell from biobricks. N Biotechnol 2017. http://dx.doi.org/10.1016/j.nbt.2017.06.014.
[139] Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, et al. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A 2006;103(2):425–30.
[140] Ruiz-Mirazo K, Pereto J, Moreno A. A universal definition of life: autonomy and open-ended evolution. Orig Life Evol Biosph 2004;34(3):323–46.
[141] Veneziano R, Ratanalert S, Zhang K, Zhang F, Yan H, Chiu W, et al. Designer nanoscale DNA assemblies programmed from the top down. Science 2016;352(6293):1534.
[142] Ducani C, Kaul C, Moche M, Shih WM, Hogberg B. Enzymatic production of 'monoclonal stoichiometric' single-stranded DNA oligonucleotides. Nat Methods 2013;10(7):647–52.
[143] Irving RA, Coia G, Roberts A, Nuttall SD, Hudson PJ. Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. J Immunol Methods 2001;248(1–2):31–45.