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Cell-free transcription–translation: engineering biology from the nanometer to the millimeter scale

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Cell-free transcription–translation (TXTL) has become a highly versatile technology to construct, characterize and interrogate genetically programmed biomolecular systems implemented outside living organisms. By recapitulating gene expression *in vitro*, TXTL offers unparalleled flexibility to take apart, engineer and analyze quantitatively the effects of chemical, physical and genetic contexts on the function of biochemical systems, from simple regulatory elements to millimeter-scale pattern formation. Here, we review the capabilities of the current cell-free platforms for executing DNA programs *in vitro*. We describe the recent advances in programming using cell-free expression, a multidisciplinary playground that has enabled a myriad of novel applications in synthetic biology, biotechnology, and biological physics. Finally, we discuss the challenges and perspectives in the research area of TXTL-based constructive biology.

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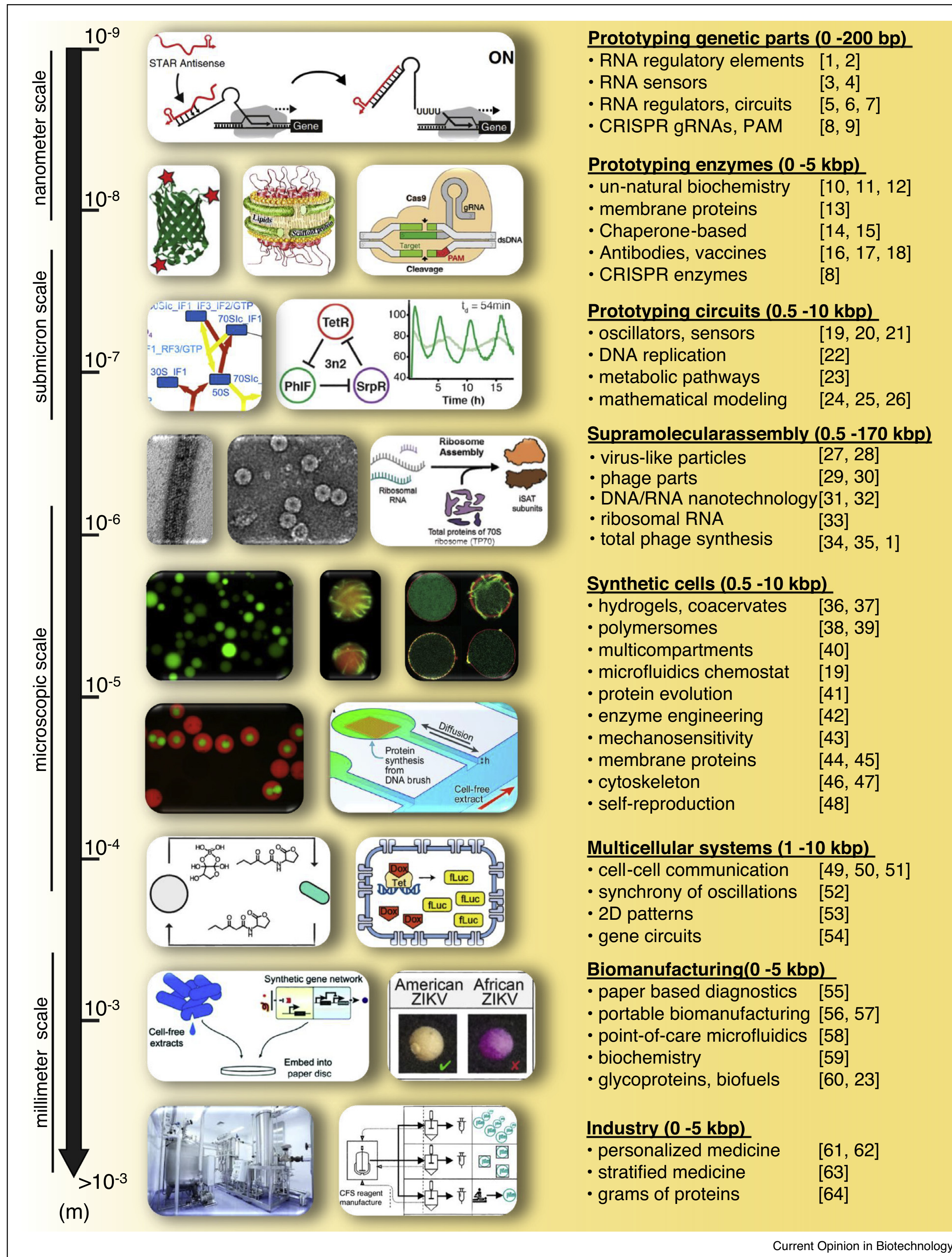
Introduction

Engineering biological systems using cell-free transcription-translation (TXTL) is arising as a major discipline. With an ever-growing scope of application, TXTL is catching the inspiration of a broader and broader research community, both in academia and industry. As a result, TXTL has gained a solid credibility in recent years as an alternative technology to perform biochemistry, biophysics and synthetic biology outside living organisms, to engineer biomolecular systems over a physical size spanning at least seven orders of magnitude, from the nanometer to the millimeter scale (Figure 1).

Prototyping regulatory elements [1•,2,3,4•,5–7,8••,9], enzymes [8••,10–18], and gene circuits [19–22,23•,24–26] has proven powerful at many levels and remains one of the major TXTL research areas. Achieving supramolecular assemblies in TXTL paves the way for the construction of complex molecular machines from scratch [1•,27–32,33••,34,35•], providing evidence as well that TXTL can process large gene sets. Cell-sized compartmentalization of TXTL reactions is utilized for a wide variety of applications [19,36–48], among which bottom-up synthetic cells have emerged as a means to isolate, dissect and engineer biological functions in cell analog settings. Creating arrays of synthetic cells, interactions between synthetic cells or with living cells opens the door to larger engineering scales and to directing cellular behavior of non-genetically modified organisms [49–54]. New methods to rapidly produce minute quantities of biologics at the point-of-care have expanded the portability and utility of TXTL [23•,55,56••,57,58]. Finally, the development of new platforms allows precise biomanufacturing [59,60], which is also geared towards and performed at the industrial level [61–64].

This post-genomic enthusiasm for cell-free gene expression relies on several key advances, by and large, accomplished for systems-based on *Escherichia coli* that remains the major cell-free chassis. The TXTL technical breadth is first due to the synthesis strength of the new generation of cell-free expression platforms. Several highly efficient metabolisms for ATP regeneration have been formulated to energize TXTL [65–67]. With at least 1 mg/ml of proteins produced in batch mode reactions, the current *E. coli* TXTL systems provide enough room to concurrently express and measure the dynamics of several genes. Second, the preparation of TXTL systems has been simplified, facilitating the accessibility of this technology to a larger community. Modern TXTL systems have become affordable, user-friendly and convenient as a safe experimental environment to carry out bioengineering, thereby turning into a material also suitable for educational purposes. Because their preparation has been demystified over the years, cell-free expression systems have become easier to customize for specific uses. The hybrid T7 cell-free platform, that couples the T7 bacteriophage transcription to the translation of an organism such as *E. coli*, has been adapted for new applications [10,60]. The PURE system, a fully purified cell-free TXTL also-based on T7, allows working in a well-defined environment and a simple biochemical background compared to extract-based platforms [26]. Third,

Figure 1



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the versatility of TXTL, at the level of transcription, in particular, has been improved by eliminating the need for bacteriophage RNA polymerases. The development, for instance, of an all *E. coli* cell-free system that uses the endogenous RNA polymerase and housekeeping sigma factor 70 [1•] has widened the transcription repertoire to hundreds of bacterial regulatory elements, thus providing a multipurpose platform to program and emulate complex dynamical behaviors *in vitro* [52,53,68], in a manner getting closer to real living cells. TXTL systems prepared from other bacteria are being developed as alternatives to *E. coli* [2,69,70]. The preparation of TXTL systems from eukaryotic cells extend cell-free expression to post-translationally modified proteins. Finally, the growing interest for *in vitro* protein synthesis owes also a lot to the scalability of TXTL reactions, spanning seventeen orders of magnitudes, from femtoliter synthetic cells to hundreds of liters in the industry for the production of grams of proteins [64]. TXTL reactions have been accommodated to a remarkably broad variety of setups as diverse as liposomes [71], hydrogels [36], microfluidics chips [68], and paper [72].

In this article, we review the current scope of cell-free TXTL applications (Figure 1), focusing on the size and type of DNA programs that have been executed in TXTL, mostly from *E. coli*, and on the physical scale at which TXTL reactions are performed (Table 1). We discuss some of the possible extensions and some of the most important limitations that could be addressed to further expand TXTL capabilities (Table 2).

Prototyping DNA programs in TXTL

The unique turnover speed of cell-free reactions setup and execution has transformed TXTL into an ideal technology for prototyping genetic elements, the major application in the field currently (Figure 1, Table 1). The properties or activity of single coding and non-coding DNA parts (promoters, untranslated regions, riboregulators, terminators, aptamers, genes) can be assessed in TXTL within a day. Accelerating the design-build-test cycle is not the only advantage. The functions and quantitative behaviors of single DNA parts in TXTL are comparable to the one measured *in vivo* to a large extent [7], a feature at the basis of TXTL's credibility for bioengineering. TXTL can accommodate other technologies. CRISPR has been recently installed in TXTL [8••,9], which can be used to rapidly test gRNAs, PAM sequences, CRISPR and anti-CRISPR enzymes. Nanodiscs, small soluble phospholipid rafts compatible with TXTL, have become one of the most convenient methods to express proteins that interact with or need lipids bilayers for proper function [13]. TXTL offers a genetically free background to test DNA programs while mimicking cytoplasmic physiological conditions (pH 7.5–8.0, ionic concentration 150–250 mM, crowding), thus facilitating the characterization of parts in isolation with less or no bias than *in vivo*. For that same reason, TXTL is useful to validate molecular mechanisms and to develop accurate models of gene circuits [6], and to study the biochemistry of TX and TL [25,26]. The knowledge gained from *E. coli*-based cell-free expression systems is now exploited to prepare TXTL platforms from other bacteria such as *Bacillus subtilis* [69]. This new line of research offers alternatives to programming in *E. coli* systems and could help the customization of TXTL for specific uses.

While prototyping DNA programs is a well-established TXTL activity, improvements and extensions could be made. The excellent agreement *in vitro*/*in vivo* observed for the performances of single DNA parts, such as CRISPR [8••], does not necessarily apply to circuits composed of three or more genes. Prototyping gene networks in TXTL, especially for *in vivo* applications, requires a much deeper evaluation and extensive quantitative mapping between the test tube and *E. coli* (Table 1). This will help engineer synthetic protogenomes necessary to program and regulate multiple biological functions for synthetic cells, for instance. TXTL could play a major role as a testbed to create standards of biological parts, a long-standing question in bioengineering (Table 2).

Self-assembly

The size and complexity of DNA programs that TXTL systems can execute are not very well gauged. This is due, to a large extent, to the many different TXTL platforms existing currently on the market and the difficulty to know what each of these systems can really process genetically speaking. It mostly depends on the strength of the metabolism fueling TL and the type of TX used. Yet, the strength of some platforms enables the expression of remarkably large natural DNA programs, a strength large enough to recapitulate complex gene regulation, self-assembly, and metabolism to deliver active living systems in a single test tube reaction. The largest DNA program executed in a one-pot TXTL mixture is the genome encoding for the bacteriophage T4 [35•]. After a few hours of incubation, infectious T4 phages are

(Figure 1 Legend) Overview of the scales at which cell-free expression is performed, emphasizing either the size of the DNA programs executed or the physical size of the setup used for TXTL reactions. Estimation of the size of the DNA processed in each category is shown in the title. In each section, the bulleted items are ranked from the smallest to largest DNA program executed. Reproduced with permission from: Prototyping genetic parts [3], enzymes [10], circuits [19,26], Supramolecular assembly [30,33••,34], Synthetic cells [20,40,46,47], Multicellular systems [49,54], Biomanufacturing [55,69], Industry [63].

Table 1

Examples of DNA programs executed in TXTL

DNA program	Mechanism, process and results	Ref.
Prototyping parts and circuits		
• Plasmids: 1 kbp 2 genes, 2 promoters	Riboregulation of catechol 2,3 - dioxygenase expression **(test tube reactions, *E. coli* TXTL,** *E. coli* **promoters, sfGFP and OD 385 nm)**	[7]
• Plasmids: 1 kbp 2 genes, 2 promoters	Temperature-dependent TX riboregulation of eGFP **(test tube reactions, *E. coli* TXTL,** *E. coli* **promoters, GFP)**	[4•]
• Plasmids: 1 - 2 kbp 4 genes, 4 promoters	Prototyping RNA network **(test tube reactions, *E. coli* TXTL,** *E. coli* **promoters, sfGFP)**	[5]
• Linear dsDNA: 4 kbp 4 genes, 4 promoters	Self-replication of phage phi29 DNA in synthetic cell **(test tubes and tested in liposomes, PURE system, T7 promoter, YFP reporter)**	[22]
• Plasmids: 2 - 6 kbp 5 genes, 5 promoters	Prototyping n-Butanol biosynthetic pathway **(test tubes reactions, *E. coli* TXTL, T7 promoter, quantification of butanol by HPLC)**	[23•]
• Linear/Plasmids: ~ 6 kbp 7 genes, 3 promoters	CRISPR and anti-CRISPR proteins, gRNAs, PAM assay **(test tubes reactions, *E. coli* TXTL,** *E. coli* **promoters, deGFP)**	[8••,9]
• Linear dsDNA: 5 - 10 kbp 5 genes, 5 promoters	5 node ring oscillators **(microfluidic chemostat, *E. coli* TXTL,** *E. coli* **promoters, sfGFP, mCherry, Citrine, Cerulean)**	[19]
Self-assembly		
• Plasmid: 0.5 kbp, 1 gene, 1 promoter	Synthesis and assembly of norovirus capsid proteins **(test tube reactions, *E. coli* TXTL, T7 promoters, TEM)**	[28]
• Plasmid: 0.5 kbp 1 gene, 1 promoter	HBV virus like particle assembly stabilized by disulfide bridges **(test tube reactions, *E. coli* TXTL, T7 promoters, TEM)**	[27]
• Plasmid: 2 kbp 1 gene, 1 promoter	Expression of gp18, formation of nanotubes **(test tube reactions, *E. coli* TXTL, T7 promoter, TEM)**	[29]
• Plasmids: 4 - 5 kbp 2 genes, 2 promoters	Assembly of hybrid RNA/protein nanostructure **(test tube reactions, *E. coli* TXTL, T7 promoters, mTurquoise2 and Ypet)**	[32]
• Plasmids: 5 - 10 kbp 3 genes, 3 promoters	TX of rRNA and active ribosome assembly **(test tube reactions, *E. coli* TXTL, T7 promoters, Firefly Luciferase and sfGFP)**	[33••]
• Linear dsDNA: 40 kbp 60 genes, 20 promoters	Complete synthesis of phage T7, DNA replication **(test tube reactions, *E. coli* TXTL, phage promoters, plaque assay (3 10^{11} PFU/ml), EM)**	[34]
• Linear dsDNA: 169 kbp 289 genes, 119 promoters	Complete synthesis of phage T4 **(test tube reactions, *E. coli* TXTL, phage promoters, plaque assay (10^9 PFU/ml), EM)**	[35•]
Synthetic cell systems		
• Plasmid: 1 kbp 1 gene, 1 promoter	Intercommunication between synthetic cells and ***E. coli*** **(liposomes,** *E. coli* **TXTL, T7 promoters, α-Hemolysin and GFP)**	[51]
• Plasmids: 1 - 2 kbp 1 gene, 1 promoter	Encapsulation of TXTL into hydrogel compartments **(hydrogel particles, PURE system, T7 promoters, fluorescence mCherry and GFP)**	[36]
• Linear DNA: 4 kbp 5 genes, 4 promoters	Synchrony and pattern formation of coupled oscillators on a chip **(microfluidic and glass chip, *E. coli* TXTL,** *E. coli* **promoters, deGFP)**	[52]
• Plasmid: 1 - 5 kbp 1 gene, 1 promoter	Independent TXTL expression in different compartments of vesosomes **(liposomes, *E. coli* TXTL, T7 promoters, RFP)**	[40]
• Plasmids: 5 kbp 3 genes, 3 promoters	Synthesis of cell division proteins FtsZ, FtsA and ZipA **(liposomes, PURE system, T7 promoters, sfGFP)**	[47]
• Plasmids: 7 - 8 kbp 2 genes, 2 promoters	2-steps circuits operating in multiple liposomes **(liposomes, *E. coli* TXTL,** *E. coli* **promoters, Firefly Luciferase)**	[54]
• Plasmids: 8 kbp 6 genes, 6 promoters	6-stage TX cascade using 5 TX factors **(liposomes, *E. coli* TXTL,** *E. coli* **promoters, deGFP)**	[1•]
Biomanufacturing		
• Plasmid: 1-2 kbp 1 gene, 1 promoter	Protein expression after rehydration of a paper-based lyophilized TXTL **(Paper, *E. coli* TXTL,** *E. coli* **and T7 promoter, GFP and mCherry)**	[72]
• Plasmid: 1-2 kbp 1 gene, 1 promoter	Glycosylation of a target protein **(test tube reactions, *E. coli* TXTL, T7 promoter, western blot analysis)**	[60]

The **left column** includes information about the type DNA (linear dsDNA or plasmid), the total length of the coding sequences composing the DNA program (promoters, operators, UTRs, genes, terminators), the number of genes and promoters. Ranked by size of the DNA program in each of the three sections. The **column in the middle** provides a short description of the work, including reaction setup, type of TXTL, type of promoters, and reporter used.

fully synthesized in a standard *E. coli* cell-free reaction supplemented with the genomic DNA. While the number of genes, out of 289 total, expressed from the 169-kbp genome is not known, at least 62 structural proteins are produced to assemble active phages. The synthesis of infectious T7 phages, although smaller, demonstrated that genome replication occurs simultaneously with gene expression, self-assembly, and packaging [34]. DNA

Table 2

Examples of limitations and possible extensions.

Current limitations and challenges	Potential improvements and extensions
TXTL	
• **Basic characteristics and working principles of TXTL systems are often not described or reported** • Biochemistry, metabolism and resource limitations of most of the TXTL platforms are not well understood yet • There are no generic TXTL platform, the TXTL landscape is broad and dispersed There are no standard TXTL preparation methods, characterization and practices • Lab to lab reproducibility is not established	• **Develop TXTL quantitative skills (e.g. data in arbitrary units are adjustable and opaque)** • Report the basic properties and characteristics (strength, limitations) of TXTL system used • Develop models that truly capture limitations (e.g from the TX and TL molecular components) • Develop a couple of universal TXTL systems (e.g. one from a prokaryote organism and one from an eukaryote organism)
Prototyping parts, enzymes, circuits	
• **TXTL-specific and calibrated gene regulation that preserve resources has not been developed** • Time-course of batch mode TXTL reactions (6–8 h) is limited for prototyping large circuits (>3–5 genes) • Agreement cell-free versus *in vivo* is not yet established for circuits larger then 3–5 genes • Long-lived TXTL reaction systems have been demonstrated but not tooled for high-throughput work • Complex parallel and/or sequential regulation of gene sets has not been demonstrated • Dynamics of gene circuits is still limited by a rather poor control of the balance between synthesis and degradation (for both mRNA and protein)	• **Develop sets of well-defined regulatory elements for efficient gene circuit dynamics preserving the energy and resources of the system** • Establish robust semi-continuous TXTL reaction system for high-throughput applications (e.g. suitable for large DNA programs on plate readers) • Engineer circuits in one plasmid backbone to integrate and emulate digital and physical DNA compaction of multiple biological functions • Use TXTL to develop standards of biological parts • Truly control the synthesis strength and the degradation rates to adjust the dynamics of circuits for biologically relevant regimes
Self-Assembly	
• **There are no high-throughput methods to rapidly produce and characterize synthetic nanostructures in TXTL from genetic parts (e.g. phage parts)** • Capabilities for DNA programmed self-assembly of large structures are TXTL-dependent and not estimated yet for most of the platforms • Rapid TXTL engineering and biomanufacturing of synthetic phages has not been demonstrated yet	• **Exploit the complete synthesis of phages for understanding the algorithmic nature of DNA information and self-assembly** • Exploit the complete synthesis of phages for biophysics studies and nanotechnologies • Exploit the complete synthesis of phages for medical applications (e.g. phage therapy and/or antimicrobial against drug-resistant bacteria)
Synthetic cells	
• **Encapsulation of TXTL reactions and DNA programs in cell-sized compartments is not fully controlled and hardly ever quantified** • Preparation of TXTL-based liposomes is TXTL-dependent and method-dependent • Cell-free expression in synthetic cells is poorly reported and rarely quantitative (e.g. arbitrary units) • Importance of the composition, chemical and biophysical properties of the lipid membrane is under appreciated and not addressed • TXTL-based synthetic cells are made in ideal laboratory conditions far from natural environments	• **Develop quantitative measurements for TXTL-based synthetic cell systems independently of the cell-free system used** • Report quantitatively the efficiency of the encapsulation method employed for sufficiently large populations of synthetic cells • Develop simple energy harvesting system to fuel TXTL (e.g. membrane-based light harvesting for ATP regeneration) • Develop mechanically robust TXTL-based synthetic cells (e.g. polymersomes) with tight and selective control of membrane permeability
Biomanufacturing	
• **Soft substrates and materials not fully exploited to host TXTL reactions** • TXTL capabilities for biomanufacturing not fully evaluated and understood yet	• **Test TXTL resilience across the whole soft matter toolkit** • Challenge TXTL systems with gene sets encoding for large metabolic pathways from various bacteria

The **left column** lists some of the current limitations and challenges in cell-free expression, also including some missing capabilities. The **right column** lists some of the possible improvements and extensions to TXTL that would bolster and expand this technology. In each column and section, one bullet is highlighted as one of the most critical features.

replication from another phage, phi29, was recently repeated in the PURE system [22]. Another notable step towards complex TXTL-based supramolecular assembly was the reconstitution of active ribosomes from transcribed ribosomal RNA and purified proteins [33••]. An alternative route towards making hybrid self-assembled molecular machines consists of expressing in TXTL some proteins that interact and assemble with DNA/RNA to form new hybrid nano-structures [32], a rather recent research direction.

The total synthesis of functional phages in test tube reactions is surprising and promising at many levels. First, the assembly of such a complex system without the whole structure of a living cell is unexpected as it reveals the existence of self-organization modes simpler than anticipated. Genetic elements of viruses can be used to put together parts of phages [29,30], to determine basic mechanisms underlying the complex assembly of whole viruses and make steps towards programming the construction of synthetic nanomachines. Second, the ability

to make phage structures outside living organisms also opens the door to TXTL-based nanomedicine. The cell-free expression and assembly of virus-like particles could serve as a vaccine or scaffold for vaccine and drug delivery [27,28]. Producing phages for therapies and as antimicrobials is another bioengineering territory where TXTL has a promising role. Strikingly, a poorly investigated research area is to exploit the cell-free synthesis of phages T7 and T4 to grasp the algorithmic nature of and the relationship between genetic information and self-assembly. This type of basic study could provide substantial information on how genetic information is digitally condensed in natural DNA programs.

TXTL-based synthetic cells

The bottom-up construction of synthetic cells using TXTL has become highly popular in the last few years. The demonstration in the early 2000s that TXTL reactions can be encapsulated and efficiently executed in liposomes was a groundbreaking step towards the so-called minimal cell system [71,73], which is one of the major synthetic cell research directions. The minimal cell approach consists of using a TXTL system and natural molecules, such as lipids, to build up a cell from scratch. TXTL stands out from the other experimental approaches because it enables the construction of cell analogs that are genetically programmable, taking advantage of other technologies like DNA assembly for instance. TXTL-based minimal cells allow isolating biological functions in a setting particularly attractive for synthetic biology applications, even if compelling realizations have yet to be delivered. The minimal cell approach is also an ideal system to reveal the fundamental links between information, self-assembly, and metabolism, especially those that require membrane proteins and functions [48].

Expressing large DNA programs to implement complex biological functions in minimal cell systems is more difficult, however, compared to test tube reactions (Table 2), and has been limited to a few genes until now, for several reasons. Characterizing cell-free expression in liposomes is harder experimentally and mostly low-throughput. Because different TXTL systems and liposome preparation methods are used across laboratories, the visibility of what is really achieved in this field is reduced. No standard methods to prepare liposomes and TXTL mixtures exist to harmonize minimal cell research because none of these experimental procedures stands out as nearly ideal nor have found consensus across the research community. The encapsulation of TXTL reactions, of the DNA program especially, in liposomes and cell-free gene expression are not well understood and hardly ever quantified. The results and claims are often difficult to interpret as they depend largely on experimental conditions and practices unique to each laboratory. As a consequence, there is an important gap between the true potential of TXTL-based synthetic cells and the current state-of-the-art, particularly scattered and unclear. It is certain, the best synthetic cells years are still to come, many critical milestones will be overcome to assemble genetically programmed synthetic cells applicable in natural environments (Table 2), far from ideal laboratory conditions. A wealth of molecular mechanisms will be decrypted en route toward minimal cells, another strong motivation to support building synthetic cells. The minimal cell approach has stimulated the development of alternative means to carry out TXTL in cell-sized volumes. Creating populations of interacting minimal cells could facilitate engineering gene circuits on larger scales [54]. The fabrication of vesosomes expands the types of TXTL synthetic cell architectures that can be assembled [40]. Integration of TXTL into solid microfluidic chips extends reaction times and allows precise control of the geometry of gene expression, an approach that revealed how a wealth of dynamical behaviors can arise even with small circuit motifs [19,52,68]. Performing TXTL in hydrogel particles could become a major route to biomanufacturing [74]. A broad variety of smart hybrid materials combining TXTL and soft matter will certainly emerge in the near future.

Biomanufacturing

The recent demonstration that TXTL works efficiently on paper substrates has considerably improved the portability of cell-free expression [72]. By lyophilizing several microliters of reactions on cellulose filter paper, stable for months at room temperature, one can perform diagnostics or produce therapeutics on-demand in just a few hours, without requiring a cold chain, and at the point of care. Shelf-stable cell-free expression opens new perspectives for biosensing and biocatalysis and is expected to have an increasing impact across many other applications [62], including education. The lyophilized approach to TXTL, complementary to the liter-scale cell-free reactions achieved in industry, facilitates cell-free expression and allows economical and calibrated biomanufacturing.

Concluding remarks

Cell-free expression has become a reliable technology to accelerate prototyping gene circuits for *in vivo* applications and to program synthetic systems like minimal cells. TXTL is also becoming a serious alternative for the biomanufacturing of biologics, at small and large scales. Cell-free expression capabilities and settings are being expanded at a fast pace, catching the interest of a growing multidisciplinary research community. Yet, improvements could be made to strengthen the credibility of this technology. The biochemical properties of most of the cell-free expression platforms are not well understood yet. Many TXTL systems are commercially available or can be prepared in laboratories creating a spectrum of platforms with broad and poorly defined characteristics.

While establishing standards for the preparation of cell-free expression system and practices seems hard to implement, some rather simple habits could be developed across the cell-free expression community to increase the visibility of TXTL studies. For instance, reporting quantitatively the basic properties of the cell-free expression system used would help to position each study in a more authentic context, facilitating the comprehension of results and scopes. Surprisingly, while TXTL systems are ideal to provide quantitative data, most of the work done in this research area is published in arbitrary units, a particularly adjustable and opaque approach to bioengineering.

Conflict of interest statement

Nothing declared.

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