Annual Review of Biomedical Engineering

The New Age of Cell-Free Biology

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Annu. Rev. Biomed. Eng. 2020. 22:51–77

The *Annual Review of Biomedical Engineering* is online at bioeng.annualreviews.org

https://doi.org/10.1146/annurev-bioeng-092019-111110

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Keywords

bottom-up synthetic biology, synthetic cell, cell-free transcription–translation, self-assembly

Abstract

The cell-free molecular synthesis of biochemical systems is a rapidly growing field of research. Advances in the Human Genome Project, DNA synthesis, and other technologies have allowed the in vitro construction of biochemical systems, termed cell-free biology, to emerge as an exciting domain of bioengineering. Cell-free biology ranges from the molecular to the cell-population scales, using an ever-expanding variety of experimental platforms and toolboxes. In this review, we discuss the ongoing efforts undertaken in the three major classes of cell-free biology methodologies, namely protein-based, nucleic acids-based, and cell-free transcription–translation systems, and provide our perspectives on the current challenges as well as the major goals in each of the subfields.

Review in Advance first posted on March 9, 2020. (Changes may still occur before final publication.)

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Contents

1. INTRODUCTION 52
2. PROTEIN-BASED SYSTEMS 54
   2.1. The Cytoskeleton 54
   2.2. The Min System 57
   2.3. The Circadian Clock and the Mitotic Oscillator 57
   2.4. Membrane Sculpting Proteins 58
3. NUCLEIC ACID SYSTEMS 58
   3.1. DNA Strand Displacement and Binding 59
   3.2. DNA Origami and Nanostructures 60
   3.3. Translation-Free Circuits 61
4. CELL-FREE TRANSCRIPTION–TRANSLATION SYSTEMS 62
   4.1. Harnessing Gene Expression In Vitro 62
   4.2. Batch Mode Transcription–Translation in Cell-Free Biology 65
   4.3. Transcription–Translation-Based Synthetic Cell Systems 66
5. CONCLUSIONS AND PERSPECTIVES 68

1. INTRODUCTION

Constructing biochemical systems outside living organisms is becoming a major multidisciplinary research area in the postgenomic era. At the technical level, assembling biochemical systems in experimental environments characterized by a reduced background enables the construction of biological systems in isolation, offering in vitro models with more quantitative control than in vivo ones. The physical and biochemical scalability of these systems allows for unparalleled control over reaction conditions. Building biological systems that are unlikely to reproduce or escape provides a high degree of safety. Finally, the enthusiasm for cell-free biology relies on a biomolecular toolbox that has become highly accessible and is rapidly expanding (1, 2). Besides the technical aspects, recapitulating biological systems in vitro by molecular assembly, either using proteins or by executing DNA programs, holds great promise as a means to explore and unravel the fundamental rules of living systems. Cell-free biology constitutes a novel approach to understanding biological systems because it aims to explore how to synthesize new biological systems unconstrained from their natural biological limits by harnessing and controlling biological matter. Cell-free environments are also adequate for interfacing synthetic components with natural ones. Modular approaches have been developed to build sophisticated biomolecular processes in test tubes, on microfluidic chips, and in liposomes that provide ample opportunities for fundamental research beyond pure engineering perspectives.

The goal of cell-free biology is to bridge the parts to construct the whole. Biology ranges across spatial scales that span many orders of magnitude. Research in cell-free biology is being done at the molecular, cellular, and population levels, with each level increasing in its degree of complexity (**Figure 1*a***). Discovering biological systems through construction in cell-free settings is generally performed at three levels of composition of informational molecules: proteins, nucleic acids, and transcription–translation (TXTL) systems (**Figure 1*b***). Each of these systems seeks to leverage a different facet of the central dogma of molecular biology outside of living organisms. Interestingly, the motivations and backgrounds necessary for working in these three research areas are remarkably different and complementary. The protein-only method is largely

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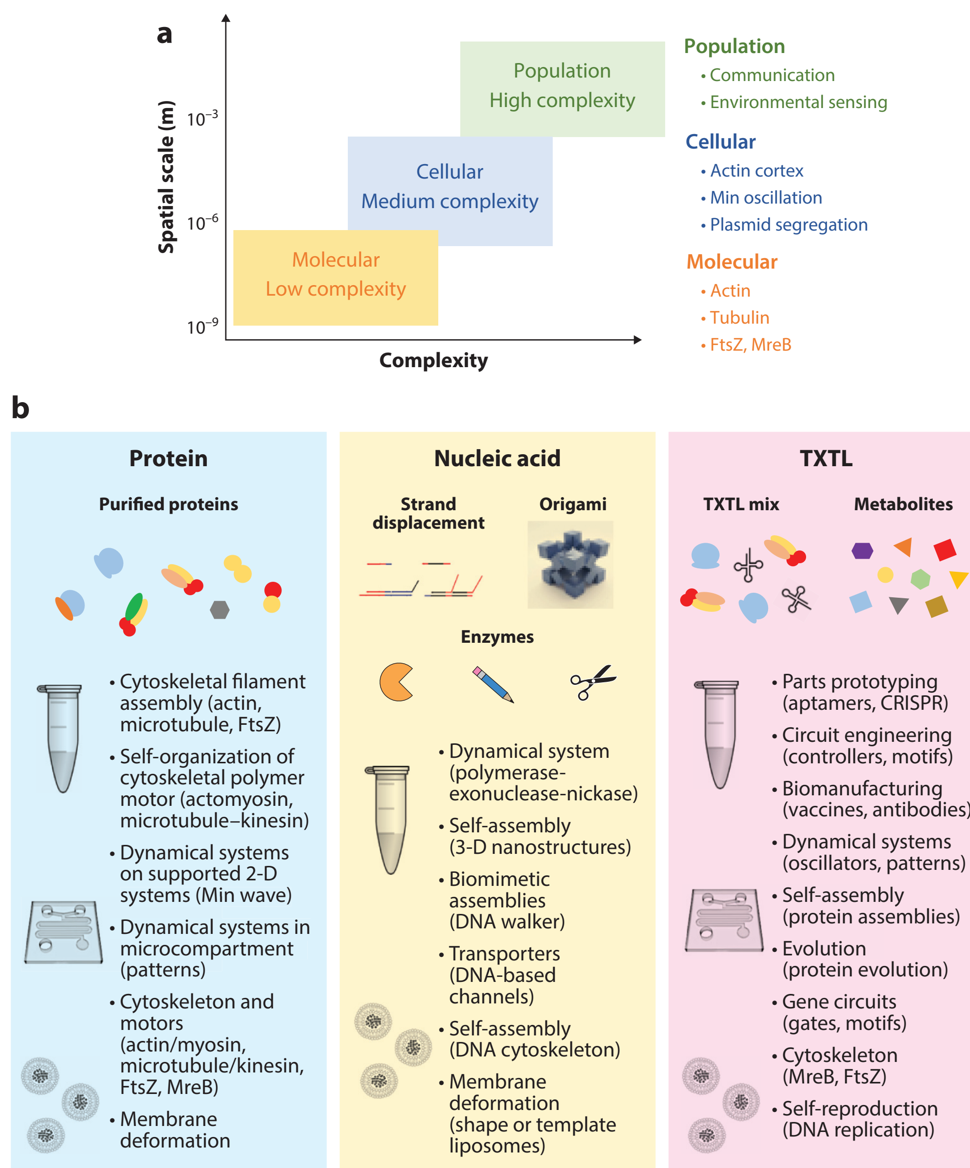

Figure 1

(*a*) Efforts in cell-free biology range from the molecular to the cellular to the population level, with complexity increasing at each level. (*b*) Cell-free biology is founded on three primary systems: protein-based, nucleic acid-based, and transcription–translation (TXTL)-based. Each of these systems is compatible with different spatial scales.

inspired by and based on pregenomic research in cytoskeleton biophysics, enzymology, and single molecules. Nucleic acid cell-free research has been stimulated by computer scientists to a large extent (3, 4), and coupling translation-free circuits to DNA nanotechnology opens the door to a whole class of synthetic systems (5, 6). The TXTL branch of cell-free biology has been mostly developed by engineers and synthetic biologists (7, 8). In recent years, DNA-dependent in vitro

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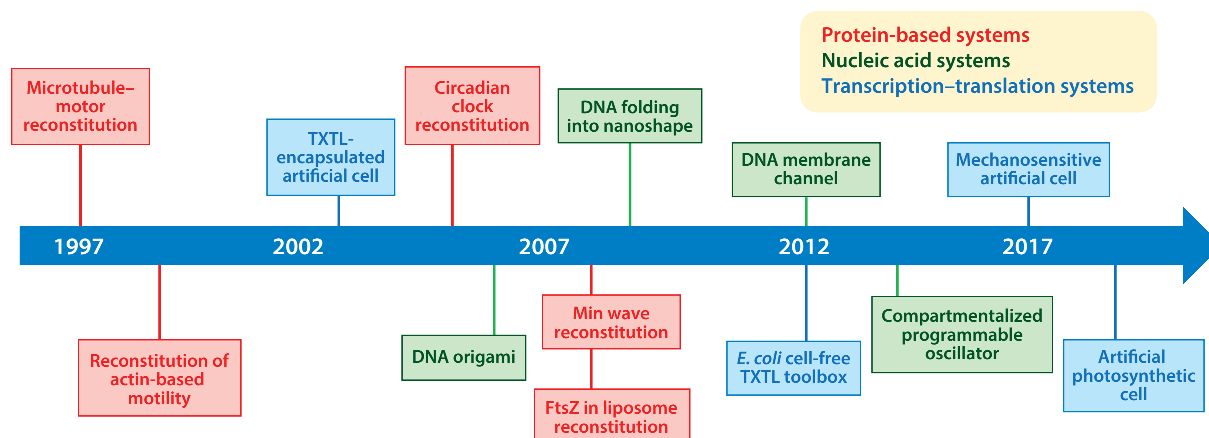

Figure 2

Timeline of salient work during the past 20 years on cell-free biology, with examples from protein-based, nucleic acids-based, and transcription–translation (TXTL)-based systems.

protein production has been reshaped into a versatile and popular technology, accommodating an ever-growing variety of applications (9).

During the past 20 years, each of the three cell-free biology approaches has demonstrated a high potential for making fundamental advances in our comprehension of biological mechanisms (**Figure 2**). The major barrier to moving forward remains a problem of integrating biomolecular mechanisms into larger and more elaborate systems so as to truly understand how complexity arises when molecules are put together, to identify the cooperative links between molecular machineries, and to grasp how robustness in biochemical systems arises. Assembling synthetic cells addresses many of these questions and motivations because it elucidates the information–metabolism–compartment global picture of unicellular life (10–12). Furthermore, working directly at the length scale of a cell is relevant at the biophysical level, especially when considering diffusion or self-organization processes (2, 13). It is not surprising that major research initiatives are being undertaken to enable the bottom-up synthesis of cell analogs. In this article, we review the key advances in cell-free biology. We organize the review by looking at the three major cell-free systems, and we include examples of work at different physical scales, ranging from molecular to network to cellular and multicellular, with the focus primarily on the synthetic cell-level systems.

2. PROTEIN-BASED SYSTEMS

Early reconstitution approaches have mainly been focused on proteins, as they can be synthesized recombinantly or isolated from various tissue sources. Biochemical reconstitution seeks to identify the minimum set of components both necessary and sufficient to recreate a biological process or phenomenon (1). This approach has produced tremendous insights into basic mechanisms (14). More importantly, self-assembly and self-organization of tiny molecular parts that give rise to large-scale cellular structures and coordinated movement provide a rich avenue for discovering how behaviors emerge (15).

2.1. The Cytoskeleton

The cytoskeleton is perhaps the most widely studied system in the bottom-up assembly of synthetic cells. The shape of a living cell is governed by the interaction between the membrane and

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internal cytoskeleton, networks that consist of actin, microtubules, and intermediate filaments. The dynamic arrangement of the actin cytoskeleton is the cornerstone of a wide range of cellular behaviors, including embryogenesis, cell migration, and wound healing. Dynamic microtubules in the form of a bipolar array of mitotic spindles underlie the proper distribution of genetic materials between two daughter cells. Motor protein kinesins and dyneins, as well as a host of microtubule-associated proteins, regulate microtubule dynamics and functions. The cellular reconstitution of cytoskeleton networks from component parts may reveal how mechanical forces organize life from molecules to cells and tissues (16). For the purpose of this section, we define protein-based systems as both purified proteins and cell-free lysates (i.e., without the flow of genetic information).

2.1.1. The actin cytoskeleton. Earlier work on liposome encapsulation of actin monomers under conditions that promoted their subsequent polymerization showed liposomes deforming into dumbbell or disc shapes (17). A major advance in actin biochemistry was achieved when Loisel et al. (18) demonstrated reconstitution of the actin-based motility of *Listeria* using purified proteins that assembled into a dynamic dendritic actin network. Reconstituted dendritic actin networks on the surface of giant unilamellar vesicles (GUVs) revealed a dynamic interplay between actin networks and membrane organization (19), as well as the unexpected discovery that filopodium-like protrusions can spontaneously emerge from a dendritic actin network (20). It was shown recently that a branched actin network on a GUV is sufficient to initiate both inward and outward membrane deformation, depending on membrane tension (21).

In addition to reconstituting a minimal actin cortex on supported lipid bilayers (22–24), major efforts in recent years have focused on encapsulating actin networks in vesicles or emulsion droplets with distinct compositions and organization (25–28), and there has been particular interest in reconstituting cortical actin networks (29). If there is a strongly anchored cortex, myosin motors contract the cortex toward the membrane, whereas a weakly anchored cortex detaches from the membrane with myosin contraction (30). Symmetry breaking of reconstituted cortical actin networks in *Xenopus* extract in droplets depends on actin network connectivity (31, 32). More recently, individual symmetric actomyosin cluster vibrations, due to nonisotropic ATP consumption, have been proposed to explain the emergence of the large-scale, directional motion of actomyosin clusters (33). Weiss et al. (34) used a high-throughput microfluidic method combined with electroinjection to introduce biomolecules into GUVs, devising a strategy to reconstitute cytoskeleton proteins, including actin filaments, in vesicles with enhanced stability. In the future, it will be interesting to develop methods for spatially controlling actomyosin networks to see how spatial variations can be coupled to morphological changes in a synthetic cell context.

2.1.2. Microtubules. It was reported more than 20 years ago that a homogeneous solution of microtubules and kinesins confined in a microfabricated glass chamber self-organized into dynamic asters that turned into vortices (35). It was shown that the final patterns of self-organization depended on the initial kinesin concentration and the size of the chamber, and they depended less on the shape of the chamber. By adsorbing microtubule filaments at a high concentration within a lipid vesicle, a nematic (i.e., with aligned filaments) film can be observed. The inclusion of molecular motors led to an active nematic system with topological defects and different dynamic states (36), highlighting the spatial complexity that can arise from microtubule and motor interaction on a membrane surface.

Kinesins can organize microtubules and also govern their dynamics. The dynamic instability of reconstituted microtubules is comparatively slower (both in terms of polymerization and transit rate) than the physiological dynamics. By adding microtubule-stabilizing protein XMAP215 and the microtubule-destabilizing kinesin XKCM1, Kinoshita et al. (37) reconstituted physiological

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microtubule dynamics that exhibited fast polymerization and high catastrophe rates in microtubule asters. A bipolar spindle has both polar (i.e., aster-like) and nematic microtubule networks, which are exclusively associated with, respectively, minus- and plus-end-directed microtubule cross-linking motors. Interestingly, by tuning the growth dynamics of microtubules, either minus- or plus-end-directed motors can produce both types of networks, suggesting the importance of the dynamic properties of microtubules (38). It was further shown that the microtubule–motor network organization is determined by two control parameters: the ratio of the number of motors to the number of microtubules and the ratio of microtubule growth speed to motor speed. The coexistence of a central nematic network with two polar networks in a bipolar spindle structure likely requires balanced motor activities and localized microtubule nucleation. Other recent in vitro reconstitution efforts have examined how microtubules could generate forces on chromatids while connected to the kinetochore. Multivalency of the NDC80 (nuclear division cycle 80) complex and its interaction with the Ska complex have been shown to be important for efficient end-on coupling of microtubules to the kinetochore (39, 40).

As with the reconstitution of actin networks, *Xenopus* egg extract is a powerful experimental cell-free platform for studying microtubule assembly and organization. By encapsulating DNA and *Xenopus* extract in emulsion droplets of different sizes, Good et al. (41) and Hazel et al. (42) analyzed the scaling of spindle size with droplet size and found that spindle size is set by cytoplasmic volume. Without DNA, microtubule bundle elongation induces large-scale, directed rotational flow in *Xenopus* extract depleted of dynein function in cell-sized droplets (43), reminiscent of the structures organized from purified microtubules discussed earlier.

Despite the fact that the reconstitution of both microtubule and actin networks leads to large-scale flow patterns and this underscores the fascinating nature of active matter, one should keep in mind that different cytoskeletal polymers interact in living cells to orchestrate complex cellular morphological changes. Although not reviewed here, both septins and intermediate filaments are well conserved in eukaryotic cells and have been reconstituted (15). The interaction between different reconstituted cytoskeletal networks, for instance, between actin and a microtubule cytoskeleton (15, 44), will be an important direction for future research.

2.1.3. Par and MreB. Prokaryotes possess a cytoskeleton just like eukaryotes do. Bacterial DNA segregating systems use three components from the *par* operon to accomplish the segregation of the R1 drug-resistance plasmid. The centromeric sequence of *parC* together with adapter protein ParR constitute the ParR–*parC* complex that interacts with ParM, a distant homolog of actin, which polymerizes into helical filaments. The reconstitution of ParM polymerization has revealed that ParM exhibits dynamic instability, similar to the behavior observed in eukaryotic microtubules, in which filament ends bound by ParR–*parC* are stabilized, and free filament ends undergo catastrophe (45). Another active partitioning system uses the Walker-type ATPase ParA, which is responsible for segregating chromosomes and plasmids in most bacterial cells. Using a DNA-carpeted flow cell as an artificial nucleoid, insights into ParA-mediated partition of the plasmid were gained by reconstituting ParB-stimulated ATP hydrolysis of ParA, which detaches ParA from the DNA carpet (46). To our knowledge, the encapsulation of Par systems has not been reported. MreB, another actin homolog, is essential for maintaining the rod shape of many bacteria, and MreB filaments move around the rod circumference instead of in a longitudinal direction. It was recently demonstrated that MreB encapsulated in small liposomes (<1 μm) can tubulate liposomes and align in the circumferential direction (47).

2.1.4. FtsZ. FtsZ is a tubulin homolog and is the main component of the bacterial Z ring that guides cell division. Using an engineered FtsZ membrane-targeting sequence that bypasses the

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actin-related protein FtsA for membrane binding, Osawa et al. (48) demonstrated that in tubular liposomes FtsZ could form a Z ring and generate constriction if it was directly tethered to the membrane. When reconstituted in giant vesicles of bacterial inner membrane lipids, FtsA was found to localize to the membrane surface, but it unexpectedly disassociated in the presence of an FtsZ polymer (49), presumably due to the low density of the filaments. Subsequently, self-organization of FtsA and FtsZ polymers on supported membranes into fast-moving filament bundles and chirally rotating rings was demonstrated (50). This phenomenon could be explained by the bundling and treadmilling of curved, dynamic FtsZ filaments. Further study revealed that the FtsZ membrane-targeting sequence could also self-organize into dynamic rotating rings without the membrane adaptor FtsA when the protein surface density was high (51). The process also depends on the concentrations of nucleotide and free magnesium ions. Interestingly, the chirality of the rotating FtsZ ring can be switched from clockwise to counterclockwise, depending on whether the membrane-targeting sequence is placed at the C or N terminus. Altogether, these studies point to a minimal filament system that when reconstituted at high density at the membrane, can harness self-organization to develop chiral vortices for membrane deformation.

2.2. The Min System

In order for *Escherichia coli* to accurately form a division ring, an oscillatory pattern in the Min system (consisting of MinC, MinD, and MinE) is required from pole to pole to position the bacterial Z ring to guide cell division (52). Briefly, MinD, another Walker-type ATPase, regulates the position of the FtsZ ring by controlling the localization of MinC, an FtsZ inhibitor. MinE is the master regulator of MinD–membrane interaction and functions by stimulating the hydrolysis of ATP-bound MinD dimers. MinC follows the movement of MinD and is not required for the oscillation. The reconstitution of the dynamics of MinD and MinE to generate oscillatory patterns, similar to their in vivo behavior, was vividly captured in 2008 by the reconstitution of MinD–MinE planar surface waves on a supported lipid bilayer (53), and other dynamic patterns have also been observed (54).

Moving away from planar-supported lipid bilayers to more controlled boundary conditions, microfabricated wells of different geometries and microfluidic chips have been used to further interrogate the pattern formation and reaction–diffusion mechanisms of pole-to-pole oscillation of MinD–MinE (55, 56). Recently, the reconstitution of MinD–MinE oscillation in GUVs was achieved (57). By encapsulating MinD and MinE into GUVs, Litschel et al. (57) observed periodic relocation of Min proteins between the lumen and the membrane, with several distinct spatiotemporal patterns (i.e., pulsing, pole-to-pole oscillation, circling, and trigger waves). Interestingly, when osmotically deflated, the GUVs became deformed in concert with the Min oscillations. It will be truly amazing when the holy grail of reconstituting Min oscillations coupling to FtsZ assembly and constriction becomes a reality.

2.3. The Circadian Clock and the Mitotic Oscillator

Oscillation between different biochemical states is a universal phenomenon frequently found in natural biological systems. As a model system, the circadian oscillation of the gene expression of cyanobacteria is controlled by three essential circadian clock proteins (i.e., KaiA, KaiB, and KaiC) within a gene cluster. Self-sustaining and ATP-dependent oscillation of KaiC phosphorylation and dephosphorylation has been reconstituted in vitro (58). The spontaneous oscillation cycle of KaiC phosphorylation, reconstituted using purified KaiABC proteins, is affected by light–dark cycles of

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different day lengths by a midday tracking mechanism, which is simulated by an externally imposed metabolic rhythm accomplished by altering the ratio of ATP to ADP (59).

Another notable example of biological oscillation is the progression of the cell cycle. Using a water-in-oil emulsion encapsulating *Xenopus* cytoplasmic extract as artificial cells, Guan et al. (60) recently succeeded in reconstituting mitotic oscillation for more than 30 cycles over 4 days. The cell cycle oscillator consists of coupled positive and negative feedback loops that govern the central cell cycle regulator, the cyclin B1–Cdk1 complex. Once the cyclin B1–Cdk1 complex is activated, the circuit drives chromosome condensation, nuclear envelope breakdown, and the degradation of securin (a substrate of the anaphase-promoting complex). The frequency and number of cycles are triggered and tunable by the addition of cyclin B1 mRNA, and the authors demonstrated the spontaneous oscillation of nucleus or DNA areas and securin levels (60). The behavior of a single droplet oscillator is size dependent such that smaller droplets had slower oscillations with a larger variance in the periods. This study further highlights the fact that bulk cell-free (protein-based) systems lack the essential single-cell information necessary to reveal heterogeneity in out-of-steady-state dynamics.

2.4. Membrane Sculpting Proteins

The generation of membrane curvature is an active means to create vesicular structures that underlies vesicle budding and membrane trafficking (61), and many membrane-interacting proteins, particularly those with roles in endocytic trafficking, are capable of sculpting membranes. Using a supported bilayer system with an extra membrane reservoir, it was shown that dynamin alone can mediate membrane fission in the constant presence of GTP (62). The budding of GUVs by clathrin and the adaptor protein epsin has been reconstituted, with the degree of membrane deformation found to depend on membrane tension (63). The endosomal sorting complex required for transport (ESCRT)-III is required for membrane remodeling in numerous cellular processes, including multivesicular body biogenesis, abscission, and viral budding. ESCRT-III-dependent budding and scission of intraluminal vesicles in GUVs has also been reconstituted (64). More recently, the main ESCRT-III component, Snf7, was shown to polymerize into spirals and induce the deformation of GUVs (65), and that encapsulation of the core ESCRT-III proteins together with Vps4 led to scission of the membrane tube in an ATP-dependent manner (66). Although not related to membrane sculpting, there is significant interest in reconstituting the enzymatic pathways for lipid synthesis in the context of synthetic cells. The pathway for phospholipid synthesis, with fatty acids as precursors, is well established and has been reconstituted in vitro with purified proteins (67), and a minimal pathway for phospholipid synthesis has also been recently devised and demonstrated (68).

3. NUCLEIC ACID SYSTEMS

In comparison to protein-based systems in cell-free biology, in which the de novo generation of completely functional proteins remains a holy grail, the construction of diverse sets of DNA nanostructures based on programmed hybridization of complementary strands is relatively well developed. This is due in large part to the specificity and predictability of Watson–Crick base-pairing, making DNA a versatile material for nanoscale engineering. Two mechanisms, DNA strand displacement and the folding of scaffold DNA by staple strands, have been used to create a range of DNA-based nanodevices and nanostructures. Several developments from structural and dynamic DNA nanotechnology have had an impact on cell-free biology in the context of synthetic cells,

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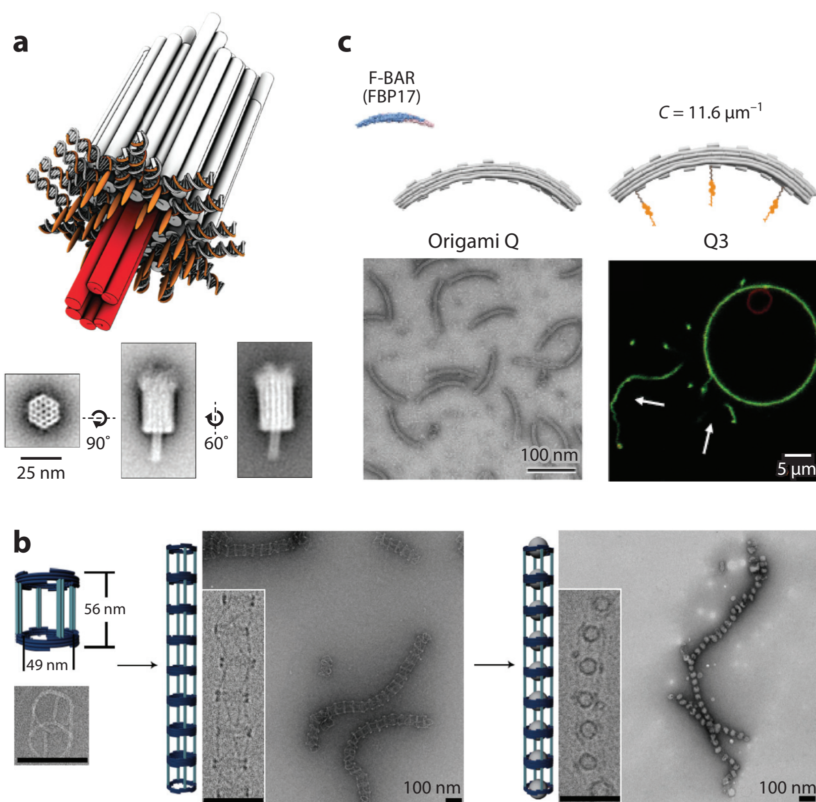

Figure 3

DNA origamis that interface with lipid bilayer membranes. (*a*, *top*) DNA membrane channel formed by 54 double-helical DNA domains. Orange ellipsoids indicate cholesterol-modified oligonucleotides, and red denotes transmembrane stem. (*a*, *bottom*) Transmission electron microscopy images of a purified DNA channel. Panel *a* adapted with permission from Reference 84. (*b*) One-dimensional liposome array made from templated liposome formed from DNA cages. Panel *b* adapted with permission from Reference 87. (*c*, *left*) Structure of origami Q (quarter) that mimics the shape of F-BAR domain proteins. (*c*, *right*) Vesicles covered by moderately curved structure Q3 that are under hyperosmotic stress have long tubular structures. Panel *c* adapted with permission from Reference 88.

especially in terms of DNA devices that interact with lipid bilayer membranes and functionally mimic proteins (**Figure 3**). Equally important, translation-free molecular toolboxes have been developed to program complex dynamic systems in vitro without relying on the synthesis of proteins by ribosomes.

3.1. DNA Strand Displacement and Binding

Although the basic design of DNA is simple, the well-controlled base-pairing has enabled researchers to use DNA strands to manipulate the spatial and temporal assembly of DNA into two- and three-dimensional (3-D) objects of varying complexity that mimic functions that are normally found in protein systems. Dynamic DNA assembly using strand displacement is required to synthesize such molecular machines. Two DNA strands that are partially or fully hybridized to each other can displace one or more prehybridized strands. Strand displacement can be initiated at

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complementary single-stranded domains, known as toeholds, and this mechanism underlies many dynamic DNA devices. For instance, a bipedal DNA walker that advances arm over arm on a DNA track (similar to how kinesin walks on a microtubule) has been demonstrated (69). The walker is fueled by the external addition of attachment and displacement strands. An autonomous bipedal DNA motor that cyclically catalyzes the hybridization of a metastable DNA fuel strand was later shown (70). Another example of a synthetic DNA molecular motor inspired by the actin-based motility of *Rickettsia rickettsii* used DNA strands to propel the motor and powered it by DNA hybridization (71).

The complementarity of DNA strands provides a convenient approach for facilitating binding between two surfaces. Using DNA-coated oil-in-water emulsion droplets and a strand-displacement strategy, a sequentially self-assembled structure with a defined assembly sequence was achieved (72). While such a programmable control scheme could potentially be useful for spatially arranging different types of synthetic cells, DNA-mediated adhesion would not require strand displacement. DNA tethers can freely diffuse on a lipid bilayer and be used to link lipid vesicles. Interestingly, a pair of DNA-linked GUVs exhibited negative thermal expansion—that is, reducing the temperature decreased the adhesion area (due to the contraction of GUVs), while the distance between the centers of a typical pair of DNA-linked GUVs increased (73). This effect can be extended to a network of DNA-linked GUVs in which the temperature can tune the porosity of the vesicle network, with higher porosity at lower temperatures.

Finally, DNA can serve as a structural material or be used for molecular communication in an artificial cell. Kurokawa et al. (74) constructed an artificial cytoskeleton based on three DNA oligomers that form a Y motif and have sticky ends that subsequently assemble into a network by annealing of the ends. The resulting DNA nanostructure that associates with positively charged membranes in a liposome mimics the function of an actin cortex and increases the stability of the artificial cell. DNA-based communication between synthetic protocells has also been reported. Protocells encapsulating DNA gate complexes can sense, process, and secrete short single-stranded DNA via toehold-mediated strand displacement (75). Although DNA nanostructures have successfully replicated certain protein machineries, their rates of assembly, dynamic control, and interfacing with natural components have room for improvement compared with the proteins that they try to mimic, and this presents opportunities for future research.

3.2. DNA Origami and Nanostructures

In 2006, Rothemund (76) introduced a one-pot method for using many short, single strands of DNA to direct the folding of a long single-stranded DNA (using viral DNA) into desired shapes, which he called DNA origami. Scaffolded DNA origami has produced a number of interesting nanostructures that may have utility in synthetic cells beyond demonstrating the complexity of nanostructures constructed from DNA building blocks. DNA double helices can be constrained to a 2-D planar or 3-D honeycomb arrangement (77) using staple-strand crossovers that bridge the scaffold at, respectively, 180° or 240° angles. By inserting or deleting base pairs from the numbers of base pairs that separate the adjacent locations of strand crossovers between neighboring helices, it is possible to introduce bending of the DNA bundle, which can lead to right- and left-handed twists, as well as to globally bent structures without a global twist (78). DNA nanostructures can also be engineered to self-assemble without using base-pairing by using shape complementarity (79, 80), similar to how protein–protein and protein–DNA/RNA binding works. This mechanism utilizes short-range nucleotide base stacking, and filaments and lattices can be produced. Furthermore, multistate, reconfigurable DNA devices that can change conformation depending on the ionic condition have been demonstrated (81). In addition to folding a long single-stranded

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scaffold, many individual DNA bricks can be designed to assemble in a completely scaffold-free manner (82, 83). These strands comprise a set of pixels in 2-D or voxels in 3-D, which allow for the assembly of highly complex structures by selecting a subset of components.

In the context of synthetic cells constructed using de novo building blocks, DNA origami has provided a unique approach, particularly with respect to interactions with the lipid bilayer membrane. Langecker et al. (84) reported a transmembrane channel made by self-assembled, scaffolded DNA origami. The DNA nanochannel was anchored to the membrane by 26 cholesterol moieties, and it exhibited nanosiemens (nS) conductivity (84) (**Figure 3*a***), similar to the conductivity of a bacterial mechanosensitive channel. More recently, a 6-nm diameter megadalton DNA origami porin was created (85). Also using cholesterol-based membrane anchoring, the nanopore was inserted in the bilayer and had approximately 40 nS conductance. In addition to being used as membrane channels, DNA origami can also shape or template liposomes. Yang et al. (86) created a DNA origami ring with handles on the inner side that can hybridize to a lipidated antihandle. Once hybridized, extra lipid and detergent are added, and the detergent is slowly dialyzed out to yield liposomes with defined and homogeneous sizes templated by the DNA origami ring. Besides using DNA origami to make spherical liposomes, DNA rings can also be connected together as a DNA cage for the formation and remodeling of liposomes with more complex shapes. This was impressively demonstrated by Zhang et al. (87) who fused liposome arrays to yield width-defined membrane tubules (**Figure 3*b***) and to generate toroidal and helical liposomes. Such DNA-templated liposomes can be reconfigured by strand displacement to bend a lipid tubule. And in addition to globally shaping liposomes, DNA origami structures have also been designed to remodel membranes in a manner similar to BAR (Bin/amphiphysin/Rvs) proteins (88) (**Figure 3*c***). BAR-mimicking DNA origami scaffolds can deform a membrane, depending on the degree of curvature. While curvature, membrane affinity, and surface density are important in tubular membrane deformation by a DNA scaffold, curvature plays the decisive role. Another notable application of a DNA origami scaffold is for positioning enzymes in a sequence to control substrate channeling (89). Such spatially addressable DNA nanoplatforms may be useful for creating artificial multienzyme systems when designing regulatory biological circuits.

3.3. Translation-Free Circuits

The realization of translation-free biochemical reaction networks, sometimes coupled to the activity of molecular devices such as DNA nanostructures, constitutes another track for cell-free biology. While a broad range of computational circuits and dynamic systems can be achieved using only nucleic acids (90–92), a remarkable wealth of dynamic systems has been emulated in vitro using small sets of molecules and mechanisms that recapitulate synthesis and degradation without relying on the synthesis of peptides and proteins by ribosomes. For instance, the combination of DNA strand displacement, transcription, and RNA degradation was the first cell-free toolbox that enabled the design and execution of relatively complex, dynamic systems. Bistable (93) and oscillatory circuits (94), comparable to the ones that have been engineered through standard gene circuit motifs, have been achieved in test tube reactions. Equally important, the demonstration that this type of circuit can be carried out in cell-sized compartments (95) and used to drive the activity of DNA nanostructures (5) increases the potential of such a molecular toolbox to develop sophisticated biochemical systems. Having transcription as an active synthesis process and a ribonuclease provides more flexibility to program genetically encoded, dynamic systems than enzyme-free environments do, and it keeps the door open to including novel RNA-based regulatory tools and devices. Similar to this toolbox, the PEN (polymerase–exonuclease–nickase) DNA toolbox (96) eliminates RNA to create another translation-free in vitro environment from only three enzymes,

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which can recapitulate the synthesis and degradation of DNA. The range of dynamic systems that can be accomplished with this toolbox is also impressive and includes oscillators (97) and patterns on millimeter scales (98).

The easier access to the biophysical and biochemical variables permitted by these cell-free toolboxes allows for much deeper descriptions of synthetic cell-free networks compared with circuits executed in living cells, thus providing quantitative information at the molecular level that is essential to building larger systems either in vitro or in vivo. Exploiting translation-free circuits to control the dynamics of DNA nanostructures, for instance, to mimic a cytoskeleton (6), could be an interesting method to implement in cell-sized compartments to create a completely artificial approach to developing synthetic cells.

4. CELL-FREE TRANSCRIPTION–TRANSLATION SYSTEMS

TXTL systems—also referred to as in vitro transcription–translation, cell-free protein synthesis, or cell-free expression systems—are becoming one of the most convenient experimental platforms on which to build genetically programmed biochemical systems outside of living organisms. The postgenomic renaissance of TXTL is the result of critical improvements and advances achieved during the past 15 years. At the technical level, TXTL enables the synthesis of scalable quantities of proteins in just a few hours, disposing of long and sometimes complicated protein purification procedures. In this section, we describe how TXTL, at the fundamental level, has become a technology suitable for cell-free biology and has uses ranging from batch mode to cell-sized compartments (**Figure 4**); we also discuss some of the current hot spots and challenges to be addressed.

4.1. Harnessing Gene Expression In Vitro

Several key advances have modernized TXTL systems to enable them to synthesize the amounts of proteins relevant for building biochemical systems. The versatility of TXTL platforms has been considerably improved at both the transcription and translation levels, expanding TXTL to larger genetic and protein territories.

4.1.1. Powering transcription and translation. The metabolic pathways energizing cell-free systems remain the most important consideration in TXTL, simply because they define the efficacy of a system. With the need for the equivalent of five ATPs per amino acid, translation is the most energy-demanding process in TXTL. No metrics exists to define the necessary strength of a TXTL system for specific applications. Considering that the average cytoplasmic protein concentration in a model organism such as *E. coli* is estimated to be 0.5–1 μM (99), a protein concentration of 1 mg/mL corresponds to about 37 μM of enhanced green fluorescent protein (eGFP) or 16 μM of firefly luciferase. Consequently, a TXTL system capable of concentrations of 1 mg/mL offers enough room to start programming biochemical systems made of a few genes.

Several ATP regeneration metabolic processes have been discovered to fuel TXTL up to at least 1 mg/mL (100–102), which has become a standard for commercial and custom-made *E. coli* cell-free systems. The demonstration that glycolysis can be activated in extract-based TXTL provided another method to fuel translation (103) or to increase the efficacy of existing systems (104). While the strength of cell-free systems seems to plateau at about 1.5–2 mg/mL, it is still worth optimizing TXTL metabolisms to enable the execution of larger DNA programs. To some extent, semi-continuous protein synthesis is a solution to this limitation (105). Semi-continuous devices have shown that the translation machinery is active for at least a day, which is remarkable

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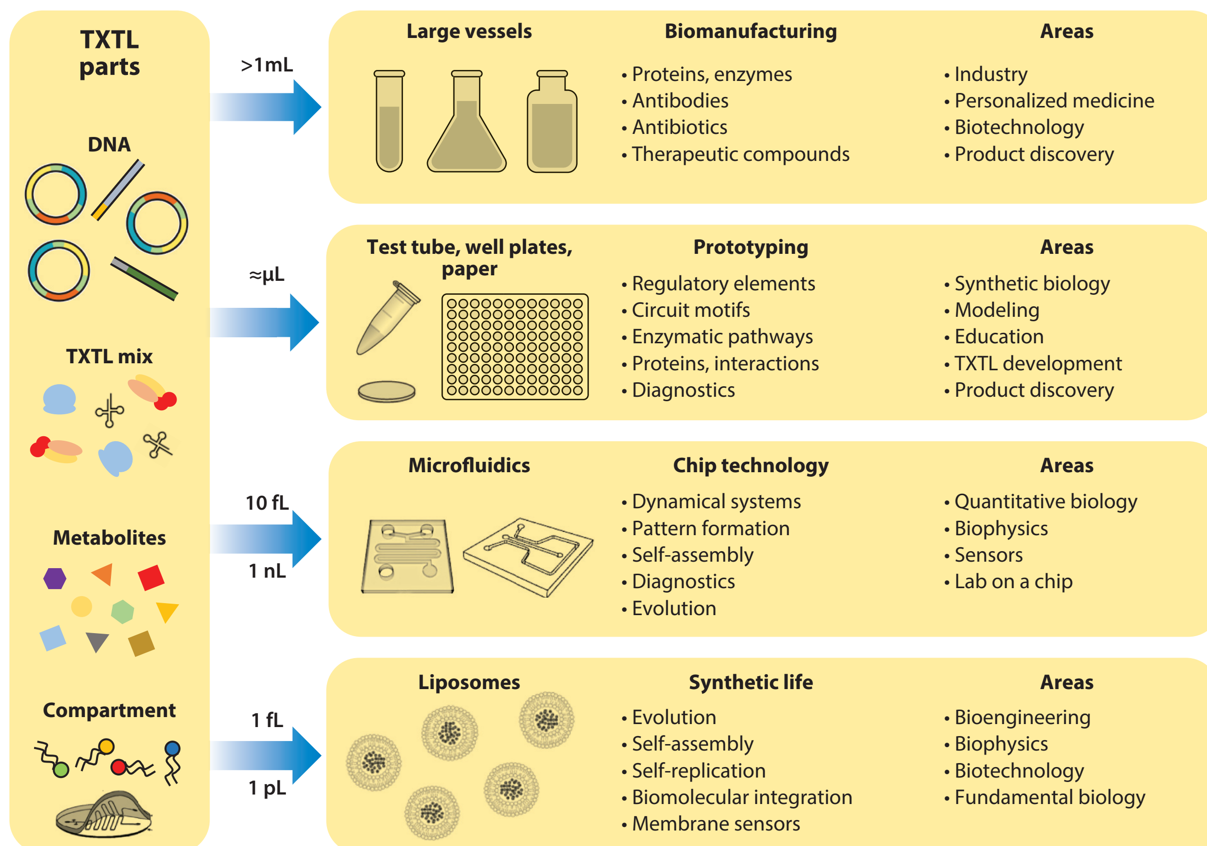

Figure 4

Cell-free transcription–translation (TXTL) systems operate at multiple length scales. TXTL mixed with metabolites and DNA can be used not only in bulk reactions at the milliliter scale but also down to pico- and femtoliter scales in encapsulated systems. The range of technologies, as well as their areas of impact, are summarized.

considering the complexity of the process. Using these fed-batch TXTL reactions, however, is not straightforward. Creating a truly easy-to-set up high-throughput continuous TXTL system would certainly be a major step forward. Otherwise, engineering strains seems to be the most promising path for increasing the productivity of TXTL systems. The recent proteomics analyses of *E. coli* lysates (106, 107) have provided a wealth of information that can be used to optimize strains. Other approaches, such as decreasing mRNA degradation, are meaningful solely for the purpose of producing proteins, but they are detrimental to circuit engineering because the mRNA lifetime is already too long, which results in a turnover of the synthesized transcripts that is too slow for executing complex dynamics (e.g., 20 min for eGFP mRNA) (108). A theoretical limit to cell-free protein synthesis in batch mode has been described only recently (109). Although the theoretical maximum that can be attained depends on the type of metabolic processes used to fuel TXTL, the description provides a quantitative analysis of the factors that limit cell-free protein synthesis in batch reactions (109).

4.1.2. Highly versatile transcription–translation systems. Until the 1990s, the optimization and utilization of TXTL systems centered on protein synthesis. With the rise of multidisciplinary

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research, synthetic biology in particular, TXTL has been reshaped into a multipurpose technology particularly adapted for cell-free biology. The new generation of TXTL systems exhibits an impressive catalog of applications that continues to grow (9). The versatility of TXTL has improved at several levels. The transcription, traditionally performed by bacteriophage RNA polymerases such as T7, has been broadened to a greater repertoire of regulatory elements (108, 110, 111), increasing the composability and size of the genetic programs that can be processed in vitro (112–114). The variety of proteins that can be expressed in TXTL has also been considerably widened. It is now possible to express integral membrane proteins either in batch mode reactions (115, 116) or directly in synthetic cell systems (117–120). Methods to express proteins with disulfide bridges have been reported (121), and glycosylation of eukaryotic proteins is now feasible (122, 123). The possibility of expressing proteins with unnatural amino acids through TXTL opens the door to building artificial systems (124, 125), thus expanding the capabilities of natural biological systems. Finally, the diversity of physical substrates that can host TXTL reactions further adds to the remarkable flexibility of this technology.

The field of cell-free biology would benefit from at least two major advances. First, the existence of many different cell-free platforms, with properties often poorly defined and understood, represents a bottleneck. None of these systems integrates the ideal set of capabilities (e.g., strength, transcription repertoire, control of TXTL dynamics, membrane proteins, posttranslational modification). Although ambitious, devising a universal platform would provide the community using this technology with the ultimate tool. Second, the reproducibility of TXTL preparations and reactions remains a challenge, even for well-established systems such as *E. coli*. Variability has also been observed between different batches for most of the commercial kits (126). Different methods are employed for custom-made TXTL systems, resulting in a wide array of systems and performance. Automation seems to offer solutions that could reduce variability (127). Standardizing procedures and protocols would also help the field reduce the rather large variability observed in reported results, especially if training and educational modules were provided.

4.1.3. Transcription–translation platforms from diverse organisms. The knowledge gained from TXTL prepared from *E. coli* cells, which is still the most-used and versatile system, serves as a solid heritage on which to develop TXTL platforms from other organisms, especially from bacteria. Several TXTL systems from microorganisms have been reported recently (128–131). However, it is not clear yet what new capabilities these alternative TXTL systems will provide. The availability of eukaryotic-based TXTL is also on the rise (132–134). The major advantage of several of these kits is that they allow for post-translational modifications, such as glycosylation, that are essential for the activity of many proteins found in higher organisms. The plant-based, high-yield ALiCE kit (LenioBio), which uses T7 transcription and recently became available, represents a major step in terms of protein production strength (i.e., 3 mg/mL).

On the one hand, the number of TXTL systems that are now available offers a larger catalog for constructing biochemical systems in vitro. On the other hand, understanding the real capabilities of each system is a challenge, as almost all of these systems are still poorly characterized quantitatively, and their basic working principles are not well understood.

4.1.4. Transcription–translation as an educational platform. Much of the success of TXTL as a highly practical system for cell-free biology relies on its fast set up and execution. For this reason, TXTL has become a handy experimental platform for education and discovery in synthetic biology, in the form of teaching modules (135); short, intensive courses (e.g., the Cold Spring Harbor Laboratory course on synthetic biology); and kits (136). These teaching materials and

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opportunities increase the accessibility of TXTL technology. More importantly, they provide information about and practice in learning the skills necessary for performing TXTL experiments.

4.2. Batch Mode Transcription–Translation in Cell-Free Biology

Constructing and programming biological systems using TXTL can be done on a remarkable range of physical scales. Although working in cell-sized compartments is the current trend, a lot of work can be done in batch mode reactions at the microliter scale. This approach has two advantages: (*a*) the setup for the reaction is fast, thus accelerating the design–build–test cycle of biochemical systems, and (*b*) high throughput assays can be implemented using liquid-dispensing equipment, which is ideal for screening hundreds or thousands of reactions in a single day. As a consequence, the reproducibility and repeatability of the experiments are also greater.

4.2.1. Building networks in transcription–translation systems. The combination of strength and versatility has transformed TXTL into a powerful system for prototyping genetic parts and programs, which remains the major activity for batch mode TXTL reactions for both basic and applied purposes. Characterizing single regulatory parts is a well-established area that maintains solid credibility based on the excellent agreement between the performances observed in vivo and in vitro (137, 138). Elementary circuits based on the traditional set of transcription factors, including the repressors and activators LacI, TetR, and AraC (110, 139), have also been validated in TXTL. One of the most recent, compelling examples is the demonstration that CRISPR technologies can be rapidly tested in TXTL, and there is high fidelity with respect to in vivo data (140, 141). This new functionality has opened the door to engineering gene circuits, such as network motifs, that include CRISPR components (114).

A persistent limitation to constructing gene circuits in runoff TXTL reactions at constant volume is the inability to precisely control the rates of degradation, for proteins in particular. Although ideal in theory, the degradation of proteins by AAA+ proteases, such as the ClpXP complex, is not easy due to the instability of ClpX (142) and because degradation demands large amounts of ATP. For these reasons, achieving genetic oscillators in TXTL requires sophisticated semi-continuous setups (143, 144). The unavailability of simple, accurate, and quantitative models is another area that necessitates substantial work. Many working TXTL principles, such as linear regimes and, more importantly, saturated regimes arising due to resource sharing, are still not well understood.

Matching the performance of parts or circuits executed in TXTL to in vivo data is not necessary for building purely synthetic biochemical systems in test tubes. Consequently, engineering new types of transcriptional regulations that are specifically adapted for TXTL offers a real opportunity to make synthetic systems independent from living organisms. TXTL could also play a major role in developing standard biological parts (e.g., any single regulatory element, such as promoters or riboregulators) due to the gene-free background setting.

4.2.2. Beyond circuits: recapitulating biological functions in transcription–translation systems. In addition to testing parts or circuits, complex biological functions are being reconstructed in TXTL that do not require cell-sized compartments. Interestingly, most of these functions contribute to the future assembly of a bottom-up synthetic cell and address challenges captured in the information–metabolism–compartment global picture (10, 11). On the information theme, the first steps in the cell-free synthesis of ribosomes have recently been taken (145). The demonstration that all ribosomal RNA can be transcribed actively is a major step, even though all of the ribosomal proteins have to be purified to assemble functional ribosomes.

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This paves the way for reproducing the translation machinery directly in a TXTL reaction. The complete synthesis of several infectious bacteriophages, especially T7 and T4 coliphages, proves that large DNA programs can be executed and that complex self-assembly can be achieved in TXTL (112, 113, 146). The replication of the T7 DNA genome concurrent with phage synthesis was the first demonstration of DNA-programmed isothermal genome amplification in TXTL. Complementary to this result, a first synthetic DNA replication circuit was proposed recently to amplify linear double-stranded DNA in TXTL (147). At the compartment level, major steps have also been taken to produce membrane proteins in batches, such as in nanodiscs (116). Although not offering a real membrane, such tools facilitate the development of functions located at the membrane. There is evidence of the synthesis of lipids in TXTL, but not at a scale large enough to be observable under a microscope (148, 149). Other biosynthesis pathways, not immediately interesting for synthetic cell systems, such as violacein, have been shown in TXTL (108). Another approach to performing metabolic functions in TXTL consists of composing cell-free reactions from lysates that have been prepared from cells expressing constitutively the enzymes involved in a pathway (150). This method, geared toward applications, suggests how biochemical systems could be assembled from different lysates.

4.3. Transcription–Translation-Based Synthetic Cell Systems

Using TXTL at the scale of living cells is not as easy as it is in test tubes, but it is appealing for many reasons. The reasons for compartmentalizing TXTL reactions are many, creating synthetic cells being the major leitmotif nowadays. Encapsulating cell-free reactions enables the recapitulation of biological functions in isolation on relevant physical scales, especially for diffusion mechanisms, and in a context appropriate for emulating biophysical and biochemical conditions close to the ones found in living cells. Although it is only a minor aspect, working in micrometer-sized compartments requires reaction volumes that are orders of magnitude smaller than those required in batch mode, and, therefore, it is cost effective.

4.3.1. Types of compartments. The variety of soft and solid microscopic compartments that can accommodate TXTL reactions is remarkable: Emulsion droplets, liposomes, polymersomes, gel particles, coacervates, microfluidics, and other solid-state chips have been successfully tested for TXTL reactions (151, 152). Two types of compartments emerge as being more advantageous and convenient for quantitative cell-free biology: microcompartments and liposomes.

Polymeric microcompartments are highly compatible with TXTL systems: Surface properties are not a problem despite the large ratio of surface area to volume; oxygenation through elastomers, necessary for most TXTL systems, occurs fast enough; high-throughput and highly parallel experiments are possible, with impressive control of the geometry. Microfluidic chips have already proven powerful for reconstituting complex, dynamic systems (143, 144, 153, 154), protein interaction networks (155), and for characterizing transcription factor binding sites (156), in addition to being effective high-throughput platforms for TXTL work geared toward biomedical applications (157, 158). Integrated microfluidics that can carry out high complexity serial and parallel processes via control of micromechanical valves could potentially extend the parallelization of TXTL testing, as protein expression from an array of more than 4,000 DNA spots on a glass substrate has been demonstrated (159). Even though it is hard to envision being able to develop properties as complex as self-reproduction, microcompartments are an ideal tool for quantitative TXTL-based cell-free biology, especially for genetically driven, dynamic systems.

Liposomes are the most commonly employed compartments because they embody the natural boundary of living cells, thus the synthesis of a self-reproducing cell can be envisioned. The

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possibilities of developing membrane functions and of using the biochemical and biophysical properties of the lipid bilayer are the principal advantages. Polymersomes are an emerging alternative to liposomes that provide greater mechanical robustness, which is particularly promising for developing biotechnologies (160). The encapsulation strategy depends on the type of TXTL system used and the composition of the membrane and surfactants (e.g., when using an emulsion-based method). The demonstration that elastin-like peptides when expressed in TXTL can form 200-nm diameter vesicles paves the way for lipid-free, programmable synthetic cells, and these may also be an alternative to other standard compartments (161).

4.3.2. Gene circuits. Sophisticated microfluidic chemostats have been fabricated to recapitulate genetically programmed, dynamic systems such as oscillators (143, 144). These chips host and keep gene circuits running for as long as 1 day. The major technical advantage is that they create an actual steady state for the synthesized proteins over long periods of time by balancing synthesis with degradation, which is emulated, respectively, by either diffusion (144) or dilution (143). This capability, which so far is not possible in batch mode reactions, provides a TXTL regime that is ideal for characterizing gene circuit dynamics in a configuration adequate for truly quantitative studies. Without such properties, constructing, running, and analyzing gene circuits over long periods of time would be intricate and hardly relevant. It is important to note that genetic oscillators, based on both transcription and translation, have not been achieved in batch mode reactions because protein degradation is not controlled and implemented well enough and because cell-free systems are not long-lived enough in runoff reactions. Multicellular TXTL-based synthetic cell systems can emulate pattern formation on millimeter scales, both in emulsion droplets (162) and on carved silicon microfluidic chips (153). The latter pioneering method for interfacing the natural protein synthesis machinery with solid-state devices could open the door to many novel applications, such as controlling cell-free gene expression by using an applied electric field (163).

Executing gene circuits in liposomes is becoming more common (118, 119, 164–168). Similar to microfluidic chips hosting multiple, interacting compartments, systems of cooperating liposomes are also being engineered. One method, based on membrane fusion of lipid vesicles, has been used to scale up the development of gene circuits in synthetic cells (167). Engineering communication between TXTL-based synthetic cells and living cells, including the use of a Turing-like test, stands out for its originality and interdisciplinary approach (169). The availability of many cell-free systems and methods for encapsulating reactions is both a bottleneck and an advantage. The advantage is that one can choose among many methods and TXTL kits. The bottleneck arises because the results obtained with a particular cell-free system and liposome preparation method may not be comparable to results obtained from other platforms and encapsulation procedures. Many of the biochemical and biophysical properties and variables of such synthetic cell systems have not been fully considered or understood. Consequently, while incredibly inspiring and exciting, the results obtained from TXTL-based synthetic cells are not generalizable. Standardization of protocols seems necessary to improve the validity and credibility of the advances made in this area.

As cell-free systems become more powerful and versatile, many fundamental questions can be addressed, such as what type of gene regulation is needed to program TXTL-based synthetic cells? What are the properties of TXTL in liposomes compared with batch mode reactions? And what is the importance of DNA physical and information compaction?

4.3.3. Self-assembly and membrane functions. In addition to reconstructing gene networks, reconstructing active macromolecular self-assembly mechanisms and membrane functions are the two most challenging goals of TXTL-based synthetic cells. The cytoskeleton is the focus of many

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of the efforts, with the objectives of creating rod-shaped bacteria and a minimal division mechanism. The *E. coli* MreB and FtsZ cytoskeletons has been expressed in liposomes, but the phenotypes have been far from the expected ones. In the case of MreB, filament assembly at the inner membrane was achieved, but no deformation was observed (170). In the case of FtsZ, filament assembly at the membrane was also achieved, but no constriction was observed (171). These findings are somewhat surprising because based on earlier studies using purified proteins (48), all of the necessary proteins are expressed that would make the FtsZ ring. These results demonstrate that recapitulating such biological functions in TXTL-based synthetic cells is far more complicated than expected and that creating self-assembly in liposomes is more challenging than previously thought.

While far from mature, using TXTL to engineer biological functions at the membrane of liposomes is forging ahead, and concrete advances have been made. Although not fully understood, many integral membrane proteins, especially from bacteria, can be expressed in a cell-free system and inserted in active forms into the membranes of liposomes. The partial reconstitution of the *E. coli* secretion system in liposomes using the PURE system (Protein Synthesis Using Recombinant Elements; New England Biolabs) (117) expands the TXTL toolbox. Some synthetic cells are capable of mechanosensitivity (118, 172). Although not reconstituted into liposomes, a mammalian TXTL system was recently used to functionally reconstitute the inner nuclear envelope proteins SUN1 and SUN2 into a bilayer membrane, highlighting the increasing use of TXTL for membrane protein reconstitution (173). Progress is undeniable, even though much work remains to be done to understand how synthetic cells that have multiple, active membrane functions can be developed. How to exploit the biochemical and biophysical properties of membranes, such as lipid composition and curvature, is also largely underexplored, with only few studies touching on these areas (174, 175).

4.3.4. Metabolism. TXTL-based synthetic cells depend on the same energy regeneration systems as do batch mode reactions, which consist of a kinase and a phosphate donor (176) and, for some kits, a carbon source. By synthesizing membrane channels, such as α-hemolysin from *Staphylococcus aureus*, one can feed, and thus extend, TXTL inside liposomes (108, 164). Yet this approach to energy regeneration, although well established and powerful, is not optimal because cell analogs are being developed only in buffers and, thus, they are far from the conditions of natural environments. More elaborate metabolic pathways must be installed to enable the harvesting and conversion of diverse types of energy sources in TXTL systems to improve the robustness of synthetic cells. The first steps toward creating TXTL photosynthetic cells have been taken recently (119), but improvements must be made to transform this approach into a robust energy source capable of fueling compartments for large DNA programs. The integration of several independent ATP regeneration pathways seems critical for developing synthetic cells capable of engaging in simultaneous biological functions and operating robustly in non-ideal laboratory conditions.

While energy regeneration is at the heart of TXTL-based synthetic cells, other metabolic functions have to be addressed concurrently to move toward unicellular self-reproduction. The synthesis of lipids, for instance, comes high on the list of processes that have to be achieved. Thus far, the programmed synthesis of phospholipids by TXTL is not strong enough to be physically visible (148, 149), but the purified protein approach to this pathway (67) supports its feasibility in TXTL.

5. CONCLUSIONS AND PERSPECTIVES

We have outlined in this review the three pillars of research in the broad area of bottom-up cell-free biology: protein-based, nucleic acid–based, and TXTL-based systems. All three branches have

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seen significant growth and interest during the past several years and will likely continue to flourish. There is now a broader interest in using the bottom-up construction of synthetic cells as a unifying experimental system. This has led to the formation of several consortia and funding opportunities in Europe and the United States. Notably, the Max Planck Research Network in Synthetic Biology (known as MaxSynBio) started in 2014 and involves nine Max Planck institutes across Germany. In 2017, the Netherlands formed a 17-laboratory group known as Building a Synthetic Cell (or BaSyC). In the United States, the National Science Foundation has funded teams of investigators across multiple disciplines who are working on high-risk, high-reward projects to build synthetic cells. There are also an increasing number of research networks, workshops, and conferences.

While we will continue to see great advances in all branches of cell-free biology, we are particularly upbeat about the utility and potential of TXTL in cell-free biology. TXTL benefits from modern DNA assembly methods to make and execute gene circuits in an environment whose programmability is more flexible than in a living cell. TXTL offers a means to construct biological systems by coupling gene expression to the final product in a manner similar to that of living cells and in a synthetic cytoplasm that emulates physiological conditions. As opposed to using a living chassis, the composition of cell-free reactions is largely accessible, enabling systems to be also interrogated at the biochemical and metabolite levels. The strength, the versatility, and the scalability of newly developed TXTL systems has made this technological tool advantageous. The preparation of lysates, the core of TXTL systems that provide the molecular machineries of transcription and translation, has been demystified, rendering TXTL more accessible to laboratories (176, 177). The rather good agreement between the performance of genetic programs executed in TXTL and in vivo measurements has strengthened the credibility of TXTL. As a consequence, TXTL has transitioned from a protein synthesis accessory to a multidisciplinary tool for engineering biochemical systems with an impressive palette of settings and physical scales (9). TXTL has expanded its scope of applications across synthetic biology (103, 125, 178), biophysics (153, 154, 179), and medicine (180, 181). Finally, the growing usage of TXTL also relies on its ability to offer unparalleled experimental turnover, on its flexibility to rapidly adapt to new settings and substrates, and on its high degree of safety.

Richard Feynman famously wrote on his blackboard in 1988 that "What I cannot create, I do not understand." This has been a driving motivation for many who work in the area of bottom-up synthetic biology. The desire to understand cellular life through a constructive approach necessitates a deep understanding of how natural cells work. Thus, the field will benefit from its close synergy with cell biology and, more broadly, the acceptance of synthetic cells as a model experimental system for engineering and understanding biology. Perhaps, a revision to Feynman's quote is fitting in this instance, "What I do not understand, I cannot create."

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review. The Noireaux laboratory receives research funds from Arbor Biosciences, a distributor of the myTXTL cell-free protein synthesis kit.

ACKNOWLEDGMENTS

The authors apologize to all investigators whose relevant work could not be cited due to space constraints. The authors acknowledge feedback from Nicholas Stephanopoulos. The authors were supported in the writing of this review by awards from the National Science Foundation to

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A.P.L. (grants MCB-1612917, MCB-1817909, CBE-1844132, and EF-1935265) and V.N. (grants MCB-1613677 and EF-1934496), and to V.N. from the Human Frontier Science Program (grant RGP0037/2015).

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