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Cell-free synthetic biology: Thinking outside the cell

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ABSTRACT

Cell-free synthetic biology is emerging as a powerful approach aimed to understand, harness, and expand the capabilities of natural biological systems without using intact cells. Cell-free systems bypass cell walls and remove genetic regulation to enable direct access to the inner workings of the cell. The unprecedented level of control and freedom of design, relative to *in vivo* systems, has inspired the rapid development of engineering foundations for cell-free systems in recent years. These efforts have led to programmed circuits, spatially organized pathways, co-activated catalytic ensembles, rational optimization of synthetic multi-enzyme pathways, and linear scalability from the micro-liter to the 100-liter scale. It is now clear that cell-free systems offer a versatile test-bed for understanding why nature's designs work the way they do and also for enabling biosynthetic routes to novel chemicals, sustainable fuels, and new classes of tunable materials. While challenges remain, the emergence of cell-free systems is poised to open the way to novel products that until now have been impractical, if not impossible, to produce by other means.

1. Introduction

1.1. Synthetic biology primer

At its core, synthetic biology is inspired by the power and diversity of the living world. It is an endeavor predicated on the idea that we can learn to more reliably and rapidly engineer biological function for compelling applications in medicine, biotechnology, and green chemistry (Andrianantoandro et al., 2006; Benner and Sismour, 2005; Endy, 2005; Fritz et al., 2010; Khalil and Collins, 2010; Purnick and Weiss, 2009; Smolke and Silver, 2011). What is unique to synthetic biology is the application of an engineering-driven approach to accelerate the design-build-test loops required for reprogramming existing, and constructing new, biological systems. This is needed because the current approach to engineering cells is often extremely laborious, costly, and difficult. In one example, economical production of artemisinin in yeast has already accounted for over 150 person-years worth of work and counting (Kwok, 2010). In other examples, it took DuPont and Genencor approximately 15 years and 575 person years to develop and produce 1,3-propanediol and Amyris Biotechnologies about 4 years and between 130 and 575 person years to make farnesene (Zach Serber, Amyris Biotechnologies, DARPA Living Foundries Industry Day, personal communication).

So far, efforts in synthetic biology have largely focused on the construction and implementation of genetic circuits, biological modules (compilations of biological "parts" that perform defined functions, e.g., ribosome binding sites, promoters, and genes (Purnick and Weiss, 2009)), and synthetic pathways into genetically reprogrammed organisms. However, a major challenge exists because of our incomplete knowledge of how life works, the daunting complexity of cells, the unintended interference between native and synthetic parts, and (unlike typical engineered systems) the fact that cells evolve, have noise, and have their own agenda such as growth and adaptation (Kwok, 2010). The guiding question, therefore, is how do we develop a new way of engineering in the face of these unique and complex features of biology? In other words, what is the suite of foundational technologies required for taming the complexity of living systems? One foundational technology that has emerged is the ability to construct new programs that carry out user-defined functions from "parts" of DNA (e.g., biological switches or oscillators). Biological computer-aided design (*BioCAD*) tools, directed evolution, and cell-free systems will also each have a role.

Although *in vivo* synthetic biology projects are dominant in the field, we focus this review on the role of cell-free synthetic biology. Without the need to support ancillary processes required for cell viability and growth, cell-free systems offer a powerful platform for accelerating the optimization of synthetic pathways and for not only harnessing but also expanding the chemistry of

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life. We begin by defining and examining cell-free systems. We then describe recent applications of cell-free biology where ensembles of cellular machines, such as the biosynthetic enzymes involved in hydrogen production (Zhang et al., 2007), have been employed to make small molecule metabolites. We do not provide a comprehensive discussion of *in vitro* synthetic biology projects, nor do we cover the rich history of *in vitro* biocatalysis (Zaks, 2001), but rather focus on a description of several recent multi-component examples in the context of metabolite production. Finally, we examine new frontiers in applying cell-free systems to re-conceptualize the way we engineer biological systems.

2. Cell-free systems

Rather than attempt to balance the tug-of-war between the cell's objectives and the engineer's objectives, cell-free synthetic biology activates, integrates, and focuses cellular resources towards an exclusive user-defined objective. Physical removal of the cell wall enables direct access to the inner workings of the cell (Fig. 1A). Because the native genomic DNA is removed, undesirable genetic regulation is no longer present and the requirement of cellular viability is also eliminated. Furthermore, transport barriers are removed, allowing easy substrate addition, product removal, system monitoring, and rapid sampling. In short, a decreased dependence on cells leads to an increase in engineering flexibility (Forster and Church, 2007).

The power of cell-free systems was first appreciated over a hundred years ago. In 1897, Eduard Buchner used yeast extract to convert sugar to ethanol and carbon dioxide (Buchner, 1897), for which he won the Nobel Prize (1907 Chemistry). Since then, cell-free systems have continued to play an important role in our understanding of fundamental biology. For example, Nirenberg's discovery of the genetic code, for which he received the Nobel Prize in 1968, shed light into how protein synthesis works (Nirenberg and Matthaei, 1961). Cell-free systems have since continued to unravel other fundamental discoveries such as the understanding of eukaryotic translation (Algire et al., 2002; Hinnebusch et al., 2007; Iizuka et al., 1994; Thompson et al., 2001).

Beyond using cell-free systems for biochemical analysis, the successful recapitulation of biological function *in vitro* has inspired attempts to use cell-free systems for product synthesis. Indeed, there are more than 30 years of successful industrial history with biotransformations *in vitro* (Zaks, 2001). For the most part, *in vitro* biocatalytic transformation has focused on single transformations, such as enabling the production of specific chirality. In another example, single-enzyme cell-free systems, such as cell-free DNA replication (the polymerase chain reaction (PCR)) are ubiquitous to molecular biology labs and have revolutionized modern biological research. PCR in combination with other common molecular tools such as DNA restriction digest and ligation reactions allow us to assemble DNA sequences at will (Gibson et al., 2009). In other approaches, crude extract cell-free systems (CECFs) (Goerke and Swartz, 2009; Jewett et al., 2008; Jewett and Swartz, 2004) and synthetic enzymatic pathways (SEPs) (Shimizu et al., 2001; Jung and Stephanopoulos, 2004; Zhang, 2010; Zhang et al., 2007) have practical applications in protein and peptide synthesis and evolution, small molecule production, cellular/metabolic pathway investigation, and non-natural product synthesis (Fig. 1B). With the recent demonstration of cost-effective cell-free protein synthesis at the 100 l scale by SutroBio (Zawada et al., 2011), cell-free systems are poised to have a significant commercial impact in addition to their well established research utility (Fig. 2).

3. Cell-free applications

Cell-free systems have been used for decades as a tool in fundamental and applied research, but are only now being

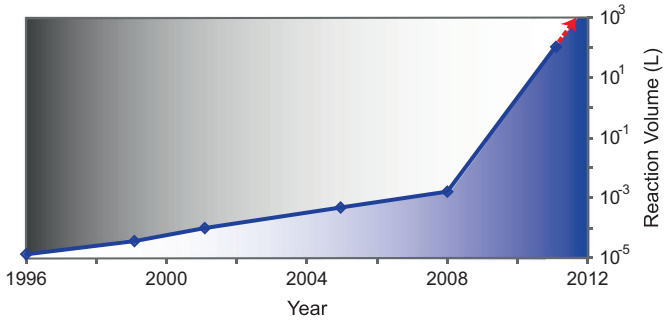

Fig. 2. Milestones in the scalability of batch *E. coli* extract cell-free protein synthesis reactions. Modern batch cell-free reactions were debuted by Kim et al. (1996) at the 20 μL scale with yields of 0.1 g L^{-1} chloramphenicol acetyltransferase (CAT). Over the last 15 years batch *E. coli* extract cell-free protein synthesis reactions have increased in reaction yields and scale to 60 μL reaction volume and 0.15 g L^{-1} CAT (Kim and Swartz, 1999), 100 μL reaction volume and 0.23 g L^{-1} CAT (Kim and Swartz, 2001), 500 μL reaction volume and 0.56 g L^{-1} CAT (Voloshin and Swartz, 2005), 2 mL reaction volume and 1.2 g L^{-1} CAT (Jewett et al., 2008), and most recently 100 L reaction volume and 0.7 g L^{-1} recombinant human granulocyte macrophage colony-stimulating factor in 2011, creating the first ever industrially relevant cell-free protein synthesis reaction (Zawada et al., 2011).

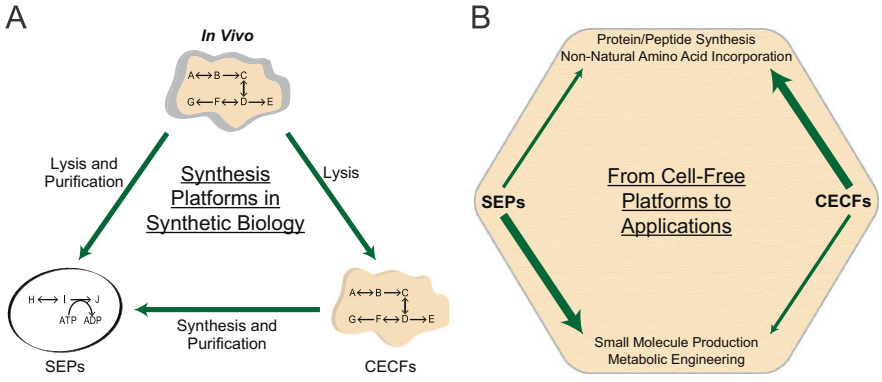

Fig. 1. (A) Synthesis platforms in synthetic biology. While the majority of synthetic biology projects are performed *in vivo*, *in vitro* systems are emerging as a complementary technology. *In vitro* systems can be further subdivided into crude extract cell-free systems (CECFs) and synthetic enzymatic pathways (SEPs). The flow diagram overviews how the different systems are related. (B) Cell-free platforms (listed in bold) can be further grouped to reflect their applications, which include translation systems and small molecule production. The strength of the arrows represents the relative amount of published papers to date, but does not necessarily represent the platform's ability and/or application potential.

transformed into factories with commercial feasibility for making therapeutics, metabolites, and non-natural products (Forster and Church, 2007; Hold and Panke, 2009; Lopez-Gallego and Schmidt-Dannert, 2010; Roessner and Scott, 1996; Swartz, 2006; Zawada et al., 2011). While penetration of cell-free technologies into industrial applications has yet to occur, cell-free factories are capable of unconstrained metabolism (in the sense that resources do not also have to sustain the life of the cell), providing advantages for optimizing pathway flux, bypassing substrate limitations through metabolic channeling, and directing resources towards a single objective.

When exploiting the biosynthetic potential of enzyme machines *in vitro*, the first design decision is choosing between a purified system built from scratch (SEPs) or an extract (cell lysate) based system (CECFs) (Fig. 1). Typically, this decision begins with considering the number of cellular "housekeeping" functions that need to be maintained, such as sustained energy generation. A major challenge of constructing SEPs stems from our incomplete understanding of fundamental biology. On the other hand, CECFs often have activities that are unwanted or difficult to characterize. Time and cost are other key issues. For cell-free protein synthesis, the expense associated with reconstituting a SEP from every factor involved in energy metabolism, respiration, transcription, translation, and protein folding is prohibitive for most commercial applications (Swartz, 2006). Although both platforms have overlapping utility (Fig. 1b), the trade-offs between SEPs and CECFs must be carefully considered. In the remainder of this section, we highlight some of the ever-expanding set of cell-free products and applications.

3.1. Protein and peptide synthesis

As one of the most common examples of CECFs (Endo and Sawasaki, 2006; Jewett et al., 2008; Carlson et al., in press), cell-free protein synthesis (CFPS) provides a useful starting point for examining the utility of cell-free systems. To produce target proteins of interest, CFPS systems utilize ensembles of biocatalysts that carry out translation from crude extracts derived from bacterial, plant, or animal cells. Upon incubation with essential amino acids and energy substrates, the ensemble of activated catalysts within the cell lysate acts as a chemical factory to polymerize amino acids into polypeptides. Although any organism can be used to provide a source of crude lysate, the most productive CECFs are derived from *Escherichia coli* extract (Jewett et al., 2008), wheat germ extract (WGE) (Takai et al., 2010), or rabbit reticulocyte lysate (RRL) (Pelham and Jackson, 1976).

Cost-effective, high-level protein synthesis ($>1\ g\ L^{-1}$ for select proteins) in the *E. coli* CFPS platform has been achieved by advances in (i) mimicking intracellular conditions (Jewett and Swartz, 2004), (ii) activating integrated biological processes (Jewett et al., 2008), and (iii) controlling cell-free metabolism (Calhoun and Swartz, 2005, 2006; Swartz, 2006). These efforts have opened the way to commercial production of protein therapeutics (Kim and Swartz, 2004) personalized medicines (Kanter et al., 2007), vaccines (Bundy et al., 2008) and classes of proteins that are difficult to produce *in vivo*, like hydrogenases (Boyer et al., 2008). A flow diagram of how CECF systems are commonly processed and utilized is shown in Fig. 3.

Beyond proteins, CECF translation systems have been used to make short antimicrobial peptides. Multiple examples have found that in order to effectively block protease degradation and obtain high yield, the peptides need to be fused with small protein segments of finely tuned lengths (Lee et al., 2010; Loose et al., 2007). When building a molecular toolkit for cell-free systems, rules like this that govern short peptide translation must be carefully considered.

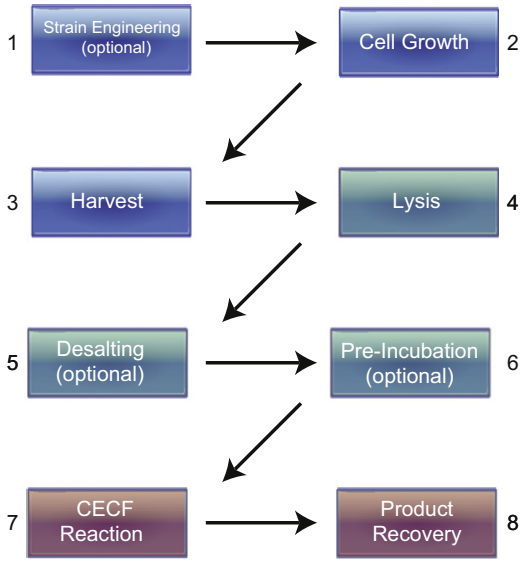

Fig. 3. Flow-diagram of typical CECF reaction. Briefly, a strain is chosen or engineered as appropriate and cells are grown and harvested; the culture is lysed, buffer is exchanged through desalting, and the extract is then normally subject to pre-incubation for translation reactions; lastly, the crude-extract is incubated under typical reaction conditions and the product is assayed or recovered. Blue boxes (#1-3) represent cellular growth and harvest, green boxes (#4-6) denote preparation of the cell-free extract, and red boxes (#7-8) denote cell-free product synthesis and recovery.

3.2. Metabolite synthesis

Over the last decade, researchers have come to appreciate that integrated metabolic functions such as central metabolism, the tricarboxylic acid cycle, and even oxidative phosphorylation can be activated in CECF systems (Calhoun and Swartz, 2005; Jewett et al., 2008; Kim and Swartz, 2001). Following suit, there has been a recent thrust in efforts to make metabolites by controlling cell-free metabolism. In one approach, Panke and colleagues devised a CECF system to achieve multi-enzyme catalysis of dihydroxyacetone phosphate (DHAP) from glucose (Bujara et al., 2010). By deleting two key enzymes from the extract source strain (Fig. 3, step 1), DHAP synthesis reached concentrations up to 12 mM, a feat not yet achieved *in vivo*. Because DHAP is itself an unstable molecule, the addition of butanal and rabbit muscle aldolase directly to the cell-free reaction (Fig. 3, step 7) converted DHAP to a more stable form. This example showcases the freedom of design in adjusting cell-free system components by modifying the genome of the host strain *and* by direct addition of new components (in this case a non-bacterial enzyme and butanal) for building synthetic multi-enzyme reaction networks *in vitro*.

Optimization of synthetic multi-enzyme networks is required for recruiting metabolism to make products of interest. By using cell-free systems, fine-tuning of network properties can be achieved without evolutionary strategies on the genome level (such as multiplex automated genome engineering, MAGE (Wang et al., 2009); conjugative assembly genome engineering, CAGE (Isaacs et al., 2011); and global transcription machinery engineering, gTME (Alper et al., 2006)) or laborious construction of variants and screening of phenotypes. This is because system properties can be monitored directly for real-time analysis that provides a comprehensive picture of network dynamics and potential bottlenecks. Building on the advances described above, Bujara et al. (2011) used such a strategy to obtain a detailed metabolic "blueprint" for optimization of *in vitro* DHAP production using a crude extract system. Their high-resolution mass

spectrometry analysis enabled metabolite profiling for identification of rate-limiting steps, which were then used for rational optimization of the synthetic network. Introduction of a synthetic operon into the extract source strain that harbored extra copies of phosphofructokinase, fructose bisphosphate aldolase, lactate dehydrogenase, and hexokinase (Fig. 3, step 1) increased DHAP yields an additional 2.5-fold over their earlier work (Bujara et al., 2010).

Complementary to efforts in cell extract based systems is the construction of synthetic enzymatic pathways for small molecule production (Zhang, 2010). Such approaches have ranged from reconstructing glycolysis from 12 purified enzymes (Itoh et al., 2004) to enabling artificial photosynthesis from 8 purified enzymes (Wendell et al., 2010), to the 12 enzyme multi-step synthesis of Vitamin B12 (Roessner and Scott, 1996). A key advantage of constructing SEPs is the flexibility that can be achieved for rational programming of biosynthetic networks. Zhang et al. (2007) have capitalized on this feature for making hydrogen. For example, a SEP for hydrogen synthesis, composed of 13 enzymes from rabbit, spinach, *Pyrococcus furiosus*, *Saccharomyces cerevisiae*, and *E. coli*, was built to make hydrogen from starch with extremely high yields of $12H_2$ per glucose (Zhang et al., 2007). When comparing this value to anaerobic fermentation at only $4H_2$ per glucose (Zhang et al., 2007), cell-free systems show a distinct advantage.

In another example from the same group, hydrogen was synthesized in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a common metabolite used in biohydrogenation, from cellobiose (Wang et al., 2011). This 12 enzyme SEP is attractive because cellobiose, which is a primary product of enzymatic hydrolysis of cellulose and dilute-acid pretreated biomass hydrolysate, is toxic to cell cultures. The researchers demonstrated the ability to achieve almost 100% theoretical yield using their synthetic platform. As *E. coli* is unable to grow in the presence of acid-treated biomass hydrolysate, this example highlights the advantage of using cell-free systems for developing biomanufacturing processes that are difficult to implement in cells because of toxicity constraints. However, the high cost of purifying stable, stand-alone enzymes is still prohibitive for large-scale commercial applications.

Outside of small molecule production, harvesting biosynthetic machinery to convert redox potential directly to electricity could lead to the creation of efficient biofuel cells (Cooney et al., 2008). For direct energy transfer biofuel cells, the electrode interacts directly with the enzyme of interest, for example glucose oxidase (Cooney et al., 2008). Here, the enzyme is able to convert the chemical signal into an electrical one, usually due to the redox potential of the active metal centers (Moehlenbrock and Minteer, 2008). Current and future work in this area is focused on activating complete metabolic pathways for increasing power density (Sokic-Lazic and Minteer, 2008), extending biofuel cell lifetime through enzyme stabilization (Moehlenbrock and Minteer, 2008), and removing product inhibitory effects, such as the effect of citrate on citrate synthase (Sokic-Lazic and Minteer, 2008).

Finally, biosynthetic enzyme systems have also demonstrated potential for site-specific isotope-labeling of small molecules (Schultheisz et al., 2008, 2010). As an alternative to chemical synthesis, both purine (ATP, GTP) and pyrimidine (UTP, CTP) biosynthesis pathways have been reconstructed *de novo* for site-specific incorporation of isotopes (Schultheisz et al., 2008, 2010). This work enables NMR detection for probing structural and dynamic features of nucleic acids. In addition, it represents a *tour de force* in synthetic pathway construction, with 28 and 18 enzymes from different species being required for purine and pyrimidine biosyntheses, respectively.

As more complex synthetic pathways and product molecules are pursued, it will be important to develop rapid techniques for going from gene sequences to proteins to pathways to functional screening. If production costs can be reduced and scaled-up effectively (Zawada et al., 2011), cell-free synthetic pathway engineering seems poised to enable an improved toolbox and faster process for harnessing the chemistry of life, analogous to the emergence of synthetic organic chemistry (Yeh and Lim, 2007).

4. Synthetic biology frontiers

How do we turn cell-free biology into a substrate for engineering? Historically, most cell-free systems have focused on obtaining and activating biocatalysts that make a desired product. Less emphasis has been given to establishing a fundamental easy-to-use molecular "toolkit" for engineering purposes. This is changing, however, with the construction of genetic circuits, protein cascades, compartmentalization, spatial organization, and minimal cells as described below. Furthermore, synthetic biology continues to push the boundary of what *does* exist to what *can* exist. To this end, cell-free systems offer a flexible platform for introducing orthogonal chemistries in order to synthesize non-natural products toward the goal of expanding the chemistry of life.

4.1. Programming circuits

A driving force in synthetic biology has been the ability to use DNA as a language to write synthetic genetic programs comprised of "parts" that rewire and reprogram organisms. Two landmark studies include the development of a synthetic genetic oscillator (Elowitz and Leibler, 2000) and a synthetic genetic toggle switch (Gardner et al., 2000). Not surprisingly, similar attempts to program circuits and logic switches have motivated new directions toward the goal of building enhanced predictable function in cell-free systems. Although, the complexity of function implemented is still in its infancy.

A particular focus area is the creation of genetic circuitry containing well-characterized promoters and regulatory sites with factors to implement logical operations using circuit-like connectivity. Multi-step reaction cascades have achieved one such type of behavior (Fig. 4A). Noireaux et al. (2003) used a CECF transcription and translation system to synthesize orthogonal RNA polymerases (e.g., T7 and SP6) in series when controlled by the previous polymerase promoter region, eventually expressing a protein of interest (e.g., luciferase). This example is analogous to linear electrical amplifiers, the major difference being the non-instantaneous signals inherent to biological systems. A cascade approach could be used to synthesize different proteins of interest that require time-delayed or coordinated expression to elicit desired function.

Other avenues of transcriptional control have included nucleic acid switches and oscillators. To this end, Kim and Winfree (2011) recently demonstrated synthetic *in vitro* transcriptional oscillators. They developed a two-switch negative feedback oscillator (Fig. 4B), an amplified negative-feedback oscillator, and a three-node ring oscillator, demonstrating high correlation with computational modeling. This exemplary example is part of a growing list of successful applications of synthetic approaches to cell-free genetic wiring that include: bistable switches using RNA transcription and degradation (Kim et al., 2006), strand displacement cascades (Zhang and Seelig, 2011), Lotka–Volterra oscillators (Soloveichik et al., 2010), limit-cycle oscillators (Soloveichik et al., 2010), chaotic systems (Soloveichik et al., 2010), and logic AND gates (Takinoue et al., 2008). Just as is observed *in vivo* (Khalil and Collins, 2010), use of DNA architectures to mediate diverse functional behavior could be extended to build more sophisticated, computing-like behavior in

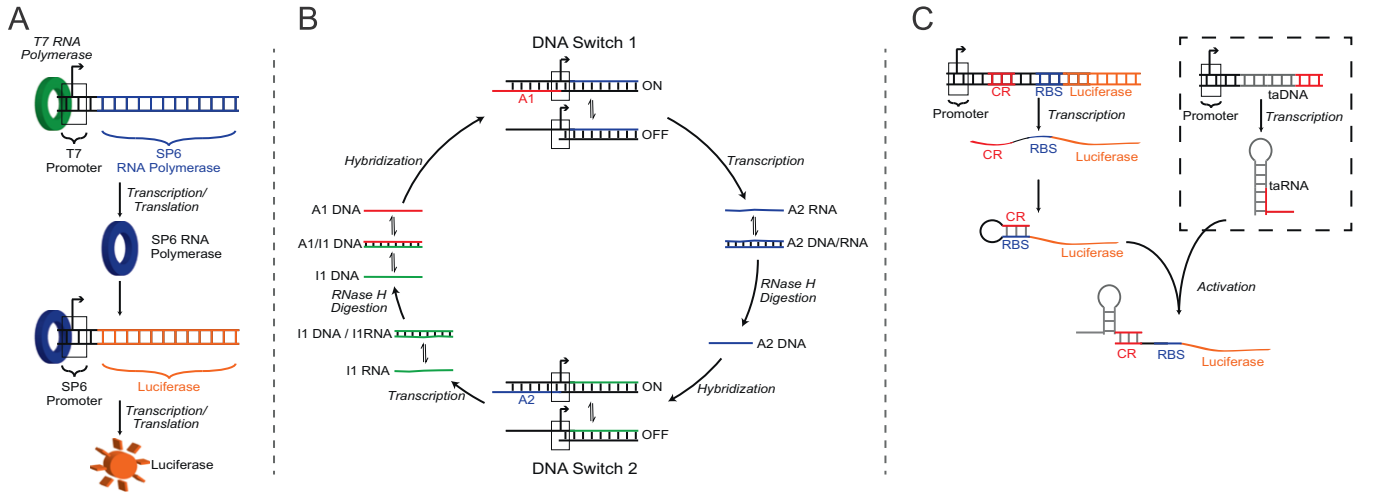

Fig. 4. Programming cell-free circuits. (A) A general schematic of a combined transcription and translation reaction cascade. Here a T7 bacteriophage RNA polymerase (T7 RNAP) is used to prime the reaction cascade by binding to its promoter region and transcribing a secondary bacteriophage RNAP (SP6). This in turn selectively binds the SP6 promoter region prefacing the expression of the protein of interest (luciferase). (B) A two-switch DNA oscillator is controlled by the addition of two activator sequences (A1 DNA and A2 DNA), which can bind to and activate a respective DNA switch, an inhibitor sequence (I1 DNA), and two enzymes RNA polymerase and RNase H. DNA Switch 1 is turned ON when A1 DNA binds to it, thus completing the RNA polymerase promoter region. Subsequently, DNA Switch 1 undergoes transcription to produce A2 RNA complementary to A2 DNA. The A2 RNA is able to initiate binding to release the activator DNA sequence A2 from the DNA Switch 2, thus turning DNA Switch 2 OFF. The A2 RNA of the A2 RNA/DNA hybrid is then digested by RNase H and releases the activator A2 DNA. DNA Switch 2 is turned ON when A2 DNA binds and subsequently undergoes transcription to produce the I1 RNA sequence complementary to I1 DNA. Binding of I1 DNA by I1 RNA releases A1 DNA thus allowing DNA Switch 2 to be turned ON. Once again, the I1 RNA of the I1 RNA/DNA hybrid is digested by RNase H and releases the DNA inhibitory sequence I1, thus turning DNA Switch 1 OFF. The process is repeated and the oscillatory behavior is sustained over time. (C) The *cis*-repressed (CR) riboswitch is designed with a complementary sequence able to bind the ribosome binding site (RBS) of the protein of interest and inhibit translation. Expression of a second regulatory RNA deemed the *trans*-activating RNA (taRNA) can bind to and release the CR region from the RBS and activate translation.

cell-free systems. In such systems, exogenously adding short DNA initiating or quenching sequences during the course of the reaction could offer precise and immediate control over functional outputs.

While lagging behind developments in transcriptional control, post-transcriptional control via RNA molecules (e.g., antisense RNAs, siRNA, and miRNA) or RNA-based sensors (e.g., riboregulators (Fig. 4C), ribozymes, and riboswitches) is also expected to provide a versatile tool for rational and model-guided construction of biological function *in vitro* (Bayer and Smolke, 2005; Isaacs et al., 2006; Muranaka and Yokobayashi, 2010).

4.2. Compartmentalization

As cell-free systems comprise of biocatalytic ensembles without walls, the reintroduction of synthetic compartments offers a versatile approach for confining enzyme systems to a specific geometry, linking functional phenotypes to the genes that encode for them, and adding new function. The two main approaches explored so far are the encapsulation of enzyme systems in phospholipid membranes (Murtas et al., 2007; Noireaux and Libchaber, 2004) and capturing enzymes in water droplets suspended in an oil emulsion, termed *in vitro* compartmentalization (IVC) (Griffiths and Tawfik, 2003). IVC has emerged as a powerful technology for directed evolution of enzymatic activity (Fig. 5) (Davidson et al., 2009; Miller et al., 2006). In one example, Griffiths and Tawfik used IVC to increase the activity of bacterial phosphotriesterase 63-fold, creating one of the fastest hydrolases ever recorded (Griffiths and Tawfik, 2003). In another demonstration, Ellington's group created streptavidin variants with altered specificities for biotin analogs (Levy and Ellington, 2008). Rather than encapsulating cell-free systems, functionality can also be added by introducing synthetic biocompatible compartments, such as DNA hydrogels called "P-gels" for activating high yield cell-free protein synthesis (Park et al., 2009) or lipid disks for membrane bound protein expression (Shimono et al., 2009).

4.3. Spatial organization of cell-free systems

A key difference between *in vitro* and *in vivo* biological systems is enzyme concentration. For example, current protocols for *E. coli* crude extract cell-free protein synthesis systems use 10 mg *E. coli* protein mL^{-1}, which is approximately 20-fold less than the cytoplasmic protein concentration (Jewett and Swartz, 2004). Since biochemical reaction networks involve multiple reactions, increasing the concentrations and hence proximity of enzymes and other mediating factors necessary for synthetic networks to function optimally may increase productivity.

For such purposes, synthetic biologists have already devised a number of methods for immobilizing or tethering enzyme pathways in a specified geometric arrangement (Fig. 6). Surface tethering is one example of these spatial organization techniques (Zhang et al., 2005). In one configuration, mRNA-protein fusion molecules produced via mRNA display are tethered to complementary DNA sequences arranged on microchips (Fig. 6A). By changing the composition of DNA on the chip, pathways can be built, optimized, and reconstructed. Jung and Stephanopoulos (2004) used this approach to construct a five-step trehalose synthesis SEP. Other on-chip biochemical circuits have also been examined (Buxboim et al., 2007). Beyond DNA chips, DNA origami nanostructures (Douglas et al., 2009; Rothemund, 2006; Yin et al., 2008) may also have potential for cell-free reaction applications such as tethering enzyme pathways together, creating ON/OFF transcriptional switches, and trapping products. Using nucleic acids to organize enzyme pathways offers an interesting toolbox that when combined with modeling efforts could enhance our understanding of exploitation of nature's biosynthetic potential.

Protein scaffolding is another technology that has shown promise for spatially recruiting enzymes in order to increase reaction rates by decreasing substrate diffusion lengths (Fig. 6B). The key idea is to localize the enzymes of a pathway using protein–protein interactions so that they function as a metabolic channel, rather than as individually dispersed enzymes. Inspired,

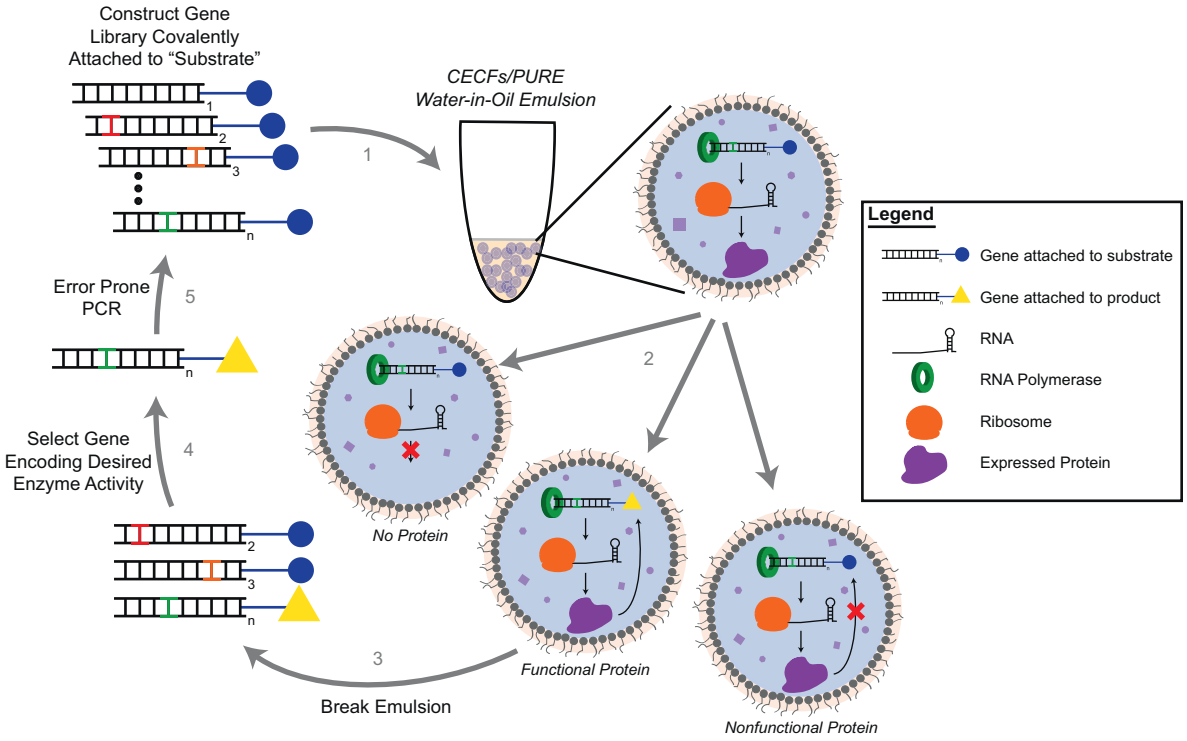

Fig. 5. Generalized *in vitro* compartmentalization (IVC) example for directed enzyme evolution. Initially, a gene library of an enzyme of interest is constructed and attached to a "substrate" in order to establish a physical link between the genotype and phenotype. For example, the substrate could be composed of a microbead displaying small molecules that interacts with the downstream enzyme of interest. The gene library is compartmentalized in a water-in-oil emulsion with on average less than one gene per water droplet where it undergoes combined transcription and translation (step 1). If successful, the synthesized enzyme will interact with the substrate to make a product (step 2). IVC prevents the synthesized protein from interacting with substrates in other compartments. The emulsion is broken (step 3) and the winning products can be recovered along with the gene (step 4), through affinity purification for example. After capture, variants can be amplified and additional cycles of selection and amplification can be carried out (step 5). For specific examples of IVC directed evolution designs see (Agresti et al., 2005; Bernath et al., 2004; Doi et al., 2004; Griffiths and Tawfik, 2003; Levy and Ellington, 2008; Levy et al., 2005; Tawfik and Griffiths, 1998), and for detailed protocols see (Davidson et al., 2009; Miller et al., 2006).

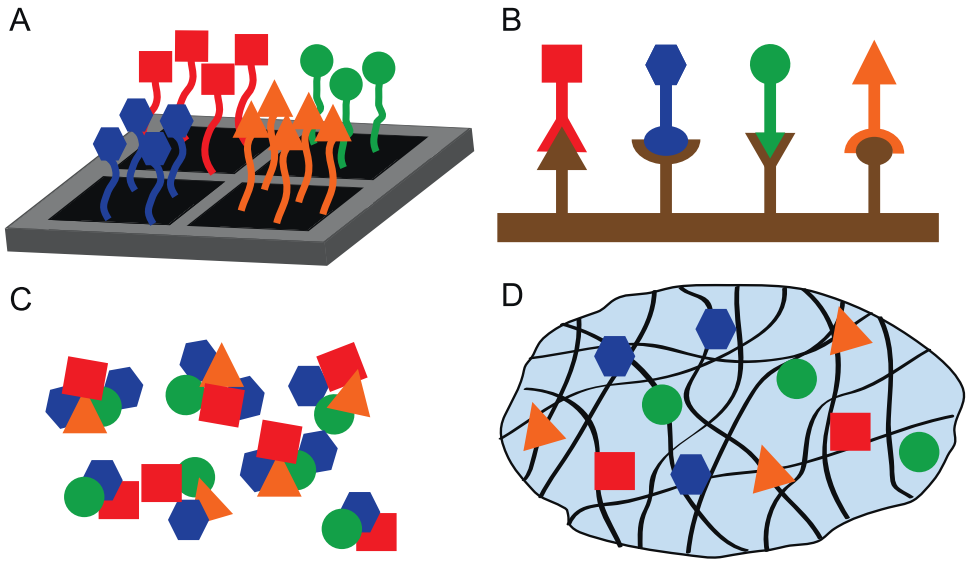

Fig. 6. Spatial organization techniques include (A) mRNA display, (B) protein scaffolding, (C) CLEA particles, and (D) foam dispersion.

in part, by modular polyketide synthases and cellulosomes, protein scaffolds can be made by a variety of methods. In one approach, pathway enzymes can be synthesized with cognate peptide ligands that bind to a protein scaffold composed of ligand interaction domains. Changes to the protein scaffold interaction domains then allows for easy stoichiometry optimization techniques. Such an approach was pioneered *in vivo* by Dueber et al. (2009). This approach was used to increase mevalonate biosynthesis yields 77-fold and also to triple the yield of glucaric acid from 0.5 to 1.5 g L^{-1}. Although protein scaffolding has yet to be reported *in vitro*, it could aid in the development of highly active cell-free systems.

A more simplified, non-specific method to immobilize enzymes is to produce covalently linked enzyme aggregate (CLEA) particles (van Rantwijk and Sheldon, 2007; Fig. 6C). In this approach, enzyme ensembles are easily precipitated in the

presence of ammonium sulfate and then cross-linked with glutaraldehyde. This method has worked well for synthesis of nucleotide analogs that are otherwise toxic to cells (Scism and Bachmann, 2010). Remarkably, the researchers found that a CLEA immobilized five-enzyme synthetic pathway was robust, reusable, and more productive than the soluble alternative.

Finally, chemical or natural detergents and surfactants such as Tween-20 (Hyo-Jick and Carlo, 2006) and Ranaspumin-2 (Rsn-2) (Wendell et al., 2010) can be used as a foam to spatially disperse enzymes and liposomes (Fig. 6D). In a recent example, an artificial photosynthesis system was built in Tungara frog nest foam made from the Rsn-2 protein (Wendell et al., 2010). Due to its unique evolutionary heritage, Rsn-2 is able to form into a foam structure at low protein concentrations without negatively effecting cell membranes and enzyme activity. In this study, the researchers were able to increase glucose production from light and carbon dioxide 3-fold relative to a bulk solution. Based on lessons learned from the examples above, increasing the local concentration of enzymes of a biosynthetic pathway greatly benefits the productivity of cell-free systems.

4.4. Minimal cells

The creation of streamlined synthetic minimal cells will improve our understanding of life and its origins and enable production of natural and unnatural chemical entities (Forster and Church, 2006; Jewett and Forster, 2010; Murtas, 2009; Szostak et al., 2001). This is being approached from two complementary methods. In the top–down reduction approach, efforts seek to minimize cellular genomes for reducing complexity *in vivo* (Gibson et al., 2010). Rather than genome reduction *in vivo*, the "bottom–up" integration of DNA/RNA/protein/membrane syntheses *in vitro* is also being pursued. In this constructive approach, the idea is to build a minimal biological system that is self-replicating, or autopoietic (Murtas et al., 2007), from individual biomolecular parts that function together. A recent example from Kuruma and colleagues (2009) used a purified translation system in liposomes to synthesize active membrane-bound proteins that convert *sn*-glycerol-3-phosphate to phosphatidic acid, a key precursor to membrane synthesis. This demonstrates a necessary step towards a self-sustaining system that can grow and maintain a bilayer membrane. A detailed update on designing and building minimal cells is given elsewhere (Jewett and Forster, 2010).

4.5. Expanding the chemistry of life

The development of technology platforms for the production of novel proteins with orthogonal chemistries serves as a foundation for making non-natural products with new enzymatic, structural, or functional properties. As an example, the translation apparatus is being used to expand the chemistry of life by introducing non-natural amino acids into proteins (Noren et al., 1989). In an illustrative example of this, Goerke and Swartz recently produced $\sim$400–600 mg L^{-1} of active chloramphenicol acetyltransferase or dihydrofolate reductase with *p*-azido-L-phenylalanine (pAz) incorporated site-specifically through amber suppression (Goerke and Swartz, 2009). In their study, the mutated synthetase and tRNA pair that incorporated pAz in response to the amber codon were constitutively expressed in the cell extract (Fig. 3, step 7) and not in the living cell, thus obviating negative effects associated with overexpression of orthogonal synthetase and tRNA during cellular growth. Here, the lack of viability constraints in cell-free systems offers an obvious advantage in reaction flexibility over their *in vivo* complements.

Because amber suppression is limited to incorporating only one type of non-natural amino acid, codon reprogramming in the Protein synthesis Using Recombinant Elements (PURE) translation system (Shimizu et al., 2001) has found utility to expand this value. The PURE system is a SEP that reconstructs active bacterial protein synthesis from His-tagged components of the translation apparatus. Capitalizing on the freedom of design, Suga and colleagues developed the wPURE system for codon reprogramming ("w" stands for withdrawn, where amino acids and aminoacyl-tRNA synthetases are withdrawn and replaced with charged amino acids of one's choice) (Kawakami et al., 2009). The wPURE system has advantages for incorporating multiple types of non-natural substrates, which can be charged using an artificial ribozyme called Flexizyme that has been shown to load virtually any amino acid onto tRNAs that bear various anticodons (Kourouklis et al., 2005). Remarkably, Suga and colleagues have been able to produce N-methylated cyclic peptides with both natural and non-natural amino acids that are both protease resistant and permeable to the cell membrane (Kawakami et al., 2008, 2009).

Finally, cell-free systems open the possibility for expanding the genetic code by introducing additional Watson–Crick base pairs (Hirao et al., 2002; Sismour and Benner, 2005; Yang et al., 2007) through rearranging the hydrogen bond donor and acceptor groups, while maintaining the original geometry. This along with changes to the functions of the translation apparatus (Barrett and Chin, 2010; Neumann et al., 2010; Wang et al., 2007) will continue to open new avenues for synthesizing non-natural molecules.

5. Conclusions

Cell-free synthetic biology is emerging as a powerful platform technology capable of complementing work in cellular *in vivo* systems. This is due to the extraordinary level of control and surprising diversity of approaches available for building biosynthetic systems without constraints that limit cellular engineering. Despite the promising features of cell-free synthetic biology, it remains unclear to what extent cell-free systems will serve as test-beds for accelerating the design of synthetic programs or as commercially relevant factories for producing therapeutics, metabolites, and non-natural products. If recent work in cell-free protein synthesis systems is an indicator (Zawada et al., 2011), it will likely be some of both. Two key areas for growth outside topics covered here are likely to include evolution of the translation apparatus and high-throughput protein synthesis of protein libraries off DNA chips. For now, an immediate challenge for cell-free synthetic biology is learning to integrate and activate predictable, highly efficient enzyme networks at low-cost and large-scale. A further challenge includes closing the apparent gap between *in vivo* and *in vitro* functional complexity that can be programmed into a single integrated platform. These challenges are both daunting and exciting, but by addressing them, we may harness the potential of cell-free systems to profoundly change how we study, control, and tune cellular ensembles for biomanufacturing *any* chemical or material from renewable resources, both quickly and on demand.

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