

Engineering microbes with synthetic biology frameworks

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Typically, the outcome of biologically engineered unit operations cannot be controlled *a priori* due to the incorporation of *ad hoc* design into complex natural systems. To mitigate this problem, synthetic biology presents a systematic approach to standardizing biological components for the purpose of increasing their programmability and robustness when assembled with the aim to achieve novel biological functions. A complex engineered biological system using only standardized biological components is yet to exist. Nevertheless, current attempts to create and to implement modular, standardized biological components pave the way for the future creation of highly predictable artificial biological systems. Although synthetic biology frameworks can be applied to any biological engineering endeavor, this article will focus on providing a brief overview of advances in the field and its recent utilization for the engineering of microbes.

Introduction

Development of biological systems that integrate synthetic biology with microbial engineering has increased rapidly in the past several years [1–3]. But what is synthetic biology? Currently, the multitude of definitions of synthetic biology often leads to ambiguous interpretations (Box 1). In many respects, this ambiguity is expected because synthetic biology is still an evolving field. At its core, synthetic biology is a concept of recasting biology into a hierarchy of abstractions, analogous to traditional engineering disciplines with the purpose of allowing biological components to be integrated by modular design into larger assemblies [1,2,4]. An example of such an abstraction is the hierarchical classification of biological systems into ‘parts,’ ‘devices,’ and ‘systems’ that are maintained in a ‘chassis’ (Figure 1). The lowest level, a *part* is defined as a single basic biological function, e.g. a ribosome binding site. These parts may then be combined to make a *device* to achieve a single human-defined function, such as an RNA sensor that can change the conformation of an actuator in response to a target ligand. *Systems* are the combination of various devices with the purpose of executing a specified task, such as a biological circuit that can emit a signal in response to explosive molecules. These systems reside within a *chassis*, a host cell, which supplies necessary resources for full functionality. Chassis might also be engineered to behave appropriately in the desired environment, for example, a

microorganism designed to thrive in a particular culture medium [1,2,4]. This abstraction necessitates standardization at each level using well-defined parts and interfaces between them to ensure reliable and compatible assembly into devices and systems of increasing complexity. Leading this effort is the Registry of Standard Biological Parts at MIT (<http://partsregistry.org>). In addition to serving as a depository of genetic materials, the Registry catalogues standardized parts and devices assembled from those parts [5].

In a simplistic view, synthetic biology frameworks should be applicable to any aspect of biological engineering for the purpose of increasing programmability and robustness. In this context, programmability can be defined as the ability to tightly control a biological system that has been engineered to generate a specified task. A highly programmable biological system enables precise quantification of an output/response, when a specified input is given (Figure 2). Robustness can be viewed as the stability of an engineered unit, with consistent performance that is independent of where it is implemented. Although a complex man-made biological system using only standardized components is yet to exist, in this review we focus on advances in the creation of synthetic biology frameworks that can be used for engineering microorganisms.

Disassembling, constructing, and rearranging DNA

The introduction of genes into microbes to achieve specified biological functions (for example, to engineer the biosynthesis of novel pharmaceuticals, chemicals, and biofuels [6–10]) is routinely achieved utilizing plasmids. One major goal of synthetic biology with respect to plasmid-based expression systems is to allow the rapid assembly and interchangeability of ‘insert’ DNA among different plasmids that have been created by different users. The addition of a second DNA fragment into an existing plasmid construct usually relies upon the availability of unique restriction sites. When alternative restriction site sequences are not available, the generation of point mutations or subcloning must typically be performed before subsequent cloning. The concept of modular plasmid constructs has been previously introduced, for example, to provide a convenient means to exchange elements such as promoters, replicons, and antibiotic resistance markers [11]. Similar to this concept, standardized plasmid formats were recently introduced to allow cloning of multiple DNA inserts with the use of only one set of restriction sites that remain preserved during the cloning process [12]

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Box 1. Synthetic biology: an evolving definition

The term *synthetic biology* was first coined in 1978 [78] in reference to recombinant DNA technology. However, it did not gain popularity until its reintroduction at the beginning of this century [79]. In this context, synthetic biology was used to refer to non-natural molecules that function as naturally occurring biological mimics. Taken more broadly, however, it can also include the disassembly and redesign of existing biological components. This ambiguity has promoted a flurry of activities to categorize synthetic biology as distinct from conventional biotechnology [1,80]. With little consensus on an all-encompassing definition [80,81], some view synthetic biology as the generation of novel functionality in a biological context. Synthetic biology is also defined as the application of engineering methodology to biology [1]. There also exists the proposition that synthetic biology can create living systems (i.e. systems subject to Darwinian evolution) from abiotic components [80,82]. Nonetheless, synthetic biology can be characterized by its methodic and quantitative approach to the design of biological systems.

(Figure 3). Therefore, additional DNA fragments can repeatedly be added adjacent to pre-existing inserts at either end without further modification. This type of vector format facilitates the use of a standardized method for modular cloning.

In addition to developing standardized methods to assemble DNA sequences, synthetic biology also aims to develop standard parts. In general, standardization is focused at the DNA level and begins with the decoupling of natural sequences from segments that are nonessential for a target function. Here, one or several nucleotides are systematically removed or reassembled to identify the minimum independent unit required to retain functionality and to limit its connectivity with other biological components [1]. Removal of sequences for standardization need not preserve the same level of activity of the target, but it should not abolish function. (This process of disconnecting DNA sequences is often termed ‘refactoring.’) Reduction of DNA components can also extend beyond the removal of extraneous nucleotides from a single fragment of interest. For instance, refactoring strategies can also be applied to entire pathways. Pathway reductions are commonly applied in metabolic engineering of microorganisms. In these cases, competing pathways are often deleted to improve the yield of target metabolites [8,9,13–17]. However, in the case of synthetic biology, pathway reduction is typically geared towards the identification of the minimal set of genes required to assemble a living cell [18]. For example, individual gene disruption in *Mycoplasma genitalium* revealed that 100 out of 485 protein-coding genes were nonessential under optimized culturing conditions [19,20]. Similarly, deletion of ~80% of the yeast genome also did not result in measurable growth rate changes in rich medium [18]. A recent high-throughput study showed that when chemical or environmental stress was applied to these yeast deletion mutants, 97% of the gene deletions resulted in measurable reduction of growth [21]. All together, these studies revealed the possibility of standardizing a ‘life unit’ through the disposal of ‘nonessential’ genes. However, the degree of nonessentiality of a gene often cannot be determined *a priori* due to the complexity of a cellular system.

De novo DNA synthesis also continues to play a key role in the efforts to standardize DNA fragments [22]. Using *de*

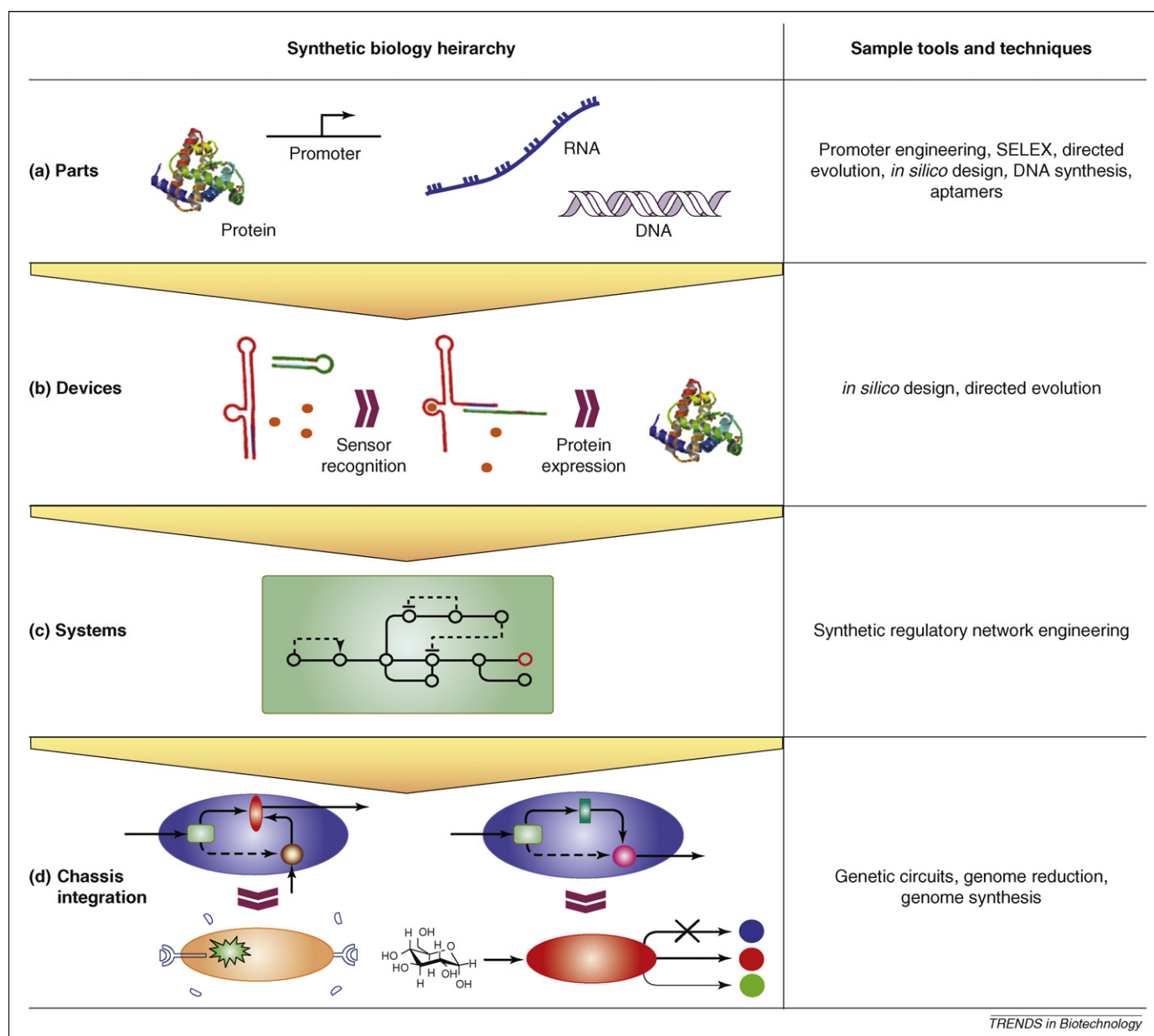
novo synthesis, DNA fragments can be generated from any known sequence, with the direct incorporation of any desired changes. Already, long DNA fragments can be successfully assembled using this approach [23–25]. Despite these successes in synthesizing DNA, the robust assembly and cloning of large DNA fragments still remains a challenge [26,27]. Recently, some of these limitations were overcome and the entire 582-kb *M. genitalium* genome was constructed [28]. In this work, synthetic DNA fragments of 5 to 7 kb in length were assembled *in vitro* before *in vivo* compilation into larger fragments. Because clones containing assemblies greater than half of the genome could not be maintained in *Escherichia coli*, the yeast *Saccharomyces cerevisiae* was used as a host, and clones carrying the entire *M. genitalium* genome could be obtained with the help of yeast homologous recombination machinery. Although this work represents a milestone for chemical synthesis of long genomes, the transplantation of a synthetic genome into an empty cell has yet to be achieved. However, methods have already been developed to swap the entire genome of a bacterial species with that of another [29,30]. Therefore, the creation of a standardized microorganism can be assumed to be technically feasible by replacement of the natural microbial genome with an entirely synthetic genome [28].

In addition to synthetic biology applications, the power of custom DNA synthesis has been exploited in the metabolic engineering of microorganisms. In this case, synthetic DNA has been used to improve heterologous expression by modifying the primary DNA sequence to match the codon utilization of a particular microbial host [17,31]. A synthetic biology framework has also been developed to control gene expression at the promoter level. Gene expression using common promoters is usually mediated by a binary state, ‘on’ or ‘off.’ To further expand the collection of promoters with varying strength, a random mutagenesis approach was recently performed to obtain a diverse promoter library [32]. Using a fluorescence screening strategy, chimeric promoters with strength spanning 196-fold range were identified. From such a library, it is possible to pick and choose a desired promoter strength that is most suitable for a particular application that requires controllable gene expression.

At the DNA level, standard biological components have been created by removing nucleotides, by manufacturing DNA fragments from scratch, and by artificially evolving natural sequences. Today, the availability of standardized plasmid systems, custom-made DNA, and promoter libraries have already been successfully implemented to increase the speed and effectiveness of engineering microbial factories to synthesize high-value chemicals [17,31,32].

Engineering RNA-based control systems

Many genetic engineering tools (e.g. antisense RNA, small interfering RNA, micro-RNA, riboregulators, ribozymes, and riboswitches) have been developed by exploiting the complex, yet malleable architecture of non-coding RNA to regulate the expression of specific gene targets at the transcriptional or post-transcriptional level [33–35]. For example, as an alternative to gene knockout strategies, antisense RNA has been used to redirect the primary



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Figure 1. The Synthetic Biology Framework. **(a)** Various tools and techniques inform the design of potential parts, such as promoters, aptamers and genes encoding novel proteins. **(b)** Through clever design, these parts are combined to form a device. The example shown here is the simple expression of a protein that performs a desired function in response to a signal. **(c)** These devices are then integrated into systems that can achieve more complex functionality through a variety of interactions. In this example, the protein device forms part of a branched reaction pathway. **(d)** Finally, these systems are assembled into an optimized chassis to accomplish an astounding array of functions, such as hunting for human tumor cells, acting as biosensors, or operating as a microbial cell factory. As an aside, a more rigorous definition would suggest that a single mRNA is a device (composed of various parts such as a ribosome binding site and protein coding sequence) that would imply the scenario presented in **(b)** is more akin to a system. This distinction is semantic as a device is distinguished primarily by its single *human-defined* function. What is important, however, is the methodical and modular approach allowing for extensibility, reusability and compatibility of designed parts, devices and systems.

metabolism of *Clostridium acetobutylicum* to increase butanol production [36]. Such a *trans*-acting RNA strategy, however, often suffers from ‘molecular cross-talk,’ the off-target binding events that hinder the expression of other genes. Several synthetic biology frameworks that engineer controllable RNA regulatory systems have been developed to increase the specificity of *trans*-acting RNAs. For example, the construction of riboswitches has enabled conformational alterations of RNA structure between active and inactive binding states in response to an effector molecule [37]. By using an engineered RNA molecule, the repression of a specified gene target can only take place in the presence of an effector molecule, thus reducing the

frequency of nonspecific target binding in the absence of the effector molecule.

The development of riboswitches often begins with the *in vitro* selection of artificial aptamer sequences using methods such as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [38] to incorporate desired characteristics, e.g. high ligand specificity, towards a variety of molecular analogues. Today, *in vitro* selection techniques have generated individual aptamer sequences that are capable of selective recognition of tetracycline over doxycycline [39], theophylline over caffeine [40], and codeine over morphine [41]. Using this selection method, the demonstrated ability of engineered aptamers to

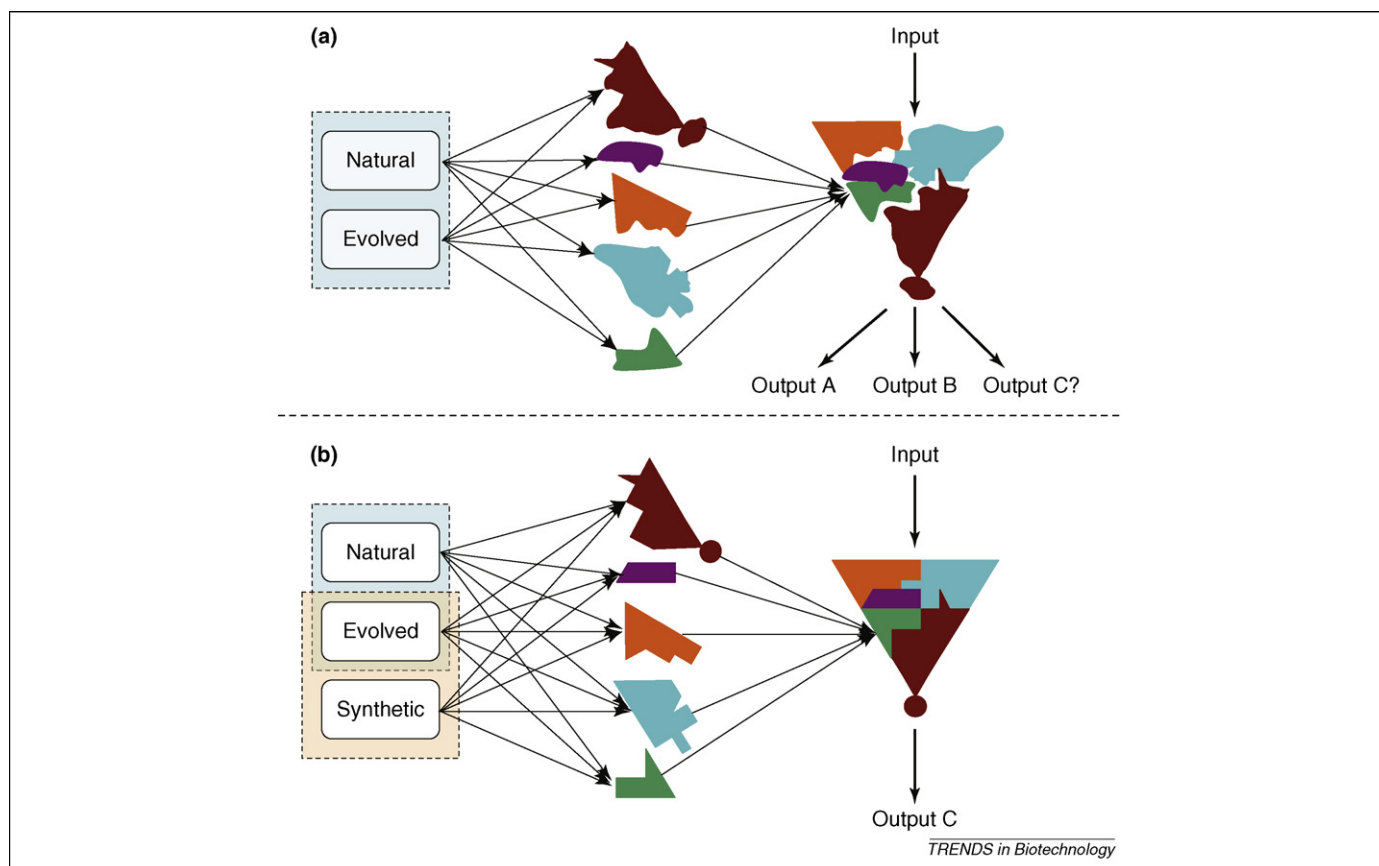


Figure 2. A representation of the design process of traditional genetic engineering (a) and synthetic biology (b). Both traditional genetic engineering and synthetic biology employ naturally occurring and modified biological components to construct an engineered system. However, because the building blocks and assembly methods of traditional genetic engineering methods are not well characterized, they often result in unanticipated interactions that can lead to suboptimal integration and pleiotropic effects when assembled into a larger system. This means that the output is frequently unpredictable and strongly dependent upon both the selection of specific parts and the methods for assembly. By contrast, synthetic biology begins with developing standardized biological components, with standardized interfaces that are fixed to promote optimal integration. The result should be highly predictable engineered systems with a single output for a single input.

discriminate between molecules with high structural similarity is encouraging for their prospective incorporation into the design of control algorithms of biosynthetic pathways, where intermediates might vary by single functional groups.

As highly programmable control systems, riboswitches have also been implemented for use as Boolean operators, and as 'ON' and 'OFF' switches [42–44]. Additionally, the construction of conditional logic operators has been aided by the recent discovery of several naturally occurring tandem riboswitches [45] (Figure 4). With riboswitches that individually respond to *S*-adenosylmethionine and coenzyme B₁₂, this multi-input configuration could function as a 'NOR' logic gate. The ability to construct additional conditional logic operators ('AND', 'OR', 'YES', and 'NOT') using oligonucleotide sensing allosteric ribozymes has also been demonstrated *in vitro* [46], and shows promise for future *in vivo* applications.

By using an RNA-type control system, it is possible to construct a device that can precisely tune the expression of a specific gene target based on its selectivity toward a particular ligand. Such a device could be programmed to respond to intracellular metabolites, enabling the molecules to exert control overexpression of genes in related biosynthetic pathways. However, currently there are only few aptamer sequences available for the recognition of endogenous metabolites. Expanding the collection of apta-

mer sequences can further expand the applicability of such systems to control cellular functions.

Modular enzyme engineering

The ability to generate enzymes that are able to catalyze the conversion of any given substrate into any given product is an important ultimate aim of synthetic biology. Comparative modeling and *ab initio* approaches have contributed to the characterization of enzymatic mechanisms and structural predictions. Recently, it was shown that *in silico* design can be used to guide the generation of enzymes that can catalyze reactions that previously did not exist in nature, such as Kemp elimination [47]. In this study, *de novo* enzymes were created by searching for protein backbones that were capable of forming an idealized active site with maximum transition state stabilization. Together with the *in silico* design, directed evolution methods were applied to improve the performance of newly obtained biocatalysts. This work represents a successful example of modularly assembling appropriate protein backbones to generate new biocatalysts for a specified chemical reaction. To date, pathway engineering of microorganisms has primarily relied on the availability of naturally occurring biochemistry. Therefore, the generation of entirely new enzymes should allow the design of *de novo* biosynthetic pathways in microorganisms and enable the synthesis of any chemical in a manner analogous to organic

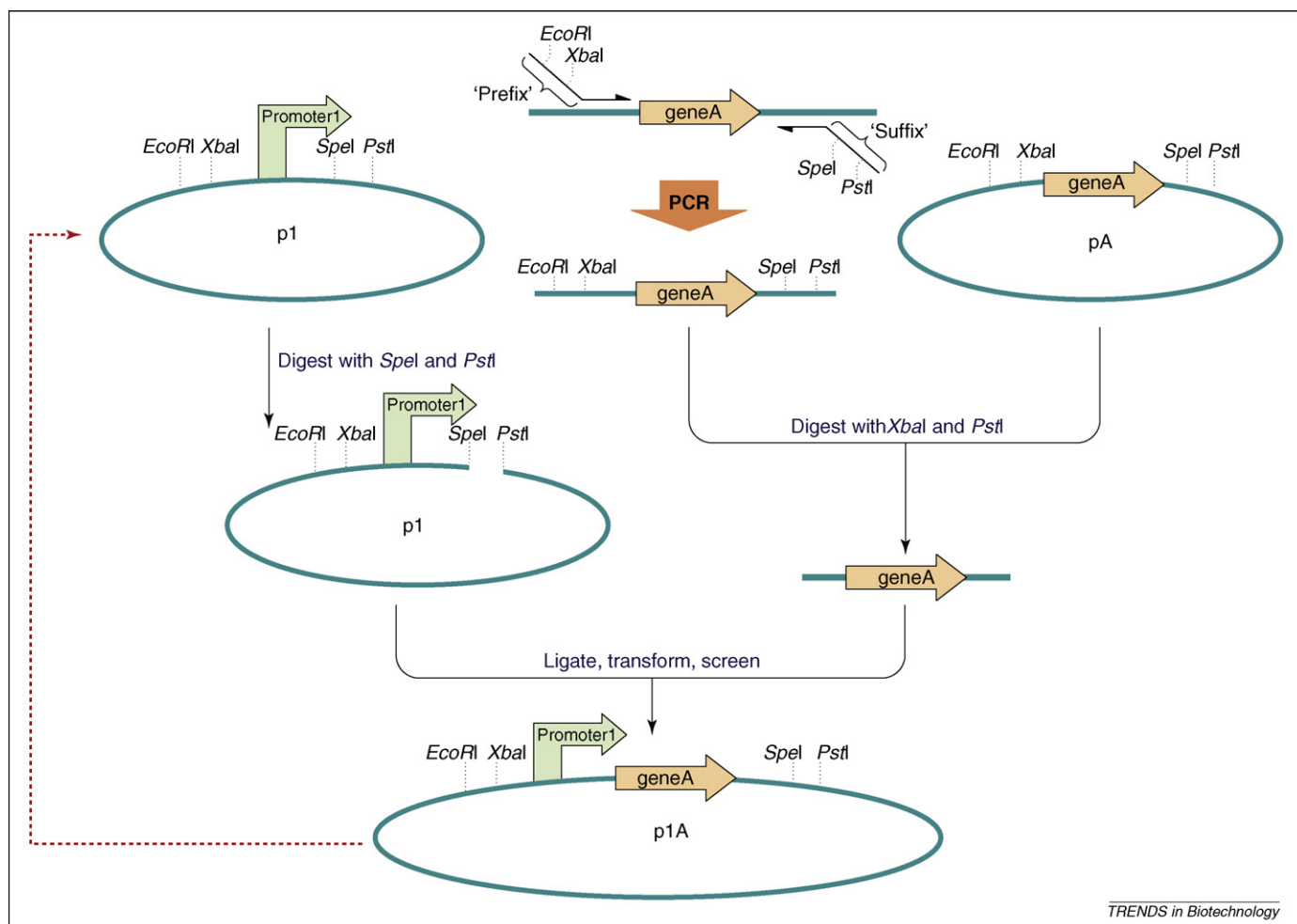


Figure 3. Assembling DNA molecules using a BioBrick standard interface. Compatible parts might be generated via PCR using oligonucleotide primers with standardized 'prefix' and 'suffix' sequences encoding the necessary restriction enzyme recognition sequences, or by digestion from a plasmid that has previously been made available in the Registry (<http://partsregistry.org>). Such parts can then be cloned into standardized plasmids (p1). The BioBricks cloning strategy ensures the regeneration of compatible restriction digest sites at the terminal end of the assembly (p1A), thus allowing additional parts to be readily added.

retrosynthesis ('retro-biosynthesis'). Towards this goal, we have recently created a publicly accessible database that catalogs enzymes based on their generalized functionality (<http://www.retro-biosynthesis.com>).

Standardization strategies at the protein level can be pursued by bottom-up assembly, as shown in the previous example. However, the identification of peptide modules within naturally existing enzymes can also be used to

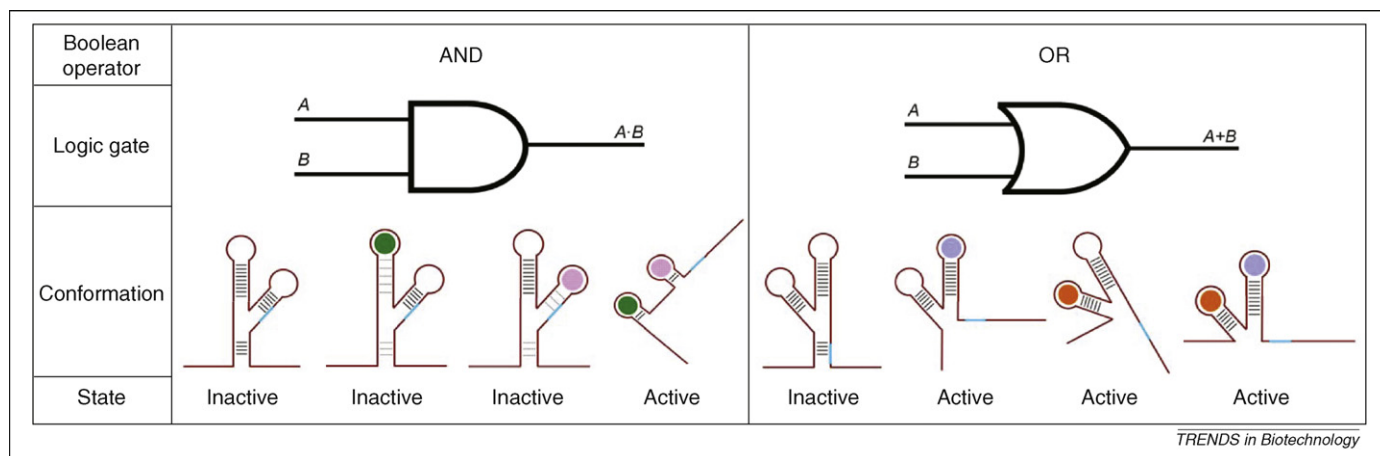


Figure 4. Programming Boolean logic operations using RNA engineered to respond to small molecule ligands. As an example, 'AND' and 'OR' commands are illustrated through the use of hypothetical riboregulator architectures that interact with two distinct ligands (indicated by the colored circles). A ribosome binding site (RBS) is indicated as a blue line. Occlusion of the RBS based on inactive secondary structures prevents translation, thereby preventing expression of the target gene. A change in conformation as the result of binding one ligand, in the case of the 'OR' gate (right panel), or of both ligands for the 'AND' gate (left panel), releases the RBS and allows translation and expression of the downstream gene.

reconstruct new enzymes with desired functions. For example, using an *in silico* algorithm to identify structurally conserved protein fragments, libraries of hybrid β -lactamases were created from recombination of peptide modules derived from two parental genes [48]. Through screening of the resultant expressed libraries in *E. coli*, novel enzymes were discovered that conferred differential antibiotic resistances. A module-based design was also demonstrated in the engineering of tightly controlled signaling proteins [49].

Modifications of naturally existing protein modules can also yield standardized peptides that allow the functional expression of some challenging proteins in microorganisms. For example, the synthetic peptide MALLAVF has been successfully employed as a leader fragment to facilitate expression of eukaryotic membrane-bound cytochrome-P450 enzymes in bacteria, such as *E. coli* [50]. This peptide was originally derived from a bovine P450 N-terminal sequence that was modified and assembled using *E. coli* codon preferences and sequences which minimize the formation of secondary RNA structures [50]. By using this synthetic leader peptide and truncating their native N-terminal membrane recognition signal peptides, many cytochrome-P450s, including those derived from plants, have successfully been functionally expressed in *E. coli*, facilitating the synthesis of functionalized pharmaceuticals [17,51].

With advances in structural biology, bioinformatics, and computational design, it is now possible to develop standardized methods to construct enzymes using module-based approaches to yield novel function. In practice, *in silico de novo* enzyme design often fails to give rise to highly functional enzymes. Therefore, experimental methods such as directed evolution coupled with powerful screening strategies will continue to play a key role in fine-tuning or increasing the robustness of synthetically assembled enzymes [47].

Redesigning cellular networks

Synthetic biology also focuses on the development of benchmark frameworks to assess the stability of intracellular networks, and to create non-natural cellular network behavior. The development of synthetic regulatory networks typically involves the systematic rearrangement of regulatory elements, (e.g. repressor proteins and promoters) to create artificial input-output responses. For example, synthetic networks that mimic naturally existing network behavior, such as oscillation [52], positive [53] and negative [54] feedback loops, have been developed. Moreover, a bistable toggle switch using a system of two mutually repressible promoter-repressor systems has also been constructed [55].

To promote the predictability of *de novo* genetic circuits, the selected parts/devices should be dynamically characterized to assess the suitability of their prospective interactions upon introduction into a chassis [56]. To this end, it has been demonstrated that the function of a simple negative feedback loop, composed of a standardized promoter-repressor pair, could be accurately predicted according to a simple model [57]. However, several exceptions to this rule were observed by constructing a library of synthetic net-

works using well-characterized components [58]. By identifying several networks composed of identical connectivity that executed different logical operations, it was demonstrated that even an *a priori* understanding of network connectivity does not fully determine its *in vivo* function. Therefore, at present it can be concluded that *in vitro* characterization of devices does not always translate into predictable *in vivo* function [59].

The creation of synthetic cellular networks still relies on naturally occurring genetic materials for assembly. Therefore, the lack of predictability of an engineered behavior is still context-dependent, despite recent evidence suggesting that the implementation of artificial networks in *E. coli* is not particularly susceptible to perturbations [60]. To some degree, the deviation in the predicted behavior of an engineered genetic circuit once inside a living cell could be rescued by applying directed evolution strategies on selected parts of the synthetic circuit [61]. However, it is anticipated that by using standardized parts, the stability and predictability of such engineered networks can be improved. Although much work is still needed to characterize the compatibility of engineered networks in microorganisms to promote performance stability, as of today, synthetic networks have found applications in microbial engineering for the purpose of mitigating metabolic imbalances caused by expression of heterologous pathways [62] and as a means of reducing the accumulation of toxic metabolites [63,64].

Perspectives

The promise of synthetic biology is in its potential to transform the means by which biological systems are manipulated. Indeed, the purpose of this field is to simplify the engineering of biology by developing experimental methods and methodologies that will lead to the predictable and reproducible assembly of biological 'machines,' organisms that perform specific and predefined functions. The assembly of these biological machines can be done in a manner analogous to the assembly of mechanical machines if there are both standardized biological parts to choose from among and standard methods for the assembly of the chosen parts. The ability to identify standardized biological parts and devices has been rapidly improved by advances in technologies needed to decode and rewrite genes, enzymes, and networks. These parts may be naturally occurring, e.g. promoters that require alterations in DNA sequence only to facilitate standardized assembly. Alternatively, the parts may be modified to alter their core functions, such as the substrate specificity of an enzyme.

DNA sequencing technology is sufficiently effective [65,66] so that it is now possible to sequence the entire human genome in a relatively short period [67,68]. This ability will facilitate the identification of new, natural parts for use in engineered biological systems. Recent advances in *de novo* DNA synthesis have also enabled the construction of natural [25,31,69] and non-natural enzymes [47], as well as viral particles from scratch [23,24]. However, the availability of parts is only one requirement for the successful execution of a synthetic biology design. It is also necessary to have tools that guide the design and assembly process. As an example, *in silico*

technologies that allow prediction of essential cellular pathways [70,71] can be used to assemble synthetic metabolomes using standardized parts.

There are several challenges that complicate the use of a synthetic biology framework for the widespread design and assembly of biological systems. One challenge is the degree to which an abstraction hierarchy can be successfully constructed. Abstraction involves the suppression of details in favor of generalized descriptions. The abstraction hierarchy is useful for understanding the core function, yet it may not sufficiently capture the context dependence that is a hallmark of biology. In other words, the device may operate differently in one particular organism, or chassis, than in another. One proposed solution to this problem is to create a streamlined chassis with minimal functions required for its survival. Such an organism would presumably behave more predictably because the potential for unidentified interactions would be reduced. An additional challenge is the ability to accurately model biological parts, both as discrete entities and as components of higher-order devices. This challenge is partly confounded again by the context dependence of biology, which can complicate the translation of experimental measurements into reliable models that are applicable to various host backgrounds.

Although the concept of synthetic biology is relatively new, the development and characterization of individual parts continues to increase, which is a necessity to build a sufficient parts catalog from which devices can be designed and assembled. Today, synthetic biology frameworks have enabled the engineering of biological components that were previously limited by constraints of 'nature.' Despite the fact that a living chassis generated by using only standardized components has yet to be created, advances in the underlying science and engineering are progressing towards this goal. In the future, a combination of synthetic biology platforms with current metabolic and cellular engineering tools [72–77] is expected to give rise to a new generation of microbes that function as highly robust and programmable biological machines.

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