Synthetic biology: applications come of age

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Abstract | Synthetic biology is bringing together engineers and biologists to design and build novel biomolecular components, networks and pathways, and to use these constructs to rewire and reprogram organisms. These re-engineered organisms will change our lives over the coming years, leading to cheaper drugs, 'green' means to fuel our cars and targeted therapies for attacking 'superbugs' and diseases, such as cancer. The *de novo* engineering of genetic circuits, biological modules and synthetic pathways is beginning to address these crucial problems and is being used in related practical applications.

Memory elements
Devices used to store information about the current state of a system.

Pulse generators
Circuits or devices used to generate pulses. A biological pulse generator has been implemented in a multicellular bacterial system, in which receiver cells respond to a chemical signal with a transient burst of gene expression, the amplitude and duration of which depends on the distance from the sender cells.

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The circuit-like connectivity of biological parts and their ability to collectively process logical operations was first appreciated nearly 50 years ago[1]. This inspired attempts to describe biological regulation schemes with mathematical models[2–5] and to apply electrical circuit analogies to biological pathways[6,7]. Meanwhile, breakthroughs in genomic research and genetic engineering (for example, recombinant DNA technology) were supplying the inventory and methods necessary to physically construct and assemble biomolecular parts. As a result, synthetic biology was born with the broad goal of engineering or 'wiring' biological circuitry — be it genetic, protein, viral, pathway or genomic — for manifesting logical forms of cellular control. Synthetic biology, equipped with the engineering-driven approaches of modularization, rationalization and modelling, has progressed rapidly and generated an ever-increasing suite of genetic devices and biological modules.

The successful design and construction of the first synthetic gene networks — the genetic toggle switch[8] and the repressilator[9] (BOX 1) — showed that engineering-based methodology could indeed be used to build sophisticated, computing-like behaviour into biological systems. In these two cases, basic transcriptional regulatory elements were designed and assembled to realize the biological equivalents of electronic memory storage and timekeeping (BOX 1). Within the framework provided by these two synthetic systems, biological circuits can be built from smaller, well-defined parts according to model blueprints. They can then be studied and tested in isolation, and their behaviour can be evaluated against model predictions of the system dynamics. This methodology has been applied to the synthetic construction of additional genetic switches[8,10–18],

memory elements[8,14,15,19] and oscillators[9,10,20–23], as well as to other electronics-inspired genetic devices, including pulse generators[24], digital logic gates[25–30], filters[31–33] and communication modules[23,31,34,35].

Now, 10 years after the demonstration of synthetic biology's inaugural devices[8,9], engineered biomolecular networks are beginning to move into the application stage and yield solutions to many complex societal problems. Although work remains to be done on elucidating biological design principles[36], this foray into practical applications signals an exciting coming-of-age time for the field.

Here, we review the practical applications of synthetic biology in biosensing, therapeutics and the production of biofuels, pharmaceuticals and novel biomaterials. Many of the examples herein do not fit exclusively or neatly into only one of these three application categories; however, it is precisely this multivalent applicability that makes synthetic biology platforms so powerful and promising.

Biosensing
Cells have evolved a myriad of regulatory circuits — from transcriptional to post-translational — for sensing and responding to diverse and transient environmental signals. These circuits consist of exquisitely tailored sensitive elements that bind analytes and set signal-detection thresholds, and transducer modules that filter the signals and mobilize a cellular response (BOX 2). The two basic sensing modules must be delicately balanced: this is achieved by programming modularity and specificity into biosensing circuits at the transcriptional, translational and post-translational levels, as described below.

Transcriptional biosensing. As the first dedicated phase of gene expression, transcription serves as one method by which cells mobilize a cellular response to an environmental perturbation. As such, the genes to be expressed, their promoters, RNA polymerase, transcription factors and other parts of the transcription machinery all serve as potential engineering components for transcriptional biosensors. Most synthetic designs have focused on the promoters and their associated transcription factors, given the abundance of known and characterized bacterial, archaeal and eukaryotic environment-responsive promoters, which include the well-known promoters of the *Escherichia coli lac*, *tet* and *ara* operons.

Both the sensory and transducer behaviours of a biosensor can be placed under synthetic control by directly engineering environment-responsive promoter sequences.

In fact, this was the early design strategy adopted for establishing inducible expression systems[37–40]. By introducing, removing or modifying activator and repressor sites, a promoter's sensitivity to a molecule can be tuned. Synthetic mammalian transactivation systems are generic versions of this strategy in which an environmentally sensitive transcription factor is fused to a mammalian transactivation domain to cause inducer-dependent changes in gene expression. Synthetic mammalian biosensors based on this scheme have been created for sensing signals such as antibiotics[41–43], quorum-sensing molecules[44,45], gases and metabolites[46–49], and temperature changes[50,51]. Fussenegger and colleagues have even incorporated this transgene design into mammalian circuits, creating synthetic networks that are responsive to electrical signals[52].

Box 1 | **Early synthetic biology designs: switches and oscillators**
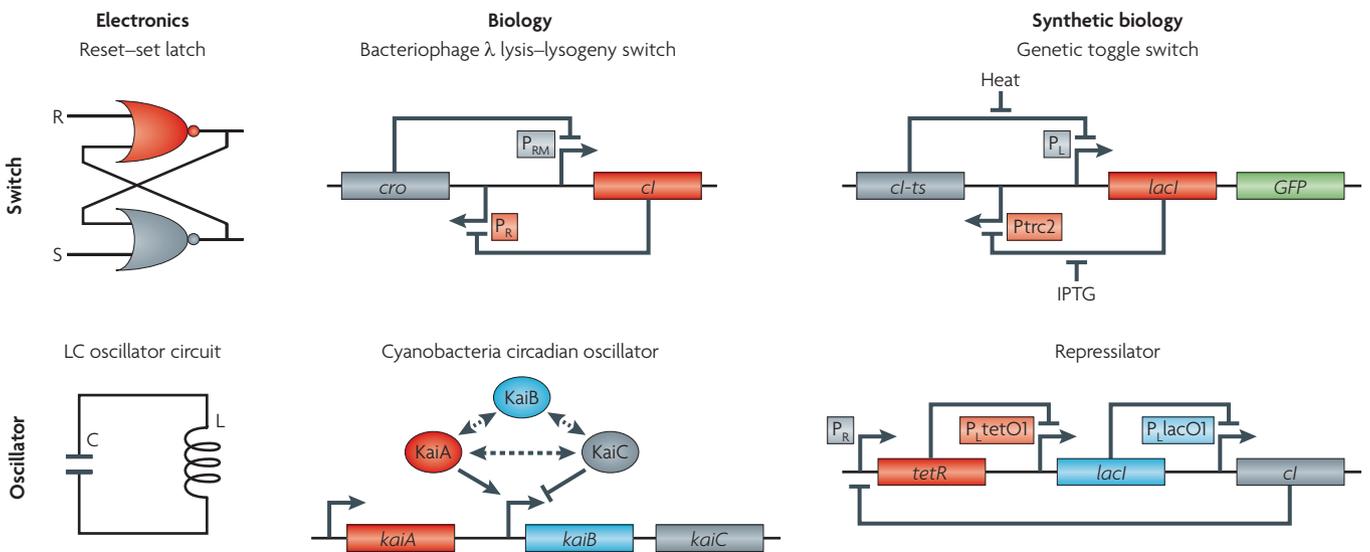
Switches and oscillators that occur in electronic systems are also seen in biology and have been engineered into synthetic biological systems.

Switches

In electronics, one of the most basic elements for storing memory is the reset–set (RS) latch based on logical NOR gates. This device is bistable in that it possesses two stable states that can be toggled with the delivery of specified inputs. Upon removal of the input, the circuit retains memory of its current state indefinitely. These forms of memory and state switching have important functions in biology, such as in the differentiation of cells from an initially undifferentiated state. One means by which cellular systems can achieve bistability is through genetic mutual repression. The natural P_R–P_{RM} genetic switch from bacteriophage λ, which uses this network architecture to govern the lysis–lysogeny decision, consists of two promoters that are each repressed by the gene product of the other (that is, by the Cro and CI repressor proteins). The genetic toggle switch[8] constructed by our research group is a synthetically engineered version of this co-repressed gene regulation scheme. In one version of the genetic toggle, the P_L promoter from λ phage was used to drive transcription of *lacI*, the product of which represses a second promoter, Ptrc2 (a *lac* promoter variant). Conversely, Ptrc2 drives expression of a gene (*cI-ts*) encoding the temperature-sensitive (ts) λ CI repressor protein, which inhibits the P_L promoter. The activity of the circuit is monitored through the expression of a GFP promoter.

The system can be toggled in one direction with the exogenous addition of the chemical inducer isopropyl-β-D-thiogalactopyranoside (IPTG) or in the other direction with a transient increase in temperature. Importantly, upon removal of these exogenous signals, the system retains its current state, creating a cellular form of memory.

Oscillators

Timing mechanisms, much like memory, are fundamental to many electronic and biological systems. Electronic timekeeping can be achieved with basic oscillator circuits — such as the LC circuit (inductor L and capacitor C) — which act as resonators for producing periodic electronic signals. Biological timekeeping, which is widespread among living organisms[120], is achieved with circadian clocks and similar oscillator circuits, such as the one responsible for synchronizing the crucial processes of photosynthesis and nitrogen fixation in cyanobacteria. The circadian clock of cyanobacteria is based on, among other regulatory mechanisms, intertwined positive and negative feedback loops on the clock genes *kaiA*, *kaiB* and *kaiC*. Elowitz and Leibler constructed a synthetic genetic oscillator based not on clock genes but on standard transcriptional repressors (the repressilator)[9]. Here, a cyclic negative feedback loop composed of three promoter–gene pairs, in which the 'first' promoter in the cascade drives expression of the 'second' promoter's repressor, and so on, was used to drive oscillatory output in gene expression.

Although the engineering of environment-responsive promoters has been valuable, additional control over modularity and specificity can be achieved by embedding environment-responsive promoters in engineered gene networks. Achieving true modularity with genetic parts is inherently difficult because of unintended interference among native and synthetic parts and therefore requires careful decoupling of functional modules. One such modular design strategy was used by Kobayashi *et al.*[34] to develop whole-cell *E. coli* biosensors that respond to signals in a programmable fashion. In this design, a sensory module (that is, an environment-responsive promoter and associated transcription factor) was coupled to an engineered gene circuit that functions like a central processing unit. *E. coli* cells were programmed to respond to a deleterious endogenous input — specifically, DNA-damaging stimuli, such as ultraviolet radiation or mitomycin C. The gene circuit, which was chosen to be the toggle switch (BOX 1), processes the incoming sensory information and flips from an 'OFF' to an 'ON' state when a signal threshold is exceeded. Because the biosensor has a decoupled, modular nature, it can be wired to any desired output, from the expression of a standard fluorescent reporter to the activation of natural phenotypes, such as biofilm formation (for example, through expression of *traA*) or cell suicide (for example, through expression of *ccdB*).

Sometimes a single signal may be too general to characterize or define an environment. For such situations, Anderson *et al.*[25] devised a transcriptional AND gate that could be used to integrate multiple environmental signals into a single genetic circuit (BOX 2), therefore programming the desired level of biosensing specificity. Genetic biosensors of this sort could be useful for communicating the state of a specific microenvironment (for example, in an industrial bioreactor) within a 'sea' of environmental conditions, such as temperature, metabolite levels or cell density.

Box 2 | Synthetic biosensors: transcriptional and translational architectures and examples
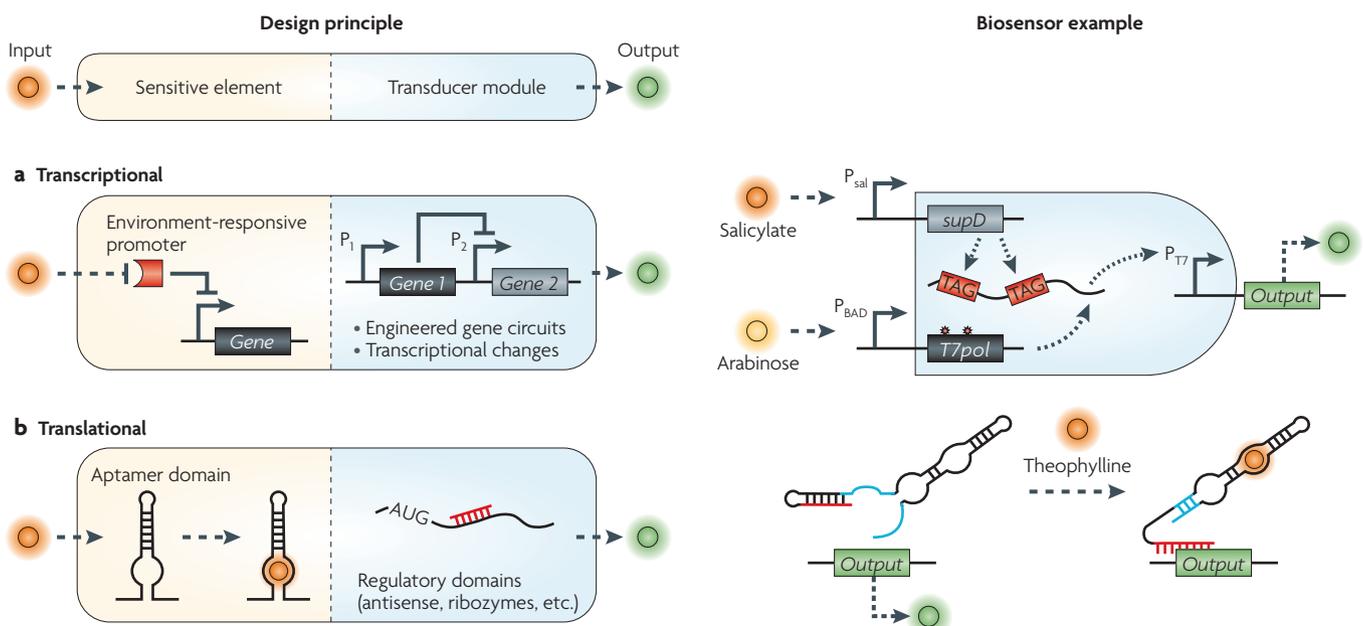
Biosensors consist of two basic modules (see the figure): sensitive elements for recognizing and binding analytes, and transducer modules for transmitting and reporting signals.

Transcriptional

Transcriptional biosensors (part **a**) are built by linking environment-responsive promoters to engineered gene circuits for programmed transcriptional changes. In the example shown, a transcriptional AND gate was designed to sense and report only the simultaneous presence of two environmental signals (for example, salicylate and arabinose)[25]. At one gate input, the researchers encoded an environment-responsive promoter (for example, P_{BAD}) that activates transcription of a T7 RNA polymerase gene in response to a single environmental signal (for example, arabinose). The gene, however, carries internally encoded amber stop codons (red spiked circles) that function to block translation of its transcript. Activation of the second gate input is the key to unlocking translation; specifically, translation can be induced when a second promoter (for example, P_{sal}) activates transcription of the *supD* amber suppressor tRNA in response to a second unique signal (for example, salicylate). In other words, only when the two environmental signals are simultaneously present can the T7 RNA polymerase be faithfully expressed and used to activate an output T7 promoter. This is an example of how sophisticated specificity can be programmed into a transducer module by creatively linking the sensory information of multiple sensitive elements. Furthermore, the design is transcriptionally modular in that different sets of environment-responsive promoters can be interfaced to the AND gate.

Translational

Translational biosensors (part **b**) are typically built by linking RNA aptamer domains to RNA regulatory domains. The example shown is an OFF 'antiswitch'. Here, the small molecule theophylline is recognized and bound by the aptamer stem of the RNA biosensor. This causes a conformational change in the molecule that liberates the antisense domain from its sequestering stem loop and allows it to inhibit translation of an output reporter[11].

Translational biosensing. RNA molecules have a diverse and important set of cellular functions[53]. Non-coding RNAs can splice and edit RNA, modify ribosomal RNA, catalyse biochemical reactions and regulate gene expression at the level of transcription or translation[53–56]. The regulatory subset of non-coding RNAs[57–59] is well-suited for rational design[60] and, in particular, for biosensing applications. Many regulatory RNA molecules are natural environmental sensors[61–69], and because of their ability to take on complex structures defined by their sequence, these molecules can mediate diverse modular functions across distinct sequence domains. Riboswitches[70], for instance, bind specific small-molecule ligands through aptamer domains and induce conformational changes in the 5′ UTR of their own mRNA, thereby regulating gene expression. Aptamer domains that are modelled after riboswitches are versatile and widely used sensitive elements for RNA-based biosensing. The choice and number of aptamer domains can provide control over specificity. Building an entire RNA-based biosensor typically requires coupling an aptamer domain (the sensitive element) with a post-transcriptional regulatory domain (the transducer module) on a modular RNA molecule scaffold.

Antisense RNAs[59,71] are one such class of natural regulatory RNAs that can control gene expression through post-transcriptional mechanisms. By linking a riboswitch aptamer to an antisense repressor on a single RNA molecule, Bayer and Smolke[11] engineered *trans*-acting, ligand-responsive riboregulators of gene expression in *Saccharomyces cerevisiae* (BOX 2). Binding of the aptamer to its ligand (for example, the small molecule theophylline) induces a conformational change in the RNA sensor that either sequesters the antisense domain in a stable stem loop (ON switch) or liberates it to inhibit translation of an output gene reporter (OFF switch). As a result of the cooperative dependence on both ligand and target mRNA, this biosensor shows binary-like switching at a threshold ligand concentration, similar to the genetic toggle design. Importantly, this detection threshold can be adjusted by altering the RNA sequence and therefore the thermodynamic properties of the structure. In principle, the 'antiswitch' framework is modular; in other words, aptamers for different ligands and antisense stems targeting different downstream genes could be incorporated into the scaffold to create new sensors. In practice, developing new sensors by aptamer and antisense replacement often involves re-screening compatible secondary structures to create functioning switches. In the future, this platform could be combined with rapid, *in vitro* aptameric selection techniques[72–75] for generating a suite of RNA biosensors that report on the levels of various mRNA species and metabolites in a cell. However, here it should also be noted that aptamers show specificity for a biased ligand space, and as a result aptamers for a target ligand cannot always be found.

Another method for transducing the sensory information captured by aptamer domains is to regulate translation through RNA self-cleavage[69,70]. RNA cleavage is catalysed by ribozymes, some of which naturally possess aptameric domains and are responsive to

metabolites[69]. Yen et al.[76] took advantage of this natural framework and encoded ligand-sensitive ribozymes in the mRNA sequences of reporter genes. In the absence of its cognate ligand, constitutive autocleavage of the reporter mRNA resulted in little or no signal. The RNA biosensor is flipped when the cognate ligand is present to inhibit the ribozyme's activity. Similar to the 'antiswitch' framework (and with the same technical challenges), these engineered RNAs could potentially be used as endogenous sensors for reporting on a variety of intracellular species and metabolites.

Post-translational biosensing. Signal transduction pathways show vast diversity and complexity. Factors such as the nature of the molecular interactions, the number of interconnected proteins in a cascade and the use of spatial mechanisms dictate which signals are transmitted, whether a signal is amplified or attenuated and the dynamics of the response. Despite the multitude of factors and interacting components, signal transduction pathways are essentially hierarchical schemes based on sensitive elements and downstream transducer modules, and as such can be rationalized for engineering protein-based biosensors.

The primary sensitive element for most signal transduction pathways is the protein receptor. Whereas environment-responsive promoters and RNA aptamers are typically identified from nature or selected with high-throughput combinatorial methods, protein receptors can be designed *de novo* at the level of molecular interactions. For instance, Looger et al.[77] devised a computational method for redesigning natural protein receptors to bind new target ligands. Starting with a 'basis' of five proteins from the *E. coli* periplasmic binding protein (PBP) superfamily, the researchers replaced each of the wild-type ligands with a new, non-native target ligand and then used an algorithm to combinatorially explore all binding-pocket-residue mutations and ligand-docking configurations. This procedure was used to predict novel receptors for trinitrotoluene (TNT; a carcinogen and explosive), L-lactate (a medically-important metabolite) and serotonin (a chemical associated with psychiatric conditions). The predicted receptor designs were experimentally confirmed to be strong and specific *in vitro* sensors, as well as *in vivo* cell-based biosensors.

Protein receptors, such as the ones discussed above, are typically membrane-bound; they trigger protein signalling cascades that ultimately result in a cellular response. However, several synthetic methods can be used to transmit captured sensory information in a tunable and desirable manner. Skerker et al.[78] rationally rewired the transmission of information through two-component systems by identifying rules governing the specificity of a histidine kinase to its cognate response regulator. Alternatively, engineered protein scaffolds can be designed to physically recruit pathway modulators and synthetically reshape the dynamical response behaviour of a system[79] (BOX 3). This constitutes a modular method for programming protein-based biosensors to have any desired response, including accelerated, delayed or ultrasensitive responses, to upstream signals.

Hybrid approaches. Combining synthetic transcriptional, translational and post-translational circuits into hybrid solutions and harnessing desired characteristics from each could lead to the creation of cell-based biosensors that are as robust as those of natural organisms. Using a synthetic hybrid approach, Voigt and colleagues[80–82] developed *E. coli*-based optical sensors. A synthetic sensor kinase was engineered to allow cells to identify and report the presence of red light. As a result, a bacterial lawn of the engineered cells could faithfully 'print' a projected image in the biological equivalent of photographic film. Specifically, a membrane-bound photoreceptor from cyanobacteria was fused to an *E. coli* intracellular histidine kinase to induce light-dependent changes in gene expression[80] (BOX 3). In a clever example of its use, the bacterial optical sensor was applied in image edge detection[82]. In this case, by wiring the optical sensor to transcriptional circuits that perform cell–cell communication (the quorum-sensing system from *Vibrio fischeri*) and logical functions (BOX 3), the researchers programmed only the cells that receive light and directly neighbour cells that do not receive light to produce a pigment, allowing the edges of a projected image to be traced. This work demonstrates that complex behaviour can emerge from properly wiring together smaller genetic programs, and that these programs can lead to unique real-world applications.

Therapeutics

Human health is afflicted by new and old foes, including emergent drug-resistant microbes, cancer and obesity. Meanwhile, progress in medicine is faced with challenges at each stage of the therapeutic spectrum, ranging from the drying up of pharmaceutical pipelines to limited global access to viable medicines. In a relatively short amount of time, synthetic biology has made promising strides in reshaping and streamlining this spectrum (BOX 4). Indeed, the rational and model-guided construction of biological

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Box 3 | Synthetic biosensors: post-translational and hybrid architectures and examples
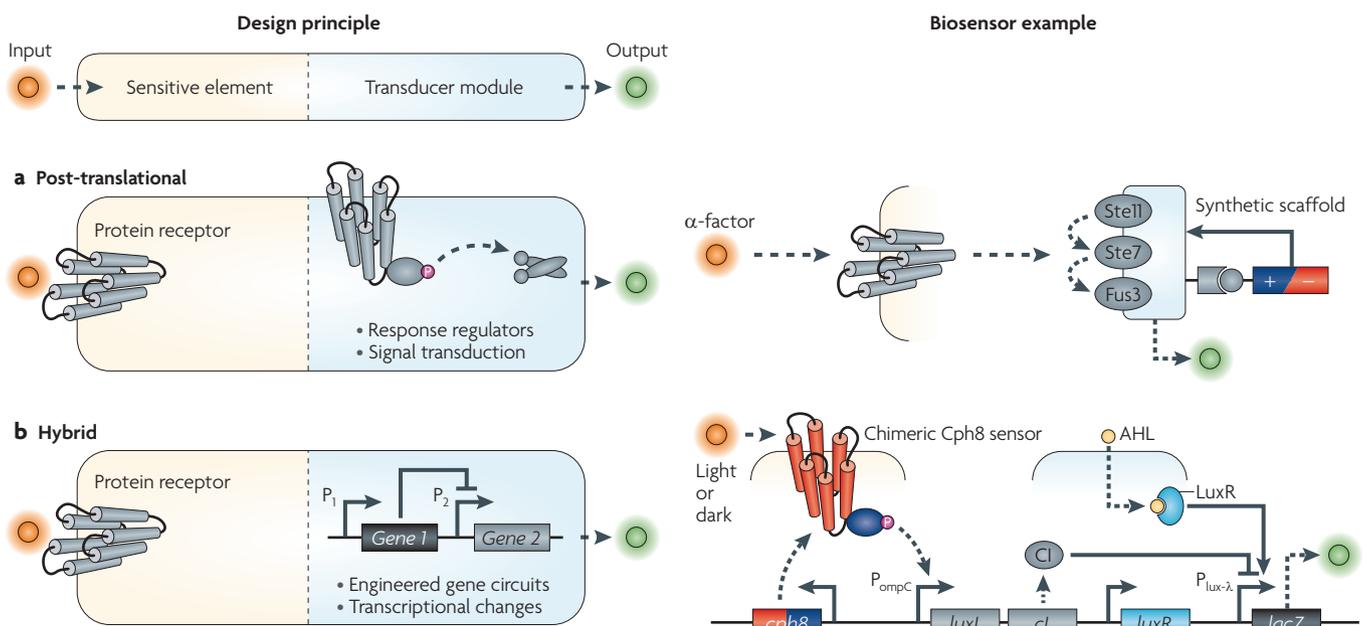
A basic biosensor has two modules (see the figure): the sensitive element recognizes and binds analytes, whereas the transducer module transmits and reports signals.

Post-translational

Post-translational biosensors (part **a**) consist of membrane-bound protein receptors that trigger signal transduction cascades through signalling proteins, such as response regulators of two-component systems. In the example shown, a synthetic protein scaffold was engineered to physically localize the pathway components of the yeast mitogen-activated protein kinase (MAPK) pathway, which here is being triggered by the mating α-factor[79]. By recruiting pathway positive and negative modulators (±) to the scaffold, the system can be tuned to enable desired responses to upstream signals (for example, accelerated, delayed or ultrasensitive responses).

Hybrid

The hybrid example (part **b**) shows a synthetic genetic edge detection circuit[82]. The sensitive element is a light–dark sensor, Cph8, made as a chimaera of the photoreceptor domain of the cyanobacteria phytochrome Cph1 and the kinase domain of *Escherichia coli* EnvZ. This synthetic sensor activates an engineered gene circuit that combines cell–cell communication (genes and promoters of the Lux operon) with a logical AND gate ($P_{lux-\lambda}$) to trace the edges of an image. Specifically, the absence of light triggers Cph8 kinase activity, which correspondingly activates the *ompC* promoter. Cells not receiving light will therefore produce the cell–cell communication molecule 3-oxohexanoyl-homoserine lactone (AHL; yellow circle) through expression of its biosynthetic enzyme LuxI. In addition, these cells will produce the transcriptional repressor CI (grey oval). AHL binds to the constitutively expressed transcription factor LuxR (light blue oval) to activate expression from the $P_{lux-\lambda}$ promoter, which is simultaneously and dominantly repressed by CI. The result is that only cells that receive light (and therefore do not express the transcriptional repressor CI) and are nearby to AHL-producing dark cells will activate the final gate and produce pigment through β-galactosidase activity (encoded by *lacZ*).

Box 4 | **The impact of synthetic biology on the therapeutic spectrum**
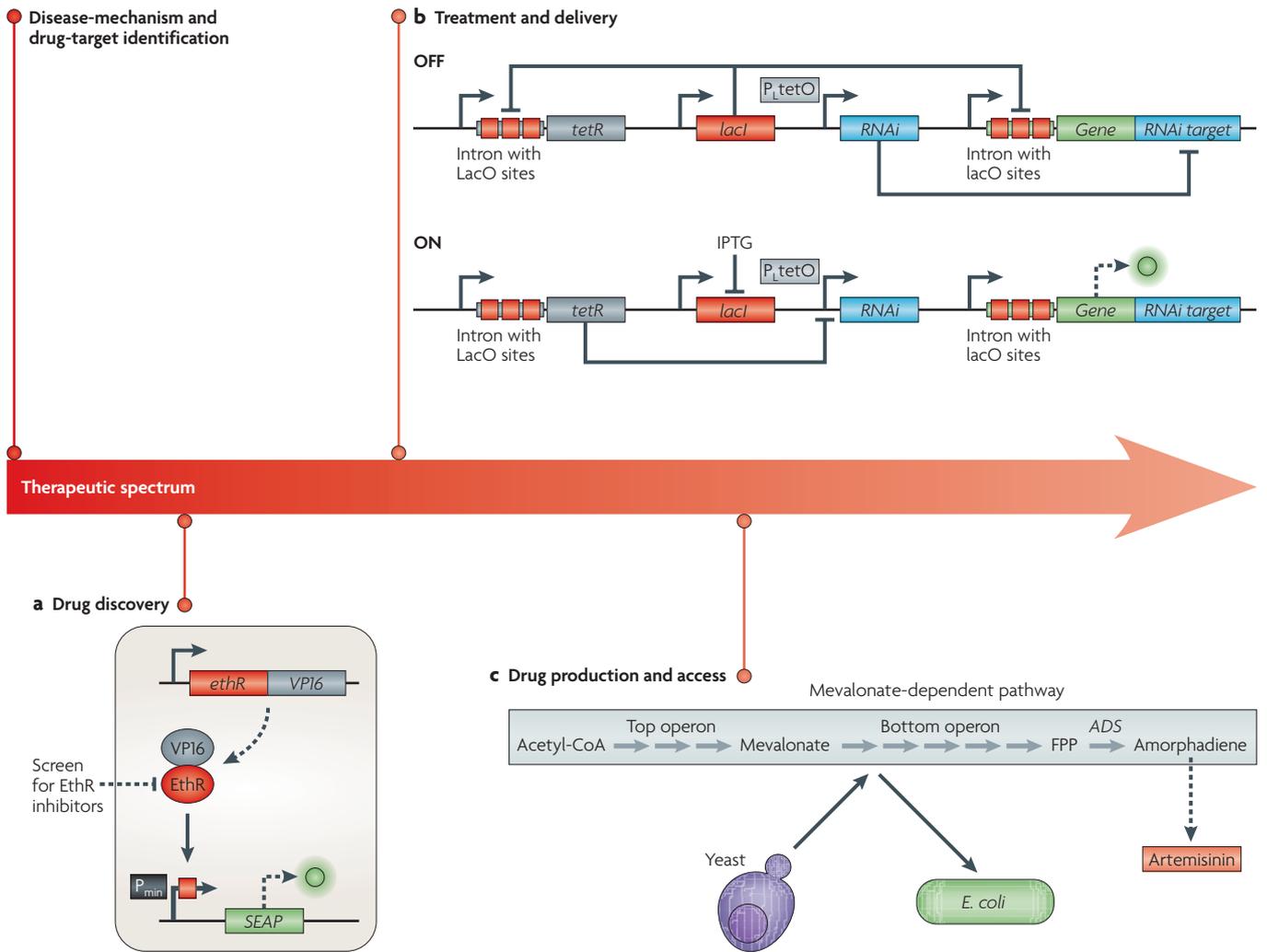
Drug discovery

Part **a** of the figure shows a synthetic mammalian gene circuit that enabled drug discovery for antituberculosis compounds[90]. The antibiotic ethionamide is rendered cytotoxic to *Mycobacterium tuberculosis* by the enzyme EthA in infected cells. Because EthA is natively repressed by EthR, resistance to ethionamide treatment is common. In the gene circuit, a fusion of EthR and the mammalian transactivator VP16 binds a minimal promoter (P_{min}) with a synthetic EthR operator site and activates expression of the reporter gene *SEAP* (human placental secreted alkaline phosphatase). This platform allows for the rapid screening of EthR inhibitors in mammalian cells.

Treatment and delivery

Part **b** shows a synthetic mammalian genetic switch for tight, tunable and reversible control of a desired gene for therapeutic or gene-delivery applications. In the OFF configuration (upper panel), expression of the gene of interest (green) is repressed at the levels of both transcription and translation. Constitutively expressed LacI repressor (red) binds to the *lac* operator sites in the transgene module of the gene of interest, therefore repressing its transcription. Any transcriptional leakage is repressed at the level of translation by an interfering RNA (blue), which targets the gene's 3′ UTR. The system is switched ON (lower panel) by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), which binds LacI repressor proteins and consequently relieves both forms of repression.

Drug production and access

The discovery of drugs does not always translate to the people who need

them the most because drug production processes can be difficult and costly. Antibiotics are industrially produced from microbes and fungi, and are therefore widespread and cheap. Conversely, many other drugs are isolated from hosts that are not as amenable to large-scale production and are therefore costly and in short supply. Such drugs include the antimalaria drug artemisinin and the anticancer drug taxol. Fortunately, global access to drugs is being enabled by hybrid synthetic biology and metabolic engineering strategies for the microbial production of rare natural products. In the case of artemisinin (part **c**), there exist two biosynthetic pathways for the synthesis of the universal precursors to all isoprenoids, the large and diverse family of natural products of which artemisinin is a member. The native isoprenoid pathway found in *Escherichia coli* (the deoxyxylulose 5-phosphate (DXP) pathway) has been difficult to optimize, so instead researchers have synthetically constructed and tested the entire *Saccharomyces cerevisiae* mevalonate-dependent (MEV) pathway in *E. coli* in a piece-wise fashion (for example, by separating the 'top' and 'bottom' operons). The researchers initially used *E. coli* as a simple, orthogonal host platform to construct, debug and optimize the large metabolic pathway[105]. They then linked the optimized heterologous pathway to a codon-optimized form of the plant terpene synthase *ADS* to funnel metabolic production to the specific terpene precursor to artemisinin. This work allowed them to build a full, optimized solution that could be ultimately and seamlessly deployed back into *S. cerevisiae* for cost-effective synthesis and purification of industrial quantities of the immediate drug precursor of artemisinin[106]. FPP, farnesyl pyrophosphate.

parts is enabling new therapeutic platforms, from the identification of disease mechanisms and drug targets to the production and delivery of small molecules.

Disease mechanism. An electrical engineer is likely to prototype portions of a circuit on a 'breadboard' before printing it as an entire integrated circuit. This allows for the rigorous testing of submodules in an isolated, well-characterized environment. Similarly, synthetic biology provides a framework for synthetically reconstructing natural biological systems to explore how pathological behaviours may emerge. This strategy was used to give mechanistic insights into a primary immunodeficiency, agammaglobulinaemia, in which patients cannot generate mature B cells and as a result are unable to properly fight infections[83]. The researchers developed a synthetic testbed by systematically reconstructing the various components of the human B cell antigen receptor (BCR) signalling pathway in an orthogonal environment. This allowed them to identify network topology features that trigger BCR signalling and assembly. A rare mutation in the immunoglobulin-β-encoding gene was identified in one patient and introduced into the synthetic system, in which it was shown to abolish assembly of the BCR on the cell surface, thereby linking this faulty pathway component with disease onset. Pathogenic viral genomes can similarly be reconstructed for studying the molecular underpinnings of infectious disease pandemics. For instance, synthetic reconstruction of the severe acquired respiratory syndrome (SARS) coronavirus[84] and the 1918 Spanish influenza virus[85] helped to identify genetic mutations that may have conferred human tropism and increased virulence.

Drug-target identification. Building up synthetic pathways and systems from individual parts is one way of identifying disease mechanisms and therapeutic targets. Another is to deploy synthetic biology devices to systematically probe the function of individual components of a natural pathway. Our group, for instance, has engineered modular riboregulators that can be used to tune the expression of a toxic protein or any gene in a biological network[86]. To achieve post-transcriptional control over a target gene, the mRNA sequence of the riboregulator 5′ UTR is designed to form a hairpin structure that sequesters the ribosomal binding site (RBS) and prevents ribosome access to it. Translational repression of this *cis*-repressed mRNA can be alleviated by an independently regulated transactivating RNA that targets the stem–loop for unfolding. Engineered riboregulators have been used to tightly regulate the expression of CcdB, a toxic bacterial protein that inhibits DNA gyrase, to gain a better understanding of the sequence of events leading to induced bacterial cell death[87]. These synthetic biology studies, in conjunction with systems biology studies of quinolones (antibiotics that inhibit gyrase)[87], led to the discovery that all major classes of bactericidal antibiotics induce a common cellular death pathway by stimulating oxidative damage[88,89]. This work provided new insights into how bacteria respond to lethal stimuli and paved the way for the development of more effective antibacterial therapies.

Drug discovery. After a faulty pathway component or target is identified, whole-cell screening assays can be designed using synthetic biology strategies for drug discovery. As a demonstration of this approach, Fussenegger and colleagues[90] developed a synthetic platform for screening small molecules that could potentiate a *Mycobacterium tuberculosis* antibiotic (BOX 4). Ethionamide, currently the last line of defence in the treatment of multidrug-resistant tuberculosis, depends on activation by the *M. tuberculosis* enzyme EthA for efficacy. However, due to transcriptional repression of *ethA* by the protein EthR, ethionamide-based therapy is often rendered ineffective. To address this problem, the researchers designed a synthetic mammalian gene circuit that featured an EthR-based transactivator of a reporter gene and used it to screen for and identify EthR inhibitors that could abrogate resistance to ethionamide. Importantly, because the system is a cell-based assay, it intrinsically enriches for inhibitors that are non-toxic and membrane-permeable to mammalian cells, which are key drug criteria as *M. tuberculosis* is an intracellular pathogen. This framework, in which drug discovery is applied to whole cells that have been engineered with circuits that highlight a pathogenic mechanism, could be extended to other diseases and phenotypes.

Therapeutic treatment. Synthetic biology devices have additionally been developed to serve as therapies themselves. Entire engineered viruses and organisms can be programmed to target specific pathogenic agents and pathological mechanisms. For instance, in two separate studies[91,92] researchers used engineered bacteriophages to combat antibiotic-resistant bacteria by endowing them with genetic mechanisms that target and thwart bacterial mechanisms for evading antibiotic action. The first study was prompted by the observation that biofilms, in which bacteria are encapsulated in an extracellular matrix, have inherent resistance to antimicrobial therapies and are sources of persistent infections. To more effectively penetrate this protective environment, T7 phage was engineered to express the biofilm matrix-degrading enzyme dispersin B (DspB) upon infection[91]. The two-pronged attack of T7 expressing DspB and phage-induced lysis fuelling the creation and spread of DspB resulted in the removal of 99.997% of the biofilm bacterial cells.

In the second study[92], it was suggested that inhibition of certain bacterial genetic programs could improve the effectiveness of current antibiotic therapies. In this case, bacteriophages were deliberately designed to be non-lethal so as not to elicit resistance mechanisms; instead, a non-lytic M13 phage was used to suppress the bacterial SOS DNA-damage response by overexpression of its repressor, lexA3. The engineered bacteriophage significantly enhanced killing by three major classes of antibiotics in traditional cell culture and in *E. coli*-infected mice, potentiated killing of antibiotic-resistant bacteria and, importantly, reduced the incidence of cells with antibiotic-induced resistance.

Orthogonal environment
A cellular environment or host into which genetic material is transplanted to avoid undesired native host interference or regulation. Orthogonal hosts are often organisms with sufficient evolutionary distance from the native host.

DNA gyrase
A type II DNA topoisomerase that catalyses the ATP-dependent supercoiling of closed-circular dsDNA by strand breakage and rejoining reactions. Control of chromosomal topological transitions is essential for DNA replication and transcription in bacteria, making gyrase an effective target for antimicrobial agents (for example, the quinolone class of antibiotics).

Biofilms
Surface-associated communities of bacterial cells encapsulated in an extracellular polymeric substances (EPS) matrix. Biofilms are an antibiotic-resistant mode of microbial life found in natural and industrial settings.

Synthetically engineered viruses and organisms that are able to sense and link their therapeutic activity to pathological cues may be useful in the treatment of cancer, in which current therapies often indiscriminately attack tumours and normal tissues. For instance, adenoviruses were programmed to couple their replication to the state of the p53 pathway in human cells[93]. Normal p53 production would result in inhibition of a crucial viral replication component, whereas a defunct p53 pathway, which is characteristic of tumour cells, would allow viral replication and cell killing. In another demonstration of translational synthetic biology applied to cancer therapy, Voigt and colleagues[94] developed cancer-targeting bacteria and linked their ability to invade the cancer cells to specific environmental signals. Constitutive expression of the heterologous invasin (*inv*) gene (from *Yersinia pseudotuberculosis*) can induce *E. coli* cells to invade both normal human cell lines and cancer cell lines. So, to preferentially invade cancer cells, the researchers placed *inv* under the control of transcriptional operons that are activated by environmental signals specific to the tumour microenvironment. These engineered bacteria could be made to carry or synthesize cancer therapies for the treatment of tumours.

Therapeutic delivery. In addition to engineered therapeutic organisms, synthetic circuits and pathways can be used for the controlled delivery of drugs as well as for gene and metabolic therapy. In some cases, sophisticated kinetic control over drug release in the body may yield therapeutic advantages and reduce undesired side effects. Most hormones in the body are released in time-dependent pulses. Glucocorticoid secretion, for instance, has a circadian and ultradian pattern of release, with important transcriptional consequences for glucocorticoid-responsive cells[95]. Faithfully mimicking these patterns in the administration of synthetic hormones to patients with glucocorticoid-responsive diseases, such as rheumatoid arthritis, may decrease known side effects and improve therapeutic response[95]. Periodic synthesis and release of biologic drugs can be autonomously achieved with synthetic oscillator circuits[9,10,20–22] or programmed time-delay circuits[96]. In other cases, one may wish to place a limit on the amount of drug released by programming the synthetic system to self-destruct after a defined number of cell cycles or drug release pulses. Our group has recently developed two variants of a synthetic gene counter[14] that could be adapted for such an application.

Gene therapy is beginning to make some promising advances in clinical areas in which traditional drug therapy is ineffective, such as in the treatment of many hereditary and metabolic diseases. Synthetic circuits offer a more controlled approach to gene therapy, such as the ability to dynamically silence, activate and tune the expression of desired genes. In one such example[12], a genetic switch was developed in mammalian cells that couples transcriptional repressor proteins and an RNAi module for tight, tunable and reversible control over the expression of desired genes (BOX 4). This system would be particularly useful in gene-silencing applications, as it was shown to yield >99% repression of a target gene.

Additionally, the construction of non-native pathways offers a unique and versatile approach to gene therapy, such as for the treatment of metabolic disorders. Operating at the interface of synthetic biology and metabolic engineering, Liao and colleagues[97] recently introduced the glyoxylate shunt pathway into mammalian liver cells and mice to explore its effects on fatty acid metabolism and, more broadly, on whole-body metabolism. Remarkably, the researchers found that when transplanted into mammals, the shunt actually increased fatty acid oxidation, evidently by creating an alternative cycle. Furthermore, mice expressing the shunt showed resistance to diet-induced obesity when placed on a high-fat diet, with corresponding decreases in total fat mass, plasma triglycerides and cholesterol levels. This work offers a new synthetic biology model for studying metabolic networks and disorders, and for developing treatments for the increasing problem of obesity.

Finally, the discovery of drugs and effective treatments may not quickly — or ever — translate to the people who need them the most because drug production processes can be difficult and costly. As discussed below, synthetic biology is allowing rare and costly drugs to be manufactured more cost-effectively (BOX 4).

Biofuels, pharmaceuticals and biomaterials

Recent excitement surrounding the production of biofuels, pharmaceuticals and biomaterials from engineered microorganisms is matched by the challenges that loom in bringing these technologies to production scale and quality. The most widely used biofuel is ethanol produced from corn or sugar cane[98]; however, the heavy agricultural burden combined with the suboptimal fuel properties of ethanol make this approach to biofuels problematic and limited. Microorganisms engineered with optimized biosynthetic pathways to efficiently convert biomass into biofuels are an alternative and promising source of renewable energy. These strategies will succeed only if their production costs can be made to compete with, or even outcompete, current fuel production costs. Similarly, there are many drugs for which expensive production processes preclude their capacity for a wider therapeutic reach. New synthetic biology tools would also greatly advance the microbial production of biomaterials and the development of novel materials.

Constructing biosynthetic pathways. When engineering for biofuels, drugs or biomaterials, two of the first design decisions are choosing which biosynthetic pathway or pathways to focus on and which host organism to use. Typically, these decisions begin with the search for organisms that are innately capable of achieving some desired biosynthetic activity or phenotype[99]. For biofuel production, for instance, certain microorganisms have evolved to be proficient in converting lignocellulosic material to ethanol, biobutanol and other biofuels. These native isolates possess unique catabolic activity, heightened tolerances for toxic materials and a host of enzymes designed to break down the lignocellulosic components. Unfortunately, these highly desired properties exist in pathways that are tightly regulated according to the host's evolved needs and


Ultradian
Periods or cycles that are repeated throughout a 24-hour circadian day.

Glyoxylate shunt pathway
A two-enzyme metabolic pathway unique to bacteria and plants that is activated when sugars are not readily available. This pathway diverts the tricarboxylic acid (TCA) cycle so that fatty acids are not completely oxidized and are instead converted into carbon energy sources.

Box 5 | Controlling metabolic flux: evolutionary strategies and rational design

Combinatorial/evolutionary

a Multiplex automated genome engineering

Rational/model-guided

b Promoter and RBS engineering
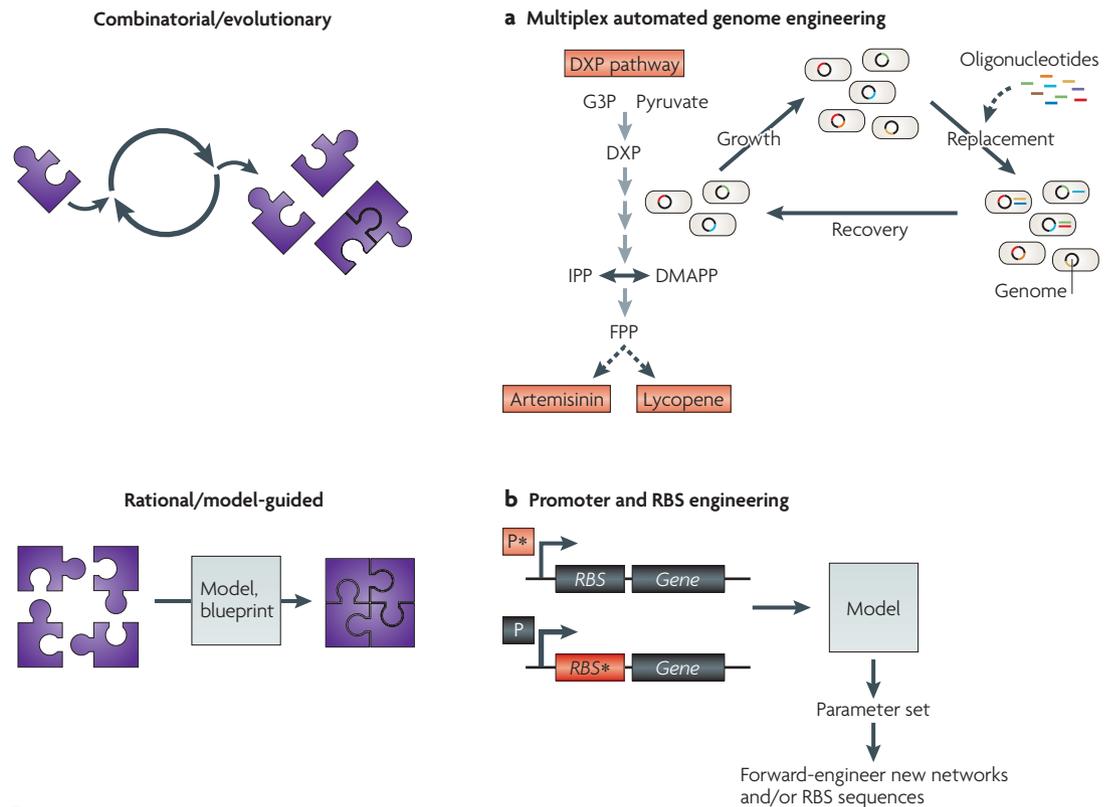
Evolutionary strategies

In the production of artemisinin precursors, the native *Escherichia coli* isoprenoid pathway (the deoxyxylulose 5-phosphate (DXP) pathway) was eschewed in favour of a heterologous pathway so as to circumvent the complex regulatory control imposed by the host (BOX 4). In an alternative method of relieving regulatory control over the large number of DXP pathway components, Wang *et al.*[121] diversified and, as a result, optimized the native DXP biosynthetic pathway in *E. coli* (see the figure, part **a**). The researchers developed a rapid, automated method for the *in vivo* directed evolution of pathways, which they termed multiplex automated genome engineering (MAGE). They then applied it to evolve the translational efficiencies of DXP pathway components to achieve maximal lycopene production. Specifically, cells were subjected to cycles of genetic modifications (through oligo-mediated allelic replacement) in an automated fashion to explore sufficient genomic diversity for optimizing biosynthetic pathways at laboratory timescales.

Rational design

At the other end of the spectrum are strategies that rely on quantitative models and blueprints for the rational design of optimized networks and pathways (part **b**). Typically, a component of interest (for example, an engineered promoter (P*) or ribosomal binding site (RBS*) sequence) will be built into a simple test network. The network and its input–output data will then be fed into a model, which attempts to determine a parameter set that optimally describes the component's dynamics within the framework of the model. Finally, the optimized parameter set will be used to forward-engineer new networks and components. For example, stochastic biochemical models have been developed to capture the expression dynamics of synthetically engineered promoters; these models were subsequently used to predict the correct *in vivo* behaviour of different and more complex gene networks built from the modelled components[122,123]. Similarly, at the level of translation, thermodynamic models that predict the relative translation initiation rates of proteins can be used to rationally forward-engineer synthetic RBS sequences to give desired expression levels[124]. Such techniques harness modelled genetic parameters (transcriptional or translational) to predict the level of expression of proteins and enzymes in a network. DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate.

therefore may not be suitable in their native state for production scale. A longstanding challenge in metabolic and genetic engineering is determining whether to improve the isolate host's production capacity or whether to transplant the desired genes or pathways into an industrial model host, such as *E. coli* or *S. cerevisiae*; these important considerations and trade-offs are reviewed elsewhere[99].

The example of the microbial production of biobutanol, a higher energy density alternative to ethanol, provides a useful glimpse into these design trade-offs. Butanol is converted naturally from acetyl-CoA by *Clostridium acetobutylicum*[100]. However, it is produced in low yields and as a mixture with acetone and ethanol, so substantial cellular engineering of a microorganism for which standard

Box 6 | Controlling metabolic flux: hybrid approaches
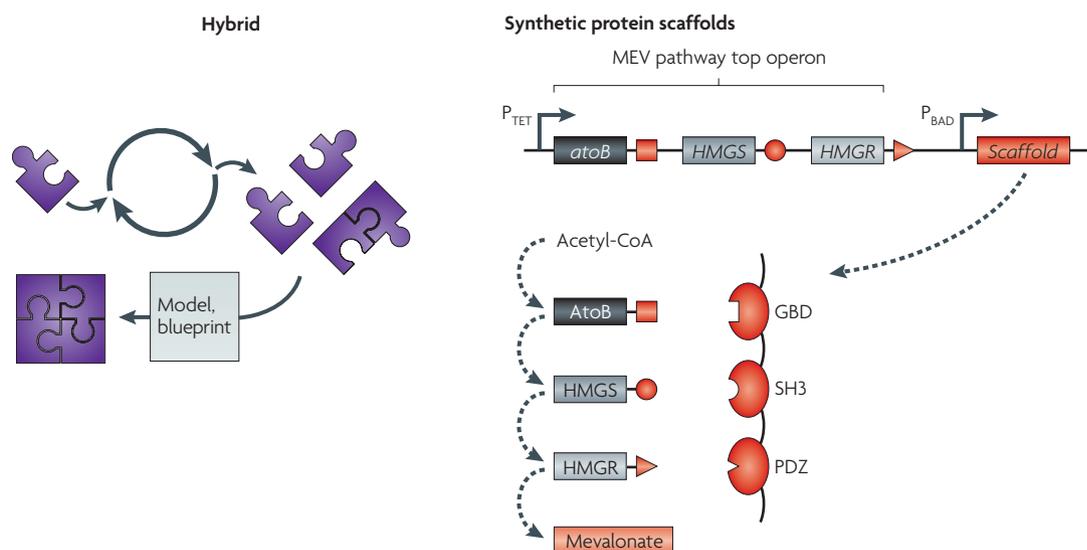
In a hybrid rational–combinatorial approach, Dueber *et al.*[125] suggested that metabolic flux could be controlled by spatially recruiting the enzymes of a desired biosynthetic pathway using synthetic protein scaffolds. To construct the enzyme scaffolding, the researchers tethered protein–protein interaction domains (for example, GBD, SH3 and PDZ domains) from metazoan signalling proteins. These domains recognize and bind cognate peptides that were fused to the enzymes to be recruited (acetoacetyl-CoA thiolase (AtoB) from *Escherichia coli* and HMG-CoA (HMBS) synthase and HMG-CoA reductase (HMGR) from *Saccharomyces cerevisiae*). By varying the number of repeats of an interaction domain, the researchers could additionally control the stoichiometry of the enzymes recruited to the complex. Using the heterologous mevalonate-dependent (MEV) pathway in *E. coli* as a model, they combinatorially (albeit, at a substantially smaller scale) optimized the stoichiometry of the three enzymes responsible for producing mevalonate from acetyl-CoA. Finally, they showed that the optimized synthetic scaffold could substantially increase product titre while reducing the metabolic load on the host; in other words, their high product titres did not require the overexpression of biosynthetic enzymes in the cell.

molecular biology techniques do not apply is needed to produce usable amounts of butanol[101,102]. Furthermore, importing the biosynthetic genes into an industrial microbial host can lead to metabolic imbalances[103]. In an altogether different approach, Liao and colleagues[104] bypassed standard fermentation pathways and recognized that a broad set of the 2-keto acid intermediates of *E. coli* amino acid biosynthesis could be synthetically shunted to achieve high-yield production of butanol and other higher alcohols in two enzymatic steps.

Indeed, complementary to efforts in traditional metabolic and genetic engineering is the use of engineering principles for constructing functional, predictable and non-native biological pathways *de novo* to control and improve microbial production. In an exemplary illustration of this, Keasling and colleagues engineered the microbial production of precursors to the antimalarial drug artemisinin to industrial levels[105,106] (BOX 4). There are now many such examples of the successful application of synthetic approaches to biosynthetic pathway construction — such approaches have been used in the microbial production of fatty-acid-derived fuels and chemicals (such as fatty esters, fatty alcohols and waxes)[107], methyl halide-derived fuels and chemicals[108], polyketide synthases that make cholesterol-lowering drugs[109], and polyketides made from megaenzymes that are encoded by very large synthetic gene clusters[110].

Optimizing pathway flux. After biosynthetic pathways have been constructed, the expression levels of all of the components need to be orchestrated to optimize metabolic flux and achieve high product titres. A standard approach is to drive the expression of pathway components with strong and exogenously tunable promoters, such as the $P_L$tet, $P_L$lac, and P_{BAD} promoters from the *tet*, *lac* and *ara* operons of *E. coli*, respectively. To this end, there are ongoing synthetic biology efforts to create and characterize more reusable, biological control elements based on promoters for predictably tuning expression levels[111,112]. Further to this, synthetic biologists have devised a number of alternative methods for obtaining biological pathway balance, ranging from reconfiguring network connectivity to fine-tuning individual components. A richer discussion of these topics, including the fine-tuning of parts, the application of model-guided approaches and the development of next-generation interoperable parts, is presented elsewhere[113]. In BOX 5 and BOX 6 we detail several synthetic biology strategies that specifically pertain to the optimization of metabolic pathway flux. These strategies range from those driven by evolutionary techniques, to those driven by rational design and *in silico* models, to those that combine both approaches.

Programming novel functionality and materials. Beyond facilitating metabolic tasks, synthetic systems

Metabolic flux
The rate of flow of metabolites through a metabolic pathway. The rate is regulated by the enzymes in the pathway.

> **Box 7 | Recommendations for improving the synthetic biology design cycle**
>
> - Scaling up to larger and more complex biological systems while simultaneously minimizing interference among parts will require an expanded synthetic biology toolkit and, in particular, libraries of interoperable parts. Eukaryotic systems are fertile grounds for discovering such parts, as many synthetic biology devices are based on a small repertoire of bacterial and archaeal regulatory elements.
> - Modelling and fine-tuning of synthetic networks should be emphasized, particularly as the network size and complexity increases. This will facilitate proper matching of input–output behaviours (that is, transfer functions) when distinct modules are connected.
> - There is a need to develop new probes and high-throughput methods for the *in vivo* measurement of circuit dynamics to rapidly characterize parts and debug networks.
> - Cellular testing platforms need to be developed to quicken the pace of identifying problematic network nodes and ease the failure-prone jumps associated with either building a more complex network or deploying a network in a more complex organism. These testing platforms could be cells engineered to have minimal genomes[126–129] or lower model organisms that have been equipped with specific machinery from higher organisms.

can infuse novel functionality into engineered organisms for production purposes or for building new materials. Early work in the field laid the groundwork for constructing basic circuits that could sense and process signals, perform logic operations and actuate biological responses[114]. Wiring these modules together to bring about reliable, higher-order functionality is one of the next major goals of synthetic biology[113], and an important application of this objective is the layering of 'smart' control mechanisms over metabolic engineering. For instance, circuits designed to sense the bioreactor environment and shift metabolic phases accordingly would further improve biofuel production. Alternatively, autonomous timing circuits could be used to shut down metabolic processes after a prescribed duration of time. Biological timers of this sort have been developed using genetic toggle switches that were deliberately rendered imbalanced through model-guided promoter engineering[112]. These genetic timers were used to program the time-dependent flocculation of yeast cells to facilitate the separation of cells from, for instance, the alcohol produced in industrial fermentation processes.

Synthetic control systems can also be used to extract and purify the synthesized product. This is particularly important in the production of recombinant proteins, bioplastics and other large biomaterials, which can accumulate inside cells, cause the formation of inclusion bodies and become toxic to cells if they are present at high titres. To export recombinant spider-silk monomers, Widmaier *et al.*[115] searched for a secretion system that would enable efficient and indiscriminate secretion of proteins through both bacterial membranes. The *Salmonella* type III secretion system (T3SS) not only fulfils these criteria but also possesses a natural regulatory scheme that ties expression of the protein to be secreted to the secretion capacity of the cell; as a result, the desired protein is only expressed when sufficient secretion complexes

have been built. To obtain superior secretion rates of recombinant silk protein, the researchers needed only to engineer a control circuit that hitches the heterologous silk-protein-producing genes to the innate genetic machinery for environmental sensing and secretion commitment.

Finally, there is an emerging branch of synthetic biology that seeks to program coordinated behaviour in populations of cells, which could lead to the fabrication of novel biomaterials for various applications. The engineering of synthetic multicellular systems is typically achieved with cell–cell communication and associated intracellular signal processing modules, as was elegantly used by Hasty and colleagues[23] to bring about synchronized oscillations in a population of bacterial cells. Weiss and colleagues[24,31] have similarly done pioneering work in building biomolecular signal-processing architectures that can filter communication signals originating from 'sender' cells. These systems, which can be programmed to form intricate multicellular patterns from a solid-phase cellular lawn, would aid the development of fabrication-free scaffolds for tissue engineering.

Future challenges and conclusions

The future of translational synthetic biology hinges on the development of reliable means for connecting smaller functional circuits to realize higher-order networks with predictable behaviours. In a previous article[113], we outlined four research efforts aimed at improving and accelerating the overall design cycle and allowing more seamless integration of biological circuitry (BOX 7).

Beyond the challenge of improving the design cycle, applied synthetic biology would benefit from once again summoning the original inspiration of biocomputing. The ability to program higher-level decision-making into synthetic networks would yield more robust and dynamic organisms, including ones that can accomplish many tasks simultaneously. Furthermore, as adaptive and predictive behaviours are naturally present in all organisms (including microbes)[116,117], synthetic learning networks built from genetic and biological parts[118,119] would infuse engineered organisms with more sophisticated automation for biosensing and related applications.

Finally, the majority of synthetic biology is currently practiced in microbes. However, many of the most pressing problems, and in particular those of human health, are inherently problems with mammalian systems. Therefore, a more concerted effort towards advancing mammalian synthetic biology will be crucial for next-generation therapeutic solutions, including the engineering of synthetic gene networks for stem-cell generation and differentiation.

By addressing such challenges, we will be limited not by the technicalities of construction or the robustness of synthetic gene networks but only by the imagination of researchers and the number of societal problems and applications that synthetic biology can resolve.

Flocculation
A specific form of cell aggregation in yeast triggered by certain environmental conditions, such as the absence of sugars. For example, flocculation occurs once the sugar in a beer brew has been fermented into ethanol.

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