

# Synthetic Biology—The Synthesis of Biology

Simon Ausländer, David Ausländer, and Martin Fussenegger\*

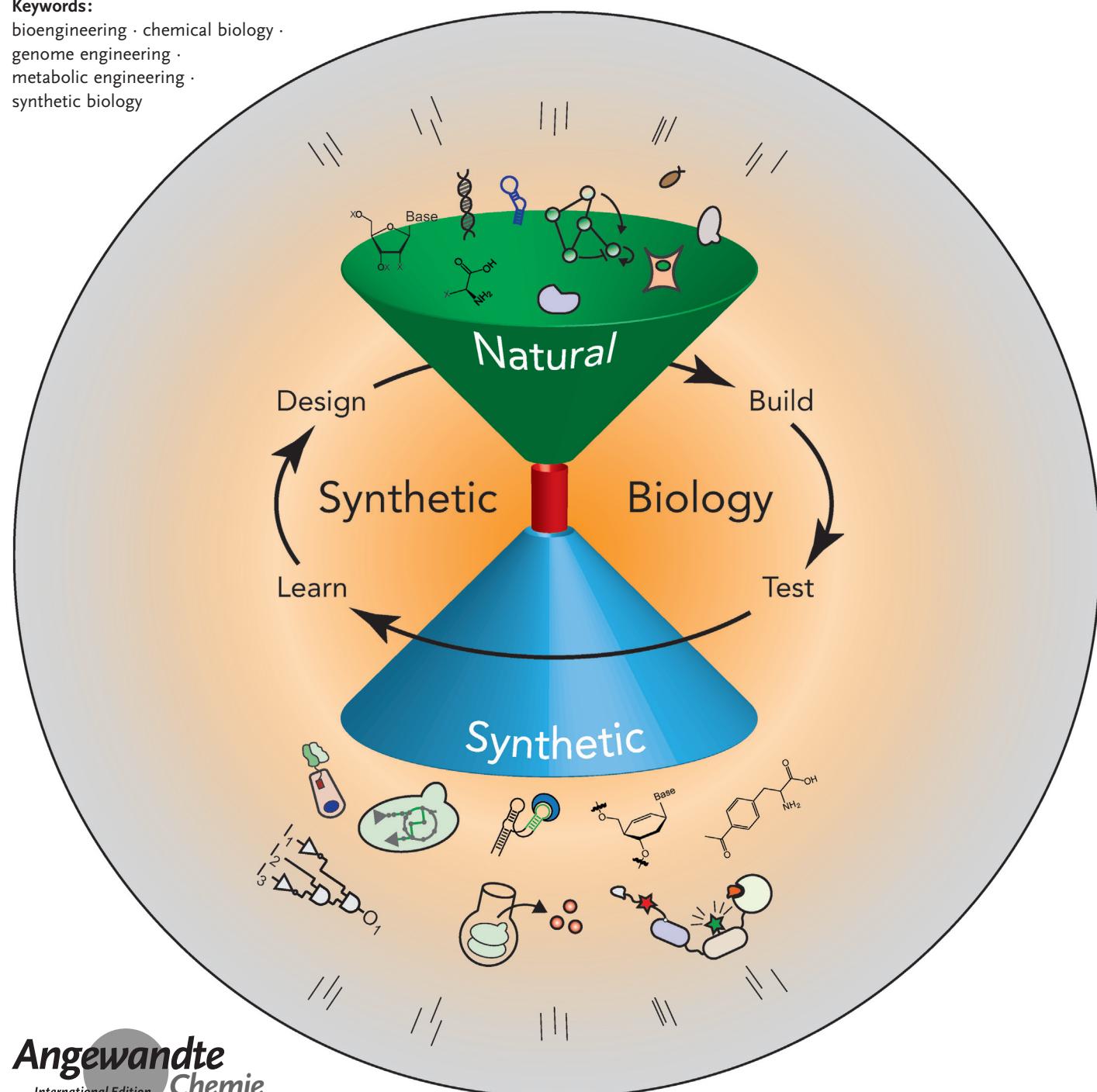
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**S**ynthetic biology concerns the engineering of man-made living biomachines from standardized components that can perform predefined functions in a (self-)controlled manner. Different research strategies and interdisciplinary efforts are pursued to implement engineering principles to biology. The “top-down” strategy exploits nature’s incredible diversity of existing, natural parts to construct synthetic compositions of genetic, metabolic, or signaling networks with predictable and controllable properties. This mainly application-driven approach results in living factories that produce drugs, biofuels, biomaterials, and fine chemicals, and results in living pills that are based on engineered cells with the capacity to autonomously detect and treat disease states *in vivo*. In contrast, the “bottom-up” strategy seeks to be independent of existing living systems by designing biological systems from scratch and synthesizing artificial biological entities not found in nature. This more knowledge-driven approach investigates the reconstruction of minimal biological systems that are capable of performing basic biological phenomena, such as self-organization, self-replication, and self-sustainability. Moreover, the syntheses of artificial biological units, such as synthetic nucleotides or amino acids, and their implementation into polymers inside living cells currently set the boundaries between natural and artificial biological systems. In particular, the *in vitro* design, synthesis, and transfer of complete genomes into host cells point to the future of synthetic biology: the creation of designer cells with tailored desirable properties for biomedicine and biotechnology.

## 1. Introduction

Engineering disciplines require standardized interchangeable parts that can be assembled into man-made systems in a modular and predictable fashion.<sup>[1]</sup> Synthetic biological systems are assembled from basic building blocks that are functional biological parts that code for gene switches,<sup>[2]</sup> genome engineering tools,<sup>[3]</sup> biocatalysts,<sup>[4]</sup> and protein switches<sup>[5]</sup> (Figure 1A). Although nature provides a plethora of parts, *in vitro* evolution techniques enable the generation of tailored versions of parts or even non-natural synthetic parts.<sup>[6]</sup> In addition, unnatural nucleotides<sup>[7]</sup> and amino acids<sup>[8]</sup> are chemically synthesized and implemented into functional parts, thereby expanding their chemical and structural space and offering new properties (Figure 1A). To maximize their actual potential, many parts rely on the functions of other parts or need to be fine-tuned, thereby resulting in interconnected synthetic systems with customized functions. Although networks were at first based on only a few parts and were assembled in a rational manner,<sup>[9]</sup> the complexity has drastically increased and is complemented by computer-assisted design strategies to construct large circuits with more than 50 parts.<sup>[10]</sup> Synthetic systems can be designed to function in cell-free environments or implemented into living cells,

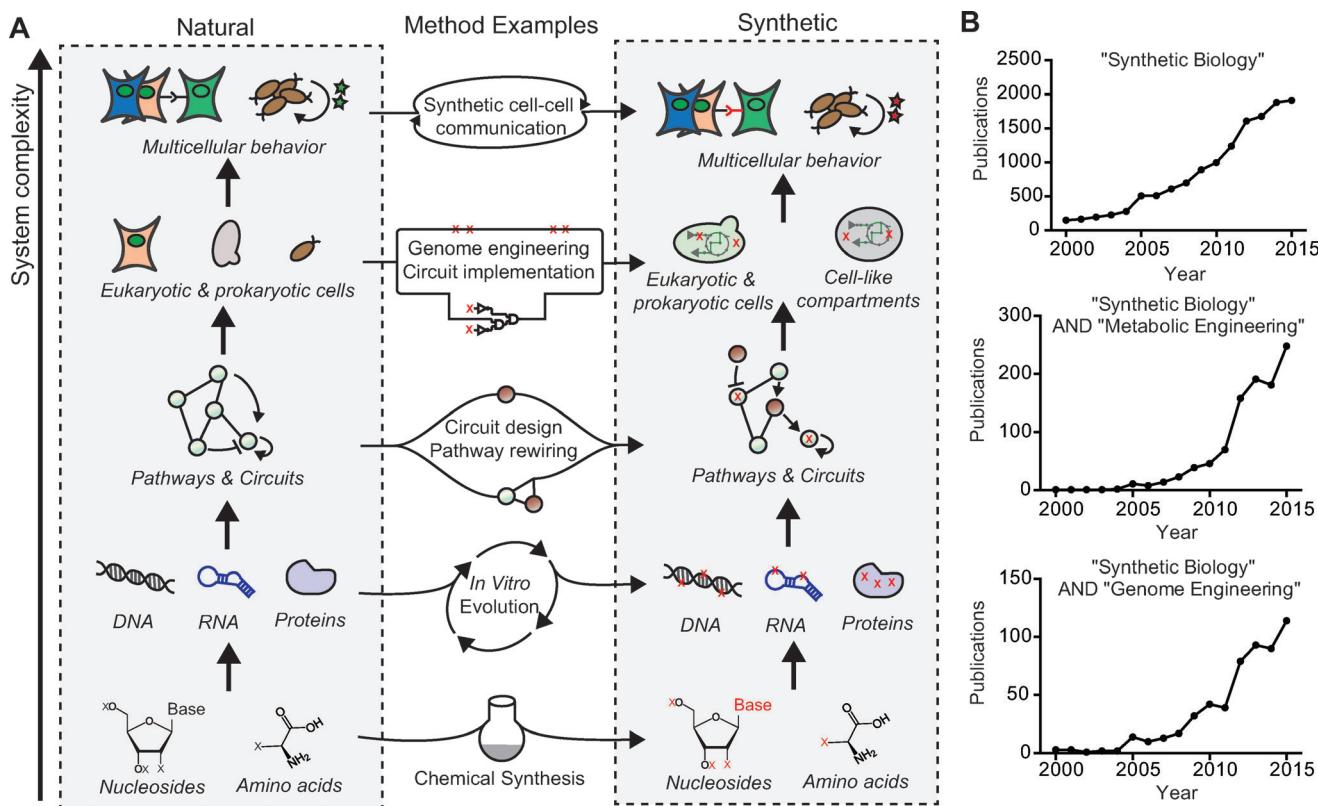
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commonly bacteria, yeast, or mammalian cells, to exploit cellular processes for desired applications. The engineering of synthetic systems in living entities allows synthetic biologists to harness cellular capacities and properties to manufacture valuable molecules,<sup>[11]</sup> transform living cells into biocomputing systems,<sup>[2a–c,e,12]</sup> and create smart cell-based therapeutics that are capable of autonomously detecting and treating diseases.<sup>[13]</sup> The engineering process, however, is often based on trial-and-error due to major gaps in basic knowledge and unpredictable

effects that may have an impact on cellular physiology. The quest for a minimal genome focuses on the understanding of the cellular complexity by defining the number of genes essential for survival of a minimal cell.<sup>[14]</sup> Advances in large-scale DNA synthesis by combining chemical synthesis with cell-based DNA assembly enabled the production of entire synthetic bacterial genomes that could boot up and program the life of bacterial ghost cells.<sup>[15]</sup> In contrast, cell-free synthetic biology seeks to greatly reduce complexity by designing predictable systems in a cell-free environment and utilizing only defined parts and parameters.<sup>[16]</sup> Furthermore, cellular phenomena are reconstituted in a test tube to find the minimal set required for functionality and to acquire comprehensive knowledge of the system.<sup>[17]</sup> In addition, the engineering of computing systems and nanomachines based

[\*] Dr. S. Ausländer, Dr. D. Ausländer, Prof. Dr. M. Fussenegger  
Department of Biosystems Science and Engineering, ETH Zurich  
Mattenstrasse 26, 4058 Basel (Switzerland)  
E-mail: fussenegger@bsse.ethz.ch  
Prof. Dr. M. Fussenegger  
Faculty of Science, University of Basel  
Mattenstrasse 26, 4058 Basel (Switzerland)



**Figure 1.** Principles of engineering in synthetic biology. A) Basic building blocks assemble into polymers such as proteins and nucleic acids, which interact with each other in functional ways to constitute pathways and circuits. Cells consist of many pathways and circuits that define their function, maintain metabolism, and ensure survival. Cell–cell interactions control multicellular behaviors and are of great biomedical importance. Synthetic biology utilizes different methods to convert natural into synthetic biological systems. B) Research on synthetic biology has dramatically increased since 2000, as illustrated by the numbers of publications. Just recently, other research fields, such as metabolic engineering or genome engineering, have started to synergize with synthetic biology. Data were derived from the Scopus database (accessed: September 2016).

on nucleic acids has emerged as a powerful cell-free technology with potential applications in smart biomaterials and therapeutics.<sup>[18]</sup>

In the last decade, different research fields have recognized the potential to tackle biological problems from an engineering perspective by using synthetic biology tools. Thus, synthetic biology can be viewed from different perspectives, for example, as a stand-alone research field or as an approach to understand biology by dissecting or creating biological systems. Certainly, there are strong synergies

between synthetic biology and many other fields, including (computational) systems biology, protein engineering, in vitro evolution, metabolic engineering, and chemical biology, to name but a few. In this Review, we attempt to give an overview of ongoing research in synthetic biology, which has grown tremendously in recent years (Figure 1B). As we are unable to cover the complete list of publications for each research topic, we refer to excellent reviews that describe synthetic biology research topics in their respective research fields (Table 1).



Simon Ausländer is a postdoctoral fellow in Martin Fussenegger's group at the ETH Zürich. He received his BSc and MSc in Life Science from the University of Konstanz, and his PhD from the ETH Zürich in 2013. His research focuses on genome engineering and synthetic biology to develop cell control systems for biomedical and biotechnological applications, including cell-based therapy. He is a member of the European Society for Animal Cell Technologies (ESACT) Frontiers group.



David Ausländer is a postdoctoral fellow in Martin Fussenegger's group at the ETH Zürich. He received his BSc and MSc in Biochemistry from the Technical University of Munich (TUM), and his PhD from the ETH Zürich in 2014. His research focuses on mammalian cell engineering based on synthetic biology, including the development of gene circuits and switches, as well as genome engineering tools. He has developed techniques in biopharmaceutical manufacturing, diagnostics, and cell therapy.

**Table 1:** A selection of reviews from different research fields.

Research field <sup>[a]</sup>	References
gene switches	[2]
genome engineering	[3]
biocatalysis	[4]
protein switches	[5]
directed evolution	[6]
unnatural nucleotides	[7]
unnatural amino acids	[8]
metabolic engineering	[11]
biocomputing	[2a–c,e,12]
cell-based therapy	[13]
minimal cells	[14]
cell-free synthetic biology	[16]
reconstruction of cellular processes	[17]
nucleic acid based nanotechnology	[18]

## 2. Engineering of Functional Biological Parts

Living cells produce all kinds of biological parts with different functions, and synthetic biology capitalizes on nature's diversity to build up a toolbox with standardized biological parts. Therefore, protein, RNA (ribonucleic acid), or DNA parts are isolated from their natural context and transferred into different cellular or *in vitro* environments to characterize their function, stability, and behavior. Traditionally, this approach is pursued by basic research, which aims to understand a biological part in as much detail as possible to answer a certain biological question. Synthetic biology greatly benefits from this wealth of knowledge and utilizes it as a source of well-characterized parts. Furthermore, synthetic biological parts not found in nature are developed by *in vitro* evolution technologies or by incorporating unnatural substrates into proteins and nucleic acids. An intrinsic property of almost every biological part is its capacity to interact with other parts. One class of such useful parts is gene switches, which are capable of sensing a signal and responding with the fine-tuning of gene expression. In contrast, protein-based switches and sensors do not rely upon the slow process of gene expression to respond to the signal, but have instead been engineered to directly report on the signal through protein engineering approaches. Another important class of parts constitutes genome engineering tools that are capable of

changing DNA sequences in living cells. Biological parts can be classified by their specific functions but, because many parts have more than one function, this classification scheme is difficult to realize. For this reason, we list and introduce biological parts on the basis of their uses in synthetic biology.

### 2.1. Gene Switches

Gene expression is the process of decoding functional genetic information into RNA and protein products. This process, called transcription, involves the production of messenger RNA (mRNA) molecules where the genetic information encoded in DNA is transcribed into mRNA molecules. In the next step, mRNA molecules serve as templates for the synthesis of protein products. Gene switches have the capacity to intervene in this process at different levels in a signal-responsive manner and enable living cells to adapt to environmental changes. Based on the level of intervention, a distinction is made between transcriptional, post-transcriptional, and post-translational gene switches. A typical gene switch consists of a sensor unit that is capable of detecting a signal of interest (input) and influencing a regulatory unit, which controls the production of a gene of interest (output).<sup>[2a]</sup> There are many natural and engineered versions of gene switches that respond to an enormous variety of chemical input signals (e.g. antibiotics,<sup>[19]</sup> metabolites,<sup>[20]</sup> and other disease-related signals<sup>[21]</sup>), biological signals (proteins<sup>[22]</sup> and nucleic acids<sup>[23]</sup>), and even physical signals (light<sup>[24]</sup> and temperature;<sup>[25]</sup> Table 2). Applications of gene switches range from basic research, biotechnology, and metabolic engineering, to diagnostics and cell-based therapies, and are discussed in Sections 4 and 5.

The architecture of transcriptional gene switches is based on four essential parts. The first is a DNA-binding part that is capable of binding to a DNA response element in a highly (sequence-)specific manner. The second is a transcriptional effector part that modulates the transcriptional activity, thereby activating or repressing the production of the mRNA product. The third is a ligand-responsive part that is responsible for the control of this process by detecting the input signal state and transmitting the information to the regulatory part. The fourth is a DNA response element that is in proximity to a promoter region that drives the expression of a target gene. Various regulatory mechanisms are possible (see Figure 2A) and include the signal-responsive 1) allosteric control of transcription factors,<sup>[5c,26]</sup> 2) dimerization of two interaction partners,<sup>[20h,27]</sup> 3) shuttling of transcription factors into the nucleus,<sup>[28]</sup> and 4) activation of signaling pathways.<sup>[21c,29]</sup> Since gene expression significantly differs between pro- and eukaryotes, gene switches need to be adapted to the particular host system. For example, trigger-inducible DNA-binding proteins such as TetR act as transcriptional repressors in bacteria, but require fusion to transcriptional effector domains such as VP16 or KRAB to efficiently activate or repress transgene expression in mammalian cells, respectively.

Post-transcriptional gene switches intervene in the process of gene expression at the mRNA level (Figure 2A). RNA

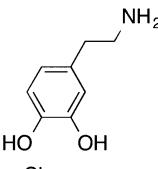
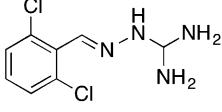


Martin Fussenegger is Professor of Biotechnology and Bioengineering at the ETH Zürich as well as the University of Basel. He graduated with Werner Arber at the University of Basel (1992), obtained his PhD in Medical Microbiology (1994) at the Max Planck Institute of Biology (Tübingen), and carried out postdoctoral studies at the Max Planck Institute of Infection Biology (Berlin, 1995), before moving to the ETH Zürich (1996) to start research in mammalian cell engineering. He is a cofounder of Synthetic Biology, a fellow of the American Institute for Medical and Biological Engineering (AIBME), and a member of the Swiss Academy of Engineering Sciences.

**Table 2:** A selection of inducer molecules of different gene switches.

Inducer molecule	Chemical structure	Sensor type	References
doxycycline		synthetic transcription factor (TetR)	[19c]
phloretin		synthetic transcription factor (TtgR)	[20a]
vanillic acid		synthetic transcription factor (VanR)	[20b]
uric acid		synthetic transcription factor (HucR)	[20c]
T3 thyroid hormone		synthetic transcription factor (TSR)	[21b]
cholic acid		synthetic transcription factor (CmeR)	[20g]
cGMP		synthetic transcription factor (GTA)	[21j]
abscisic acid		synthetic heterodimerized transcription factor (PYL1/ABI1)	[20h]
theophylline		RNA aptamer	[20k]
histamine		G protein-coupled receptor (HRH2)	[29c]

Table 2: (Continued)

Inducer molecule	Chemical structure	Sensor type	References
dopamine		G protein-coupled receptor (DRD1)	[20i]
guanabenz		G protein-coupled receptor (cTAAR1)	[21d]

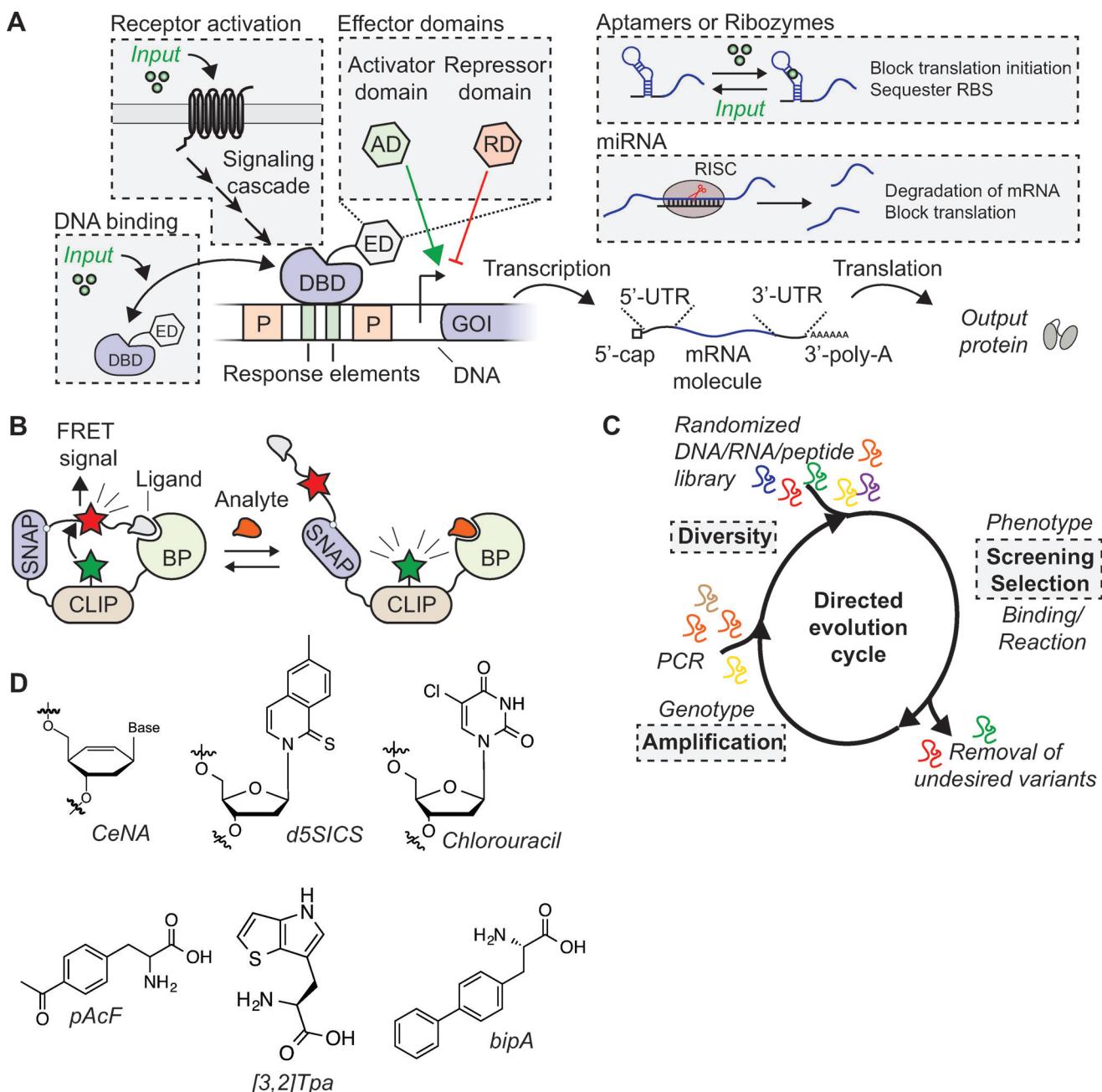
molecules can fold into complex tertiary structures that perform different functions. RNA aptamers, for example, are small RNA structures that have the capacity to specifically bind to proteins or small molecules and, therefore, can be used as ligand-responsive motifs.<sup>[6c]</sup> When placed into different RNA types, aptamers can control mRNA splicing,<sup>[22a,30]</sup> mRNA translation,<sup>[22b,31]</sup> short hairpin RNA maturation,<sup>[32]</sup> and transcription factor functions.<sup>[33]</sup> Ribozymes are another class of functional RNAs that cleave or ligate RNA, DNA, or peptide bonds. The implementation of aptamers into ribozyme structures results in engineered RNA molecules whose ribozyme activities are dependent on the ligand-bound state of the aptamer.<sup>[34]</sup> These so-called aptazymes have been used to regulate other functional RNA molecules, such as tRNA,<sup>[35]</sup> mRNA,<sup>[20e,36]</sup> shRNA,<sup>[37]</sup> and rRNA<sup>[38]</sup> in living cells. As a consequence of the four-letter code of RNA, complementary nucleotide sequences can be programmed to construct sequence-specific interactions between RNA molecules. This process can be used to block essential RNA sequences required for gene expression, such as the ribosome-binding site (RBS) in the 5'-untranslated region of a bacterial mRNA molecule. A second RNA molecule or an aptamer serves as a trigger molecule to unblock the sequence by inducing a conformational change and releasing the RBS, thereby activating gene expression.<sup>[23a,d]</sup> Similarly, synthetic trans-acting RNAs, such as small RNAs (sRNAs), have been engineered to control bacterial transcription by preventing the formation of integrated terminator hairpins within the mRNA molecule of interest.<sup>[39]</sup>

Post-translational switches act on the protein product itself and regulate its function by influencing protein stability, localization, and splicing.<sup>[40]</sup> Several ligand-responsive protein-degradation technologies have been developed that are based on tagging the protein of interest with a destabilization domain called a degron.<sup>[41]</sup> In a ligand-dependent mechanism, the degron localizes the protein of interest to the endogenous proteasome, where it is degraded. Recently, a fluorescent destabilizing domain has been developed based on the fluorescent protein UnaG that can be fused to a protein of interest to control its stability. The engineered UnaG protein displays bilirubin-dependent stability and fluorescence, thus enabling tracking and quantification of the protein of interest.<sup>[42]</sup> Another approach is based on proteolysis-targeting chimaeras (PROTACs), which are bifunctional compounds with the capacity to specifically bind to both a target

protein of interest and an E3 ligase, thereby inducing rapid protein degradation.<sup>[43]</sup> As PROTACs do not require modifications of target proteins, these compounds are also promising drugs for mediating oncogenic protein depletion in diseased cells.<sup>[44]</sup> Ligand-responsive dimerization systems are used for controlling protein localization in living cells. In particular, light-inducible dimerization systems enable the rapid localization of proteins to specific cell compartments.<sup>[45]</sup> In addition, protein splicing technologies allow the covalent connection of two protein parts to complement a functional protein.<sup>[46]</sup> When two proteins are connected to light- or ligand-inducible dimerization domains, they can be covalently linked together through split intein mechanisms.<sup>[23c,47]</sup>

## 2.2. Protein-Based Sensors and Switches

Proteins are involved in almost every process in living cells through coordinating localization, performing biochemical reactions, and transmitting, replicating, and translating genomic information. Furthermore, proteins fold into complex tertiary structures and are organized in multiple functional domains, where each domain performs a distinct function. Allostery is a common feature of proteins, where the binding of ligands can lead to structural rearrangements and influence the function of other domains.<sup>[5a,c]</sup> With the aim of creating proteins with desired properties, synthetic biology exploits a protein's modularity and flexibility.<sup>[5b,48]</sup> A general architecture consists of a sensor domain that is coupled to a functional domain in a way that allows for its allosteric regulation. Prominent examples are protein-based biosensors that have the capacity to rapidly report on the binding states of specific ligands.<sup>[5b,48]</sup> Proteins, mainly fluorescent but also enzymatic reporters, are combined with ligand-binding domains or proteins. For example, genetically encoded calcium indicators (GECIs) and genetically encoded voltage indicators (GEVIs) are widely used tools to image calcium and voltage dynamics in living cells and animals.<sup>[49]</sup> Many different architectures have been developed for both types of biosensors, thus highlighting the diverse engineering possibilities in biology to achieve similar results.<sup>[49]</sup> For example, intracellular calcium receptors (e.g. calmodulin<sup>[50]</sup> and tropo-nin C)<sup>[51]</sup> or cell surface voltage receptors (e.g. *Ciona intestinalis* voltage sensor containing phosphatase (Ci-VSP))<sup>[52]</sup> have been fused to two fluorescent proteins to enable readout



**Figure 2.** Engineering of functional biological parts. A) Gene switches control the process of gene expression. Cell-surface receptors can be modulated by external ligands to activate signaling pathways that control the transcription of target genes. Additionally, ligand-responsive transcription factors consisting of a DNA-binding domain (DBD) and coupled to activation (AD) or repression domains (RD) can be used to regulate gene expression. RNA-based gene switches control translation by regulating, for example, mRNA stability or ribosome accessibility. B) Protein-based biosensors such as the SNAP-tag-based indicator with a fluorescent intramolecular tether (SNIFITs) change their FRET signal intensity depending on the analyte concentration. The SNAP-tag is labeled with a ligand which binds to the binding protein (BP) and a molecule containing a fluorophore (red star). The CLIP-tag is fused with a second fluorophore (green star). C) A randomized library of DNA/RNA or peptides is challenged with the target molecule. Specific conditions during the selection process modulate the binding capacities of the molecules and enable elimination of nonbinders. The elution of binders and their subsequent amplification by using error-prone PCR increases diversity and enriches molecules with the desired binding capacities, which can be used for follow-up directed evolution cycles. D) Examples of the chemical structures of three unnatural nucleotides (cyclohexene nucleic acids (CeNA), d5SICS, and chlorouracil) and three unnatural amino acids ( $\alpha$ -acetyl-L-phenylalanine (pAcF), L- $\beta$ -(thieno[3,2-*b*]pyrrol-1-yl)alanine) ([3,2]Tpa), and L-4,4'-biphenylalanine (bipA)).

of signal events based on Förster resonance energy transfer (FRET). Similarly, circular permuted fluorescent proteins (cpFPs) containing shuffled protein sequences have been integrated into receptor proteins, thereby providing an

alternative to FRET and resulting in improved performance characteristics.<sup>[53]</sup>

SNAP-tag-based indicators with fluorescent intramolecular tethers (SNIFITs) are engineered protein-based biosen-

sors that make use of the SNAP-tag technology to enable protein conjugation through a chemical reaction (Figure 2B).<sup>[54]</sup> SNIFITs are based on an engineering concept that utilizes fusion proteins consisting of three modules: a ligand-binding domain followed by a fluorescent protein that is further fused to a SNAP-tag, which is covalently connected to a fluorophore and a small-molecule binder capable of binding to the ligand-binding domain.<sup>[55]</sup> In the absence of the ligand, the fusion protein displays a closed conformation caused by the intramolecular interaction and resulting in FRET. Upon addition of the ligand, the intramolecular small-molecule binder is displayed, thereby opening the fusion protein and impeding FRET. SNIFITs have been further engineered to integrate two synthetic fluorophores and to be compatible with surface display.<sup>[54]</sup> The integration of a light-emitting nanoluciferase protein enables a simplified fluorescent readout based on bioluminescent resonance energy transfer (BRET), which results in protein-based diagnostic biosensors capable of detecting several drugs, including methotrexate and cyclosporin A in human whole blood.<sup>[56]</sup>

Other strategies to engineer protein-based biosensors are based on split reporter proteins that are engineered to ligand-binding domains, thus allowing for ligand-responsive complementation of the reporter protein.<sup>[57]</sup> In addition, the reversible binding of autoinhibitory domains to respective enzyme domains is used for the design of reporter- and protease-based biosensor architectures.<sup>[5d, 58]</sup>

### 2.3. Directed Evolution of Biological Parts

Evolution generates diversity and provides biological systems with the capacity to adapt to their environment, develop solutions for exploiting alternative nutrient sources, and evolve new techniques to escape from predators. Natural evolution is, however, a relatively slow process that is based on random mutagenesis, genetic transpositions, and natural selection strategies that benefit the best-adapted system. When combined with engineering approaches, directed evolution techniques utilize this concept to generate parts with desired functions in a short amount of time.<sup>[6b, 59]</sup> In principle, a typical directed evolution cycle consists of three steps: generation of diversity, selection, and amplification of desired molecules (Figure 2C). First, a DNA sequence library encoding the desired parental protein or nucleic acid molecule is generated that optimally covers the complete sequence space of randomized nucleotides. Second, functional assays are performed using display-based or *in vivo* based selection or screening systems, where the genotype and phenotype are linked with each other. Only genotypes with the desired phenotype (e.g. a specific function) are isolated from the library. Third, the selected genotypes are amplified, thereby resulting in a library that can be fed into another round of the evolution cycle to introduce more diversity or to enrich for variants with adapted functions.

On a basic level, this concept is used for the generation of target-specific binding proteins, for example, antibody fragments or designed ankyrin repeat proteins (DARPin)s that

are indispensable molecules in basic research, biotechnology, and therapy.<sup>[60]</sup> Depending on the selection pressure or functional assay that reports upon the desired phenotype, *in vitro* evolution allows the integration of new enzymatic activities or altered biochemical properties of existing enzymes.<sup>[6a]</sup> New computational methods enable the *de novo* design of enzymes that are able to perform custom biocatalytic reactions. A promising strategy is based on the synthetic incorporation of transition-metal catalysts into proteins to give artificial metalloenzymes. By using the noncovalent biotin–streptavidin interaction, biotin-coupled metal catalysts have been linked to streptavidin proteins, thereby enabling diverse biocatalytic reactions with improved productivity.<sup>[61]</sup> More recently, this approach has been coupled to the directed evolution of periplasm-compartmentalized streptavidin to isolate mutants with improved activity when combined with biotinylated metal catalysts.<sup>[62]</sup>

In addition to enzymes and binding proteins, many other protein classes have also been customized using *in vitro* evolution technologies. An exciting example is the evolution of a Cre recombinase that is redirected to recognize a conserved HIV-1-related DNA sequence.<sup>[63]</sup> In this study, 145 directed evolution cycles were performed to isolate variants with increased specificity and reduced off-target genome cleavage. The resulting Brec1 recombinase was able to efficiently deplete HIV-1 provirus in patient-derived CD4+ T cells in cell culture and in mouse models.

Another example is based on the directed evolution of gene switches to adapt their ligand-binding site to recognize alternative ligand entities.<sup>[64]</sup> Based on the bacterial repressor protein LacI, which is responsive to the inducer molecule isopropyl-β-D-1-thiogalactopyranoside (IPTG), different methods were used to generate LacI-encoding libraries. These libraries were further screened for variants with alternative ligand-responsiveness by utilizing a screening system based on a gene switch in *Escherichia coli*. Depending on the inducer identity, variants could be isolated and further adapted to exclusively respond to either gentiobiose or sucralose, but not IPTG. This strategy enables simultaneous expression of different proteins by using distinct inducer molecules in an orthogonal manner in a single host cell.

In contrast to the twenty amino acids that serve as building blocks for proteins, functional nucleic acids are only made from four nucleotides, which enables the *de novo* synthesis of small libraries covering a complete sequence space. Functional nucleic acids can be efficiently isolated from large libraries *in vitro* in a procedure called systematic evolution of ligands by exponential enrichment (SELEX).<sup>[65]</sup>

### 2.4. Expansion of the Genetic Code

The incorporation of unnatural nucleotides significantly increases the chemical diversity of aptamers and results in new binding properties with improved functionalities.<sup>[7a]</sup> Nucleotides can be modified at the backbone, the sugar, and the base moieties or mirror-imaged to produce chiral counterparts of aptamers (called spiegelmers). As a consequence of their L stereochemistry, spiegelmers are not

targeted by nucleases and are, therefore, promising therapeutic agents.<sup>[7d]</sup> Existing polymerases, however, cannot be used for the production of spiegelmers because natural polymerases are stereoselective for their natural building blocks. Instead, solid-phase chemistry is used for the synthesis of spiegelmers. The directed evolution of RNA and DNA polymerases, however, resulted in variants that allow for the synthesis of synthetic genetic polymers (XNAs).<sup>[7b,c,66]</sup> Polymerase variants of the Stoffel fragment of the Taq polymerase, for example, are able to synthesize and amplify C2'-OMe-modified oligonucleotides, thereby resulting in dramatically increased serum stability and a great potential for the development of therapeutic aptamers.<sup>[67]</sup> The number of unnatural nucleotides, such as cyclohexene nucleic acids (CeNAs) and d5SICS (Figure 2D), is constantly growing, and combinatorial approaches, which include two or more chemical modifications, are examined first. Moreover, XNA molecules can be replicated and produced to generate functional XNA molecules capable of performing binding, cleavage, and ligation reactions.<sup>[68]</sup> Recently, even plasmids containing unnatural base pairs were shown to be propagated in living cells, thus pointing the way towards artificial cells with a synthetic genetic code.<sup>[69]</sup>

Whereas the enzymatic synthesis of nucleic acids relies on a single polymerase, protein synthesis is a much more complex process that involves the ribosome, a multiunit machinery consisting of RNA and protein parts, and transfer RNAs (tRNAs), which are loaded with their respective amino acids by aminoacyl tRNA synthetases (aaRSs). One strategy to incorporate unnatural amino acids consists of auxotrophic screening for cells that have evolved an expanded amino acid repertoire.<sup>[8e]</sup> For example, bacteria could be selected to have tryptophan in their proteome replaced by thienopyrrolylalanine (see Figure 2D).<sup>[70]</sup> Another strategy is based on the targeted, site-specific insertion of unnatural amino acids into proteins by using orthogonal aaRS/tRNA pairs, which are found in organisms other than the host cell and ensure the absence of cross-talk with endogenous aaRS/tRNA pairs.<sup>[8a,d]</sup> Loaded tRNAs are finally redirected to their specific codon (e.g. the amber stop codons or 4-bp codons)<sup>[71]</sup> within the mRNA target of interest and can, therefore, be exactly placed within a polypeptide sequence by appropriate programming of the desired genetic instruction. Orthogonal ribosome/mRNA pairs<sup>[72]</sup> have also been used to introduce multiple, different unnatural amino acids into a single protein.<sup>[73]</sup> By linking both ribosomal subunits through a flexible linker, tethered versions of ribosomes have been engineered that enable the design of completely orthogonal ribosome/mRNA pairs.<sup>[74]</sup> Thus, current systems are based on four orthogonal (O) parts, the O-ribosome, O-mRNA, O-tRNA, and O-aaRS, which operate in parallel to the endogenous protein synthesis machinery. Depending on the chemical modification, the introduced unnatural amino acid (e.g. *p*-acetyl-L-phenylalanine (*p*AcF)) equips the produced proteins with custom functions (Figure 2D). For example, these modifications can be used to investigate protein functions and are based on amino acids containing 1) a photo-cross-linker that can be used to identify protein interaction partners, 2) post-translational modifications to study their influence on the protein's

structure and behavior, 3) photocaged moieties blocking a function that can be rapidly activated by a pulse of light, and 4) chemical or biophysical tags that are used to label a protein with fluorescent moieties or chemical or biological structures.<sup>[8b]</sup> In addition, the site-specific introduction of protein modifications also holds great promise for obtaining novel therapeutic entities that are based on antibody-drug conjugates or bispecific antibodies.<sup>[8c]</sup> Cells can also be engineered to produce orthogonal amino acids by introducing the corresponding biosynthetic pathways. Important representative examples include bacterial production and the incorporation of azidohomoalanine,<sup>[75]</sup> *p*-aminophenylalanine,<sup>[76]</sup> and pyrrolysine derivatives<sup>[77]</sup> into proteins.

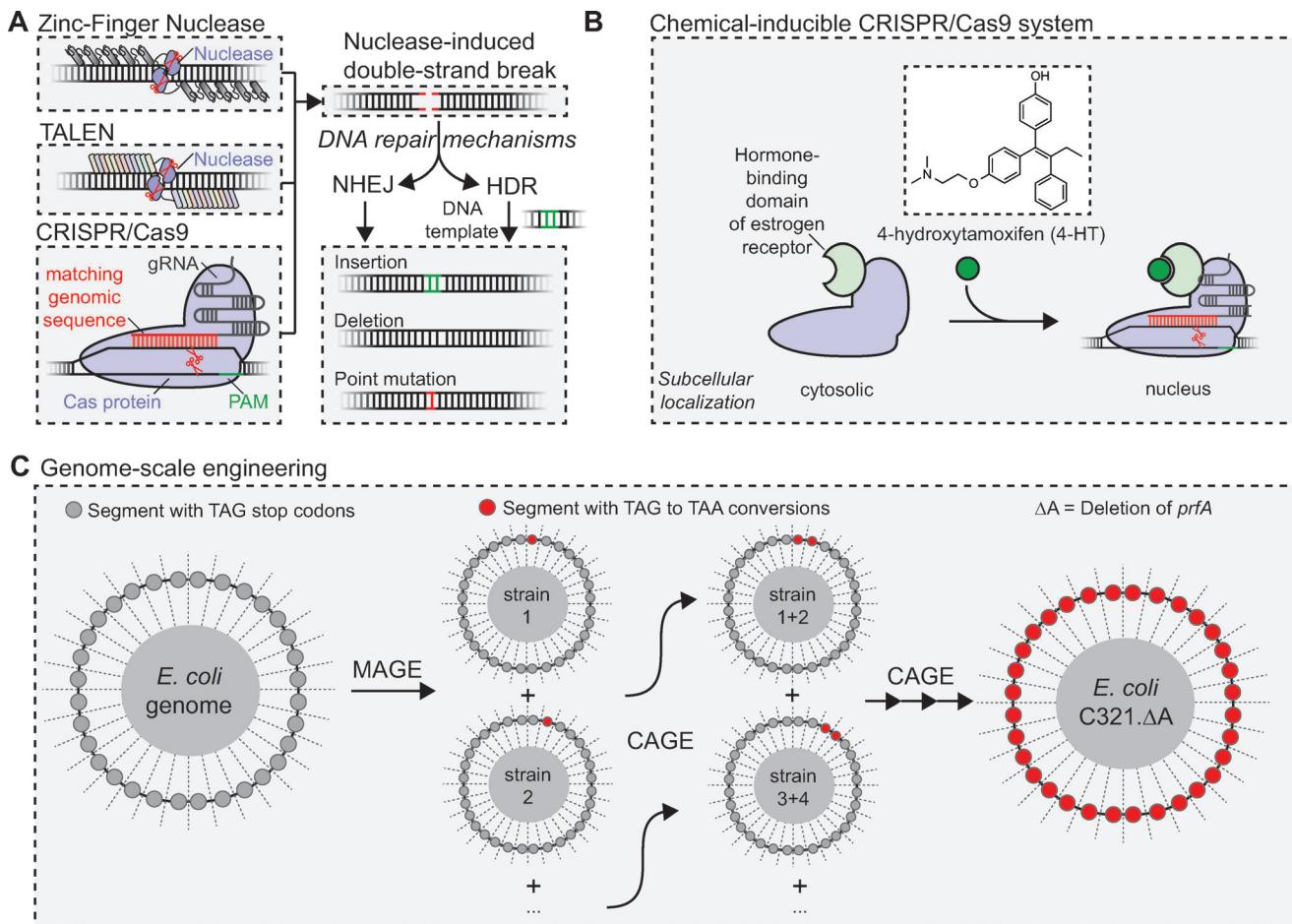
### 3. Genome Engineering

#### 3.1. Site-Specific Genome-Editing Technologies

Recombinases are proteins that bind to specific DNA recognition sites and perform diverse DNA alterations, such as reversible excisions, inversions, and integrations, depending on the orientation and architecture of the DNA recognition sites. In particular, two systems based on the site-specific recombinases Cre/lox and Flp/FRT have become important tools in genome engineering and enable precise and predictable genomic changes in cultured cells.<sup>[78]</sup> In principle, these systems require the targeted integration of the DNA recognition sites (e.g. lox and FRT) to flank the genomic region of interest. The flanked region can be replaced with a customized DNA sequence by means of recombinase-mediated cassette exchange (RMCE). In addition, light<sup>[79]</sup>- and ligand-responsive<sup>[80]</sup> Cre recombinases allow for temporal control of this event. Recombinases can also be used to efficiently control gene expression levels when stop cassettes consisting of transcriptional terminator sequences flanked by respective DNA recognition sites are placed into the 5'-untranslated region of a target gene.<sup>[81]</sup>

Changing the genome with high precision at a specific genomic site offers unprecedented opportunities in basic research, translational research, and synthetic biology.<sup>[3b]</sup> Two types of engineered proteins, zinc finger nucleases (ZFN)<sup>[82]</sup> and transcription activator-like effector nucleases (TALEN),<sup>[83]</sup> can be programmed to specifically bind to a custom DNA sequence (Figure 3A). Usually, two proteins of either the ZFN or TALEN type are programmed to bind to DNA sites in proximity within the genome, and each is coupled to the catalytic domain of the endonuclease *FokI*. The local proximity of two ZFN or TALEN proteins induces *FokI* dimerization and activation, thereby leading to a DNA double-strand break at the target site. Endogenous DNA repair pathways are recruited to the break site, which results in either error-prone nonhomologous end-joining (NHEJ) causing insertions or deletions, or recruitment of the homology-directed repair (HDR) machinery, which enables the introduction of new genetic sequences with reduced error rates (Figure 3A).

Although these powerful genomic scissors have already been available for more than ten years, a new technology



**Figure 3.** Genome engineering tools. A,B) Genome engineering tools are based on proteins (e.g. zinc finger nucleases (ZF) or transcription-activator-like effector nuclease (TALEN)) or RNA-guided endonucleases (e.g. clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated)) which induce a double-strand break at the targeted DNA locus. DNA repair mechanisms such as nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) enable DNA alterations, for example, insertion, deletion, or the introduction of point mutations, to the targeted DNA loci. C) Genome-scale engineering enables the removal of all coding TAG stop codons from the *E. coli* genome. The genome is segmented into 32 parts, each encoding 10 stop codons. The TAG stop codons are converted into TAA stop codons by multiplexed automated genome engineering (MAGE). The resulting strains are further merged to finally produce an *E. coli* strain (C321.ΔA) that harbors all the TAG-to-TAA conversions as well as the deletion of release factor 1 (*prfA*).

called the CRISPR-Cas system was the breakthrough that enabled genome editing for the broader scientific community.<sup>[84]</sup> Although the engineering of sequence-specific ZFN and TALEN proteins is an elaborate process, RNA-guided endonucleases (RGENs) based on CRISPR-Cas systems (CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated) make use of the ease with which RNA can be programmed to bind to a complementary nucleic acid sequence (Figure 3B). In principle, CRISPR-Cas systems consist of an engineered RNA part called single-chain guide RNA (sgRNA), which binds the protein part Cas and forms a complex that locates to the target site of interest by recognizing a specific target sequence, where it cuts the nucleic acid sequence. Various CRISPR-Cas systems have been adapted for genome engineering approaches that differ in their protein and RNA compositions as well as their protospacer adjacent motif (PAM) requirements at the target site. The first reports are based on a system derived from *Streptococcus pyogenes* (Sp), which includes the SpCas9

protein and 5'-NGG-3' PAM-binding specificity.<sup>[85]</sup> The RGEN system from *Staphylococcus aureus* (Sa) differs by the smaller size of the SaCas9 protein and the 5'-NGRRN-3' PAM-binding specificity.<sup>[86]</sup> More recently, the Cpf1 protein (CRISPR from *Prevotella* and *Francisella* 1) has been adapted for use in mammalian cells and showed improved characteristics, including smaller protein size, lower off-target cleavages, and staggered end cuts.<sup>[87]</sup> Furthermore, there are many more Cas9 orthologues that require further characterization of their functionality and PAM-binding specificity.

There have also been tremendous efforts to improve the CRISPR-Cas9 systems through optimization of the gRNA design,<sup>[88]</sup> development of FokI dimerization-based CRISPR-Cas9 variants,<sup>[89]</sup> nickase mutants exerting single-strand breaks,<sup>[90]</sup> and methods to improve HDR efficiency.<sup>[91]</sup> Additionally, the engineering of a 4-hydroxytamoxifen-inducible Cas9 variant enabled trigger-inducible gene editing in human cells (Figure 3B).<sup>[92]</sup> Other approaches exploit the genome localization capacity of a nuclease-deficient mutant of Cas9

(dCas9) to guide functions other than DNA cleavage to specific genomic sites, thereby resulting in novel genome imaging techniques<sup>[93]</sup> and epigenetic alterations to regulate endogenous target genes.<sup>[93a, 94]</sup> For example, the fusion of dCas9 and the transactivation domain VP64 to the light-inducible heterodimerization proteins CRY2 and CIB1 enabled the design of a blue-light-activated CRISPR-Cas9 effector (LACE) system. The LACE system was used to dynamically regulate the expression of endogenous genes with spatial and temporal control.<sup>[24d]</sup> CRISPR multiplexing technologies, which enable the simultaneous usage of multiple gRNAs, have the particular potential to drastically advance cell engineering for biotechnological and biomedical purposes. For example, the ease with which RGEN systems can be used compared to previous methods for generating mouse models to study disease promises to accelerate the growth of knowledge, which could eventually lead to new therapies.<sup>[95]</sup> The system can also be used to correct genes associated with genetic disorders either ex vivo in somatic and induced pluripotent stem cells or in vivo in animal models. For example, there are well-characterized mutations in the fumarylacetoacetate hydrolase (FAH) gene, which cause the fatal hereditary tyrosinemia type I (HTI) disorder. Successful correction of the HTI mutations and associated symptoms in a HTI mouse model following nonviral delivery of CRISPR/Cas9 components into hepatocytes exemplifies the potential of in vivo genome editing for therapeutic purposes.<sup>[96]</sup> The ability to manipulate the genome of any species by using RGEN technology triggered diverse ethical and regulatory debates. The prospect of correcting mutations in diseased cells, however, has initiated the race to find the perfect gene editing technology and an efficient delivery method to revolutionize medicine in the 21st century.

### 3.2. Genome-Scale Engineering

The development of efficient procedures for de novo DNA synthesis and assembly techniques on a large scale has paved the way for the synthesis of gene cluster and even whole genomes.<sup>[97]</sup> Through the design, synthesis, and assembly of a customized >1 mega-base-pair genome from *Mycoplasma mycoides* called JCVI-syn1.0 and its functional transfer into a *Mycoplasma capricolum* host cell, synthetic biologists demonstrated the possibility of synthesizing genomes from scratch.<sup>[15a]</sup> In the search for the minimal genetic set, which required the cells to replicate and survive, the same group of researchers minimized JCVI-syn1.0 to JCVI-syn3.0, with a halved genomic size (531 kilobases) and 473 genes (compared to 901 in JCVI-syn1.0).<sup>[15b]</sup> Although most of the remaining genes were attributed to specific functions, JCVI-syn3.0 contained 149 genes with unknown functions (some of the genes were assigned with a putative function).<sup>[98]</sup> On the one hand, a reduction of cellular complexity may result in a minimal cell that could be more controllable and predictable and, thus, more suitable for engineering purposes. On the other hand, the reduction of cellular complexity may be accompanied by the removal of genetic redundancy, thus resulting in less robust minimal cells.

Recently, an engineered version (synIII) of the *S. cerevisiae* chromosome III was synthesized from scratch and shown to be functional in its host cell.<sup>[99]</sup> The design of synIII included many genomic interventions, such as the insertion of recombinase recognition sites and the deletion of tRNAs and intronic and repetitive sequences, as well as TAG to TAA stop-codon conversions. In silico modeling and systems biology approaches may identify the essential set of genes for a living system and reveal how minimal genomes function.<sup>[98, 100]</sup>

In contrast to in vitro genome synthesis, multiple genome-wide engineering can also be introduced precisely and simultaneously by editing existing genomes through homologous recombination-based multiplex automated genome engineering (MAGE) technology.<sup>[101]</sup> In combination with conjugative assembly genome engineering (CAGE), *E. coli* strains containing small sets of changes are merged to eventually harbor hundreds of DNA alterations in a single *E. coli* strain. The potential of this approach has been demonstrated by engineering *E. coli* genomes to replace the complete set of relevant TAG stop codons in the genome (314 in number) with TAA stop codons (Figure 3C).<sup>[102]</sup> The use of multiple MAGE cycles allowed 32 *E. coli* strains to be developed that collectively coded for all 314 conversions: 31 contained 10 stop codon conversions, and one strain contained four conversions. In successive CAGE cycles, which enable the transfer of genomic regions by an engineered version of conjugation, the resulting strains were finally merged to four strains, with each coding for 80 conversions. It took some troubleshooting and experimental design changes to achieve the final step in a follow-up study and to merge the four strains into a single *E. coli* strain containing 321 TAG-to-TAA conversions (during this time, additional stop codons were found) and a gene deletion coding for release factor 1 (RF1), which terminates translation at UAG.<sup>[103]</sup> As the strain is cleared of potential TAG stop codons, it represents a suitable host to study the re-introduction of TAG codons into desired genes and to reassign them to incorporate unnatural amino acids. Therefore, orthogonal translation systems composed of orthogonal aaRS-tRNA pairs were genetically integrated into the TAG-depleted strain; this enabled the site-specific incorporation of unnatural amino acids into the desired proteins to expand their chemical diversity and hence offer enhanced functions. Such strains are useful to improve orthogonal translation systems by the directed evolution of aaRS mutants with enhanced properties, thereby allowing the efficient incorporation of up to 30 unnatural amino acids in a single protein.<sup>[104]</sup> Biocontainment strategies are used to prevent the escape of such highly engineered bacteria into the environment.<sup>[105]</sup> Therefore, auxotrophic strains have been engineered, which depend on the incorporation of unnatural amino acids into essential genes to ensure survival.<sup>[104]</sup>

### 4. Cell-Based Applications

As synthetic biological systems are often encoded on silent information storage carriers, cellular machineries, for

example, DNA polymerases, and ribosomes, are required to activate and run the systems. Plasmids, artificial chromosomes, and RNAs are suitable information carriers to deliver the encoded parts into living cells. In addition, genome engineering technologies allow the site-specific and multiplexed integration of large DNA cargos into the genomes of host cells, thereby enabling the stable expression of encoded systems.<sup>[106]</sup> When integrated, synthetic systems hijack the cellular resources to produce and maintain system-encoded functional parts, such as RNA molecules and proteins (e.g. enzymes or transcription factors), which might result in potential side effects, such as energy depletion, metabolic imbalance, and eventually cell death.<sup>[107]</sup> Indeed, our limited understanding of biological systems and the complexity of living cells make it difficult to predict the influence of synthetic biological systems on cellular processes.

The interplay of multiple parts determines the function of the synthetic system. Nature provides us with parts that can execute a plethora of functionalities, such as enzymes capable of synthesizing proteins, RNAs, carbohydrates, and metabolites, or biosensors with the capacity to detect not only biomolecules but also physical signals such as light or temperature. As a result of the universal language of biology, these functional elements can be isolated from their existing biological context and adapted to function in a synthetic system (Figure 4A). In a design-build-test cycle, synthetic biology develops new compositions of metabolic, genetic, and signaling networks that are composed of modules derived from different taxonomical origins. When implemented into living systems, the synthetic systems enable the production of drugs (e.g. artemisinin),<sup>[108]</sup> the control of input-programmable biocomputing gene circuits,<sup>[2b]</sup> or the detection of disease states<sup>[109]</sup> in living cells.

#### 4.1. Metabolic Engineering

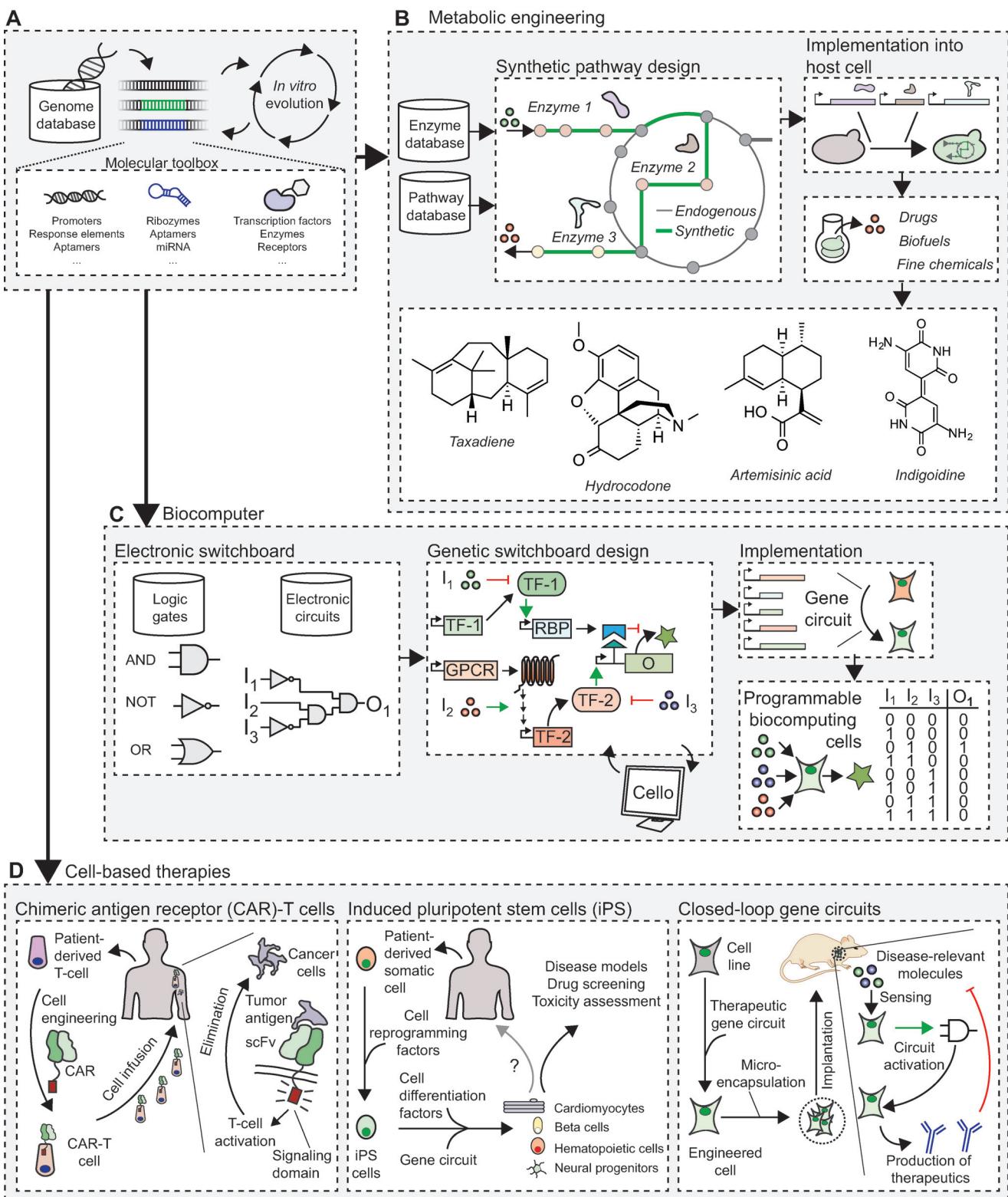
Metabolic engineering pursues a systemic approach that takes all factors into account to convert a host cell into a powerful living factory.<sup>[11a,b,d]</sup> It is based on the idea of converting (inexpensive) renewable carbon sources into (expensive) valuable products by harnessing nature's diversity of biosynthetic pathways, which are capable of producing a wealth of different molecules. Biosynthetic pathways or their individual parts are specialized and adapted in distinct organisms to optimally function in a specific, often ambient environment. Metabolic engineering is based on the design of biosynthetic pathways, which includes the engineering of individual enzymes, the assembly of heterologous parts to create a novel composition, the redirection of metabolic fluxes, and the adaption of ingredients in the medium (Figure 4B). The product portfolio covers simple chemicals, unnatural compounds, and large biological molecules with complex stereochemistry that could not be easily realized by chemical synthesis.

It is the coordinated interplay of multiple functional parts that together resemble a production pathway and lead to efficient product synthesis. Novel biosynthetic pathways are designed by incorporating parts from different organisms to

build an optimal system and then transferring the components into desired host cells (Figure 4B). The performance, however, is often not predictable due to possible side reactions, cross-talk with endogenous parts, or unbalanced expression levels of enzymes, which may hamper the production process. Thus, many design-build-test cycles are required to efficiently channel the host cell's metabolism to product synthesis. For example, the gene expression levels of distinct enzymes can be adjusted to find the optimal balance to improve metabolic flux and to reduce metabolic burden or other side effects.<sup>[110]</sup> The ribosome binding site (RBS) sequence can be engineered to adjust the protein synthesis rate in bacterial cells.<sup>[110c,111]</sup> For example, the algorithm RedLibs enables the simultaneous optimization of the RBS sequences of multiple genes to identify the optimal expression pattern of a biosynthetic pathway.<sup>[110c]</sup> In contrast to the strict determination of the constitutive expression strengths for each part, genetic circuits can be used that allow dynamic gene control of key enzymes during the manufacturing process. Therefore, gene switches are used that sense and respond to important intermediates or external inducers to adjust the enzyme levels to reduce the intermediate's toxicity or balance cell growth and product manufacturing periods in living cells.<sup>[110e,112]</sup> Additionally, gene switches are useful tools for the directed evolution of enzymes because they are capable of reporting on the rate of product manufacture. For example, an RNA-based theophylline ON switch was used to control the expression levels of the fluorescent reporter depending on the enzymatic activity of a caffeine deaminase in yeast cells.<sup>[113]</sup> Improved variants of caffeine deaminase proteins within the library were capable of efficiently converting caffeine into theophylline, which resulted in high expression levels of the intracellular fluorescent reporter, which could be measured by means of fluorescence-activated cell sorting (FACS) to isolate mutants with improved performance. LysR-type transcriptional regulators (LTTRs) were also successfully used as trigger-inducible biosensors for screening engineered yeast cells that produce high amounts of *cis,cis*-muconic acid.<sup>[114]</sup> Importantly, the production capacity of the desired metabolite correlated with the biosensor output, thus revealing a useful strategy for optimizing and fine-tuning engineered metabolic pathways.

The production of a range of biofuels from renewable carbon sources in engineered microbial strains promises to be a green alternative to the petroleum-based production process. *E. coli* and *Saccharomyces cerevisiae* are mainly engineered to produce alcohols, alkanes, and alkenes. The molecular biology approach includes the modification of the host metabolism by the selective knock-out of specific enzymes or the overexpression of endogenous or heterologous variants of enzymes to improve the product yield. Examples of biofuels produced on an industrial scale are 1,3-propanediol<sup>[115]</sup> and 1,4-butanediol.<sup>[116]</sup>

In nature, organisms produce a variety of secondary metabolites of high value for the food, cosmetic, and pharmaceutical industries. Many are flavors, fragrances, or biologically active molecules and have complex chemical structures that complicate chemical synthesis. Therefore, many of these compounds are extracted from producing



**Figure 4.** Cell-based applications. A) The synthetic biology toolbox contains DNA, RNA, and protein parts from genomic information from different environmental sources. The directed evolution of parts expands their properties and increases the diversity of the toolbox. This toolbox forms the basis for subsequent cell-based applications. B) Metabolic engineering seeks to design novel compositions and synthetic variants of metabolic pathways to manufacture valuable products in cellular factories. To optimize the production process, different approaches are used, such as protein engineering, fine-tuning of product expression levels, and insertion of heterologous enzymes into the synthetic pathways. For example, yeast was engineered to produce hydrocodone, taxadiene (a precursor of the anticancer drug taxol), and artemisinic acid (a precursor of the antimalarial agent artemisine). Mammalian cells have been engineered with enzymes to produce the nonribosomal peptide indigoindine. C) Biocomputing devices perform logic operations akin to their electronic counterparts such as Boolean logic gates and electronic circuits.

organisms in a laborious process. Metabolic engineering seeks to harness the diversity of natural products by discovering and cataloging gene clusters of the biosynthetic pathways that are involved in the production of specific molecules.<sup>[117]</sup> The transfer of these biosynthetic pathways into industrially relevant producer cell hosts requires further adaptation and optimization steps. This effort resulted in the successful production of therapeutic drugs in host cells, such as anticancer compounds (e.g. taxadiene<sup>[118]</sup> and noscapine),<sup>[119]</sup> drugs (e.g. artemisinin),<sup>[120]</sup> and narcotics (opioids).<sup>[121]</sup> Moreover, combinatorial approaches that are based on pathway shuffling are pursued to further explore the chemical diversity of natural products.<sup>[11c]</sup> In addition to pharmaceuticals, cells are also engineered to produce industry-relevant biomaterials, such as spider silk<sup>[122]</sup> or cellulose.<sup>[123]</sup>

#### 4.2. Biocomputing in Living Cells

Gene circuits are rationally designed signal-processing systems composed of interconnected gene switches that perform logic functions in input-output relationships.<sup>[2a–c,e]</sup> The advantage of cellular biocomputing systems lies in their capacity to simultaneously sense various different input molecules and to respond with a biological output, thus making them perfectly suited for control systems in cell-based therapeutic, diagnostic, or biotechnological applications. The connectivity of multiple gene switches defines the circuit's topology and determines the logic operation performed by the synthetic system (Figure 4C). The first gene circuit was a toggle switch in living bacteria.<sup>[9]</sup> Soon after, Boolean logic gates followed, which perform logic operations according to the rules of predefined truth tables. Transcriptional gene switches responding to external cues were used to build a set of different logic gates in bacterial<sup>[124]</sup> and mammalian<sup>[125]</sup> cells. Simple logic gates can already be used for sophisticated applications in living cells. For example, a genetic edge detector has been designed to recognize the transition line between light-exposed and masked bacterial cells by logically combining a light-responsive gene switch with a quorum-sensing system.<sup>[126]</sup> In addition, RNA-based gene switches have been used for the construction of basic logic gates in yeast<sup>[127]</sup> and bacterial<sup>[128]</sup> cells. By layering several logic gates, more complex gene circuits can be designed, such as half-adder,<sup>[129]</sup> 2-1 multiplexer,<sup>[129b]</sup> and four-input<sup>[23a,130]</sup> circuitries. Different strategies are pursued to minimize the complexity

of the systems and to improve the performance of gene circuits. First, combining transcriptional and translational gene control elements enables the regulation of individual genes at different gene expression levels to minimize the complexity of the gene circuit.<sup>[131]</sup> Second, complex computational function tasks can be distributed to several cellular units that eventually communicate with each other to respond to different input combinations.<sup>[129b,132]</sup> Third, advanced functional parts, such as recombinases, can be used to reduce a circuit's leakiness and to enable the design of more scalable gene circuits.<sup>[133]</sup>

Recently, the development of electronic design automation (EDA) software called Cello pushed gene circuit design to a new level.<sup>[10]</sup> Cello supports users in the design of custom gene circuits by assembling basic logic gates to complex networks. To show its functionality, transcriptional gene switches were used to design 60 gene circuits, from which many circuits were based on more than three bacterial repressor proteins. Cello delivers the DNA sequence information and a performance simulation of the suggested circuit design. When implemented and tested in bacterial cells, 45 circuits were determined to be functional, thereby showing the potential of using algorithms to improve gene circuit design. State machines emerged as another computational concept in bacterial cells.<sup>[134]</sup> State machines have the capacity to transit from a current state to different states depending on the identity of the received trigger. Thus, a sequential state-dependent logic can be produced, which is of particular interest in cell-based applications. To realize the complexity of a two-input five-state program at the molecular level, recombinase recognition sites were logically placed around three fluorescent reporter genes, and two input-inducible gene regulation systems were used to control recombinase gene expression. The recombinases modulate the fluorescent gene expression cassettes in response to the applied trigger molecule, thereby resulting in five states defined by distinct fluorescent protein expression patterns.

There are many other gene circuit topologies that execute different functions. For example, oscillators enable dynamic reporter gene expression patterns in bacterial<sup>[135]</sup> and mammalian<sup>[136]</sup> cells. Significant progress has been made to improve bacterial oscillators by synchronizing populations using quorum-sensing modules<sup>[137]</sup> and post-translational coupling,<sup>[138]</sup> which eventually resulted in a bacterial cell lysis oscillator with the capacity to release a toxin and induce cancer cell death *in vivo*.<sup>[139]</sup> Synthetic gene circuits that are

Biocomputing gene circuits are designed to sense different input compounds ( $I_1$ – $I_3$ ) using sensor molecules such as G protein-coupled receptors (GPCR), trigger-inducible transcription factors (TF), or RNA-binding proteins (RBP) that are logically rewired to produce output proteins (O) according to a truth table. The design process can be supported using computer-aided design programs such as Cello. This leads to biocomputing cells performing input-programmable operations. D) For adoptive cell therapies, autologous immune cells are isolated and engineered *ex vivo* for expression of chimeric antigen receptors (CARs). Upon injection into patients suffering from tumors with the matching antigen, these CAR-engineered T cells detect the cancer cells by the single chain variable fragment (scFv) domain of the CAR, which activates the engineered T cell and kills the tumor cells. Patient-derived somatic cells can be reprogrammed *ex vivo* to induced pluripotent stem cells (iPSs) by using a set of specific factors. iPS cells have the capacity to differentiate into diverse cell types in the presence of a cocktail of specific differentiation factors or following transfection of gene circuits encoding these differentiation factors. Lineage-controlled iPS cells are used for disease models, drug screening, and toxicity studies; in the future, they may also be used for cell-based therapies. The implantation of cells engineered with closed-loop gene circuits inside immunoprotective vascularizing microcontainers into mice suffering from experimental diseases resulted in the closed-loop gene circuits autonomously detecting and quantifying the disease-relevant compounds, processing the information according to the circuit's logic, and producing therapeutic molecules to correct the disease state.

capable of remembering an experience from a stimulus are called memory devices. They can be used to remember the output state of a logic gate or to track a cell's experience to a stimulus.<sup>[140]</sup>

RNA interference (RNAi) is a post-transcriptional gene control mechanism in eukaryotes and is based on short RNA molecules (small-interfering RNAs (siRNAs) or micro-RNAs (miRNAs)) that can be programmed to be complementary to target mRNA molecules. Gene circuits can be designed to respond to different combinations of siRNAs to compute a range of logic operations.<sup>[141]</sup> For example, based on their unique miRNA pattern, it is possible to engineer gene circuits that have the capacity to determine cell line identities.<sup>[142]</sup> In addition, such gene circuits are useful for therapeutic applications. Depending on the presence or absence of multiple endogenous miRNAs, a pro-apoptotic protein is produced to induce cell death solely in cancer cell lines.<sup>[142a]</sup>

Synthetic cell–cell communication systems allow multiple cell populations to transmit information and to coordinate tasks in an organized manner. Secreted molecules can serve as communication signals and diffuse from one population to another. For example, a two-way communication system based on the amino acid L-tryptophan and the chemical compound acetaldehyde has been designed that utilizes appropriate synthases and gene switches based on bacterial transcription factors for the production and sensing of the communication signal, respectively.<sup>[143]</sup> Whereas secreted communication signals provide tissues or organisms with the capacity to send and receive signals over a long range, the precise detection of cell–cell contacts plays crucial roles in cell development and the immune system to determine a cell's environment. Recently, synthetic cell–cell interaction systems have been designed that are based on engineered versions of the Notch receptor (syn-Notch), a highly conserved cell–cell interaction system in nature.<sup>[29d]</sup> The syn-Notch receptor is highly modular and allows for the determination of ligand specificity by swapping the extracellular binding domain and simultaneously also allows for an intracellular signaling domain to be chosen based on synthetic transcription factors. As a consequence of their modularity, customized syn-Notch receptors can be designed that signal in an orthogonal manner, thus enabling the use of multiple systems in individual cells. The binding event triggers intramembrane proteolysis, thereby leading to the intracellular release of the synthetic transcription factor, which locates into the nucleus, where it binds to a respective response element to modulate gene expression.

#### 4.3. Engineered Cells for Biomedicine

The engineering of living cells for therapeutic applications has many advantages, including their capacity to closely interact with human cells. Genetic circuits encoding for sensors, chemotaxis, therapeutic effectors, and reporter proteins are powerful programs that turn engineered cells into smart living designer cells for diagnostic and therapeutic applications.

Bacterial cells utilize quorum-sensing systems for the communication and coordination of population-dependent processes in response to the density of the cell population. The produced signaling molecules are secreted and, depending on the number of cells, a certain concentration threshold of the signaling molecule is reached, which leads to the activation of the targeted gene's expression. *E. coli* cells were engineered to sense the signaling molecule 3OC<sub>12</sub>HSL from pathogenic *Pseudomonas aeruginosa* and to respond with the production of the bacteriocin pyocin S5 and lysis E7 proteins. Accumulation of the lysis E7 protein leads to membrane perforation of *E. coli*, thereby causing the release of pyocin S5, which results in the selective killing of *P. aeruginosa*.<sup>[144]</sup> In a similar study, *E. coli* cells were engineered to secrete a chimeric, secretion tag engineered bacteriocin FlgM-CoPy to destroy *P. aeruginosa* in response to 3OC<sub>12</sub>HSL.<sup>[145]</sup> In another study, nanofactories were created that are composed of two enzymes called LuxS and PfS that together synthesize AI-2 from S-adenosylhomoserine (SAH) and an IgG-binding domain (protein G), thereby enabling the coupling of these engineered nanofactories to any antibody of interest,<sup>[146]</sup> for example, a mouse epidermal growth factor (EGFR) antibody.<sup>[147]</sup> These engineered anti-EGFR nanofactories were selectively targeted to EGFR-overexpressing human cells and increased the local AI-2 concentration. When combined with engineered *E. coli* cells that have been equipped with an AI-2-responsive gene switch, these *E. coli* cells could be targeted to nanofactory-bound EGFR-overexpressing cells by chemotaxis to induce a desired gene response. A significant advantage would be to engineer custom chemotaxis systems that guide bacteria towards diseased areas. Recently, such a synthetic chemotaxis system was engineered into *E. coli* cells to direct their motility towards N-acylhomoserine lactone (AHL) releasing *P. aeruginosa*.<sup>[148]</sup> Thus, a gene circuit has been designed to modulate chemotaxis behavior and to induce secretion-tagged toxins in an AHL-responsive manner.

Disease progression is often accompanied by abnormal expression levels of key metabolites, hormones, or proteins that are normally regulated by the organism to maintain a balanced physiological health state. In diagnostics, such biomarker patterns are important measures for the detection and classification of specific diseases. Furthermore, nature provides biological sensors that are capable of sensing these biomarkers, and these serve as optimal building blocks for the design of synthetic gene switches with therapeutic applications. A prototype closed-loop gene circuit consists of sensor modules that detect disease-relevant input signals and report their state to a regulatory unit, which produces an appropriate therapeutic effector on demand (Figure 4D).<sup>[149]</sup> Based on this concept, mammalian cells have been engineered with such gene circuits to autonomously treat gout,<sup>[20c]</sup> diabetic ketoacidosis,<sup>[21a]</sup> psoriasis,<sup>[21c]</sup> Graves' disease,<sup>[21b]</sup> and obesity<sup>[150]</sup> in relevant mouse models. To protect engineered cells from the host immune system, an encapsulation step is required before implantation into the organism.<sup>[13a]</sup> The psoriasis-specific gene circuit, for example, integrates the two disease-associated cytokines tumour necrosis factor (TNF) and interleukin-22 (IL22) in an AND logic manner.

First, TNF activates the transcription of the IL22 receptor (IL22R), which serves as a sensor module for the second input IL22. Hence, only in the presence of both inputs is IL22R-dependent JAK/STAT3 signaling activated to drive the expression of two secreted cytokines (IL4 and IL10) to repress inflammation and to counteract the psoriasis-related skin rash. Whereas therapeutic effector expression levels are controlled by endogenous, unbalanced biomarkers in closed-loop gene circuits, other gene switches responding to metabolites,<sup>[151]</sup> cosmetics,<sup>[20a]</sup> food additives,<sup>[20b]</sup> or physical signals<sup>[20b]</sup> can serve as exogenous triggers for gene control in mice.

Induced pluripotent stem cells (iPSCs) generated from adult human donor cells can be reprogrammed to distinct cell types and are, therefore, important tools for basic research and drug discovery (Figure 4D).<sup>[152]</sup> Moreover, there is also great potential to use autologous, reprogrammed iPSCs for regenerative medicine and cell-replacement therapy because they provide an unlimited number of therapeutic-relevant cells. For example, iPSCs can be reprogrammed into pancreatic  $\beta$ -like cells and have the potential to restore glycaemic control in patients suffering from type I diabetes mellitus when used for cell-replacement therapy. Significant progress has been made to efficiently reprogram human embryonic stem cells (hESCs) and human-derived iPSCs to functional  $\beta$ -like cells.<sup>[153]</sup> When encapsulated into modified alginate spheres, such  $\beta$ -like cells were able to control normoglycaemia for six months in a diabetic mouse model,<sup>[154]</sup> thus highlighting their potential for human therapy. Custom-designed gene circuits provide an alternative or complementary concept to reprogram iPSCs by coding for programs that enable dynamic expression patterns of key transcription factors required for efficient and selective differentiation of iPSCs into specific cell types or tissues. Recently, a gene circuit was developed that programmed the dynamic expression of three essential transcription factors (Ngn3, Pdx1, and MafA) in a vanillic acid responsive manner to generate  $\beta$ -like cells from iPSC-derived pancreatic progenitor cells.<sup>[155]</sup> The procedure is based on the timely coordination of three different appearance patterns of the respective transcription factors depending on increasing concentrations of vanillic acid. In another study, a doxycycline-inducible gene switch controlling the expression of the key transcription factor GATA6 was used to generate liver organoids from iPSCs.<sup>[156]</sup>

The engineering of autologous T cells for cancer treatment is an emerging concept that has proven to be efficient and safe compared to alternative treatment options (Figure 4D). Patient-derived T cells are equipped with chimeric antigen receptors (CARs) or engineered T-cell receptors (TCRs) that direct these genetically modified cells to target specific epitopes on cancer cells and induce a cancer-specific immune response.<sup>[157]</sup> Adoptive cell therapy with CART cells showed impressive performance in clinical trials with response rates of up to 80% and low remission rates. However, serious adverse events, such as cytokine release syndrome, have been observed in some cases; therefore, many efforts have been made to render engineered cells smarter and safer.<sup>[158]</sup> One strategy is based on the introduction of control systems that regulate the CAR-induced activation of

the immune response. For example, a split CAR design enables ligand-responsive dimerization of the CAR, which renders the activation of this system dependent on two signals according to an AND gate logic.<sup>[27e]</sup> In principle, the dimerization ligand and the CAR antigen must be present to complement and activate CAR signaling. Such systems significantly increase the safety of adoptive cell therapy by adding an additional control layer to adjust the immune responses in the body to engineered CART cells. In another study, the specificity was increased by the combinatorial use of two antigen-responsive receptors that are functionally coupled to each other.<sup>[158]</sup> A syn-Notch receptor is expressed on the cell surface of an engineered T cell, and upon binding to antigen A it induces the expression of the second receptor, a CAR. Only in the presence of antigen B, which selectively binds to the CAR, is the T cell activated and induces a cancer-specific response. As a consequence of its modularity, different systems can be envisaged that enable the activation of CART cells in specific environments or in response to cancer cells expressing two antigen targets. Similarly, CART cells can be engineered to release immune modifiers, such as IL-12, into the tumor microenvironment, potentially boosting its antitumor activity by recruiting other immune cells.<sup>[159]</sup> CART cells engineered with sophisticated control systems may lead to significantly improved safety in adoptive cell therapy.

In addition to therapeutic applications, living cells are also useful for diagnostic setups to report on the medical states of patients. An orally delivered engineered probiotic *E. coli* variant colonizes and reports on liver metastasis by releasing luciferin into the urine.<sup>[160]</sup> In a different study, *E. coli* cells were engineered with gene circuits consisting of transcriptional gene switches that are capable of sensing disease-relevant signals, such as nitrogen oxides (NOx) and glucose levels in human blood serum and urine.<sup>[161]</sup> Importantly, the gene circuit design is based on signal-inducible recombinases that activate completely repressed fluorescent reporter genes and result in a digitalizing step to produce a clear biosensor output. The intrinsic property of mammalian cells to functionally express any GPCR of interest on their cell surface makes them perfectly suited to measure biomarkers in a disease-relevant concentration range. GPCRs are a huge family of sensor proteins covering a wide range of ligands, including various disease markers. In a proof-of-concept study, an allergy profiler based on mammalian cells was engineered that is able to discriminate between allergic and non-allergic reactions in allergen-exposed human whole blood.<sup>[29c]</sup> The exposure of different allergens to human-derived whole blood triggers the release of the major allergy mediator histamine only in the corresponding allergic patients. A designer cell line consisting of a constitutively expressed histamine-responsive GPCR HRH2 and a reporter gene cassette that is exclusively activated by an HRH2-dependent signaling pathway was able to precisely measure the released histamine. This allergy test has been further developed to detect allergic reactions to seven common allergens covering a concentration range of five orders of magnitude and including important quality control measures that, together, generate a personalized allergy profile with

high information density when compared to the skin prick test, the current gold standard of allergy diagnosis.

## 5. Cell-Free Synthetic Biology

In contrast to the approach of engineering living cells by implementing synthetic systems, the bottom-up strategy aims to build artificial systems from scratch in a cell-free environment.<sup>[16a,c,17a,b,162]</sup> In contrast to the top-down approach, which relies on uncontrollable cellular processes and unknown cellular parameters, this approach brings the engineering principles much closer because it provides unprecedented control. A cell-free system consists only of defined parts, and the environmental parameters can be freely set. When sufficient biological information regarding the function, behavior, and biochemical properties of each part are available, the mathematical modeling is more precise and is, thus, helpful in the design of a synthetic system.

Cell-free synthetic systems are designed to reconstitute and minimize cellular processes to understand their mechanisms and ultimately allow for the design of a custom version of these cellular processes with adapted functions. Other approaches are application-driven, such as the cell-free protein synthesis system PURE, which is based on purified versions of all of the components that are required for transcription and translation.<sup>[163]</sup> PURE is used for protein synthesis and for running gene circuits in cell-free environments. Moreover, the construction of *in vitro* biosensors, gene circuits with various topologies, and cell-free manufacturing platforms<sup>[16b]</sup> are only a few of the many examples of promising applications in cell-free synthetic biology.

### 5.1. Cell-Free Applications

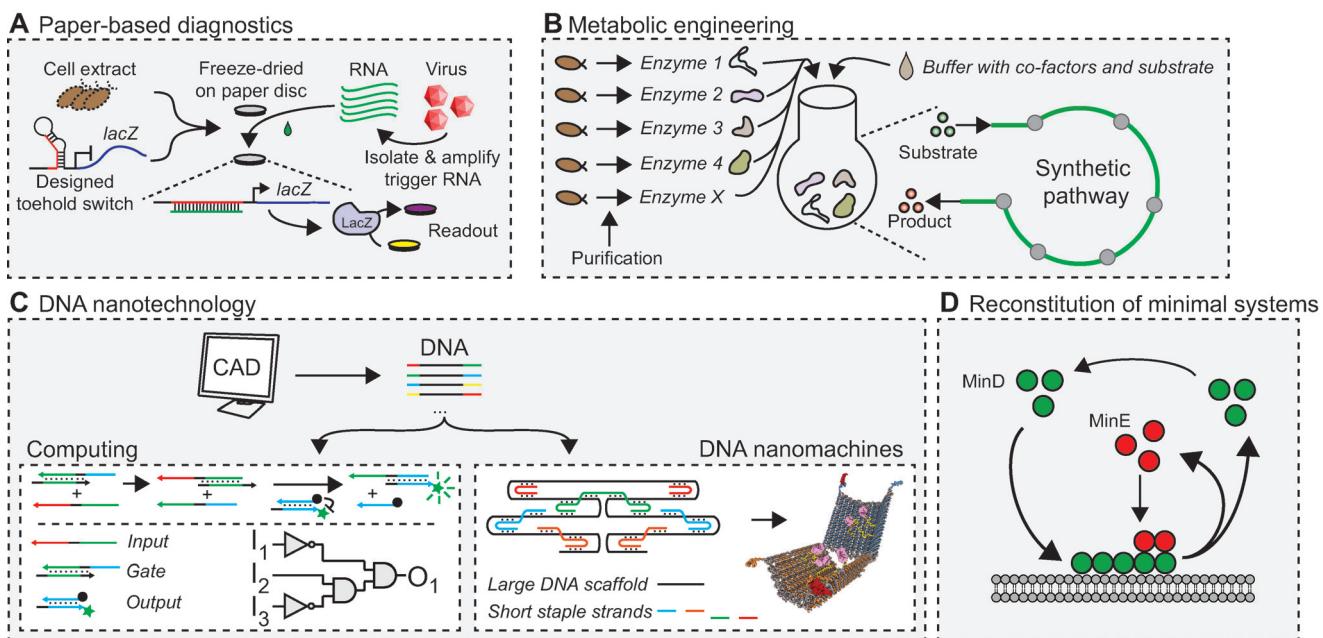
Purified transcription factors are capable of detecting environmental signals and serve as biosensors when added to cell extracts.<sup>[164]</sup> Two transcription factors, TetR and MerR, which sense tetracycline and mercury, respectively, were used to drive the expression of a luciferase reporter output gene in response to the input signals. Such systems are simple but efficient detection systems that make use of cell extracts to run the systems. Recently, a modular biosensor system was developed that utilizes different transcriptional, translational, and protein switches in combination with bacterial or mammalian cell extracts to measure a wide range of diagnostic-relevant molecules, including viral RNAs (Ebola<sup>[165]</sup> and Zika-specific<sup>[23b]</sup> sequences), mRNA levels encoding for antibiotic resistance proteins, and clinically relevant glucose levels (Figure 5A).<sup>[165]</sup> Importantly, all the components can be freeze-dried and applied on paper disks for easy handling and storage at ambient temperature. Rehydration activates the diagnostic device, which is able to produce fluorescent or colorimetric signals depending on the output, which is coupled to the molecular switch. Recently, RNA-based fluorogenic aptamers have been developed and used as an alternative output module in cell-free systems. Based on this aptamer, several biosensors have been designed

that are capable of measuring c-di-GMP,<sup>[166]</sup> RNA sequences,<sup>[167]</sup> and ribozyme cleavage performances.<sup>[168]</sup>

Similar to cell-based gene circuits, ligand-responsive gene switches are important building blocks for the design of programmable circuits by providing control over transcription output.<sup>[16d,169]</sup> For example, a cell-based oscillatory gene circuit, the repressilator,<sup>[170]</sup> was reconstructed to run in a cell-free system.<sup>[169b]</sup> Based on an expanded set of transcription factors, improved versions of oscillators were designed and tested *in vitro* and in *E. coli* cells. This work highlights the potential to accelerate the design-build-test cycle by using cell-free systems before implementing the best performing circuit into living cells. Similarly, the performance of ligand-responsive RNA-based gene switches was first tested and optimized in cell-free systems by using cell extracts before their implementation into living cells.<sup>[171]</sup>

Cell-free metabolic engineering seeks to generate standardized and predictable production systems based on defined parts and parameters to circumvent cell-based disadvantages.<sup>[16a-c]</sup> These disadvantages include energy depletion by cell growth and other cellular processes, product toxicity that may limit yields, potential substrate or product impermeability to reach the intra- or extracellular space, and competing metabolic pathways that may accumulate unwanted side products. In principle, cell extracts from engineered cells containing heterologous enzymes and endogenous metabolic pathways or purified enzymes can be used for the construction of cell-free production systems (Figure 5B). Such approaches resulted in the production of DHAP from glucose by using four heterologous expressed enzymes and cell extracts,<sup>[172]</sup> hydrogen from cellulose by using twelve purified enzymes,<sup>[173]</sup> and farnesene from acetyl-CoA by using nine purified enzymes.<sup>[174]</sup>

Nucleic acids are perfect building blocks for programmable circuits and nanomachines because of their programmability and simple manufacturing compared to proteins.<sup>[18a-c,e]</sup> Cell-free circuits based on enzymatic DNA cleavage<sup>[175]</sup> or DNA<sup>[176]</sup> or RNA<sup>[177]</sup> ribozymes could be designed that are able to perform logic operations in response to oligonucleotide input sequences. Toehold-mediated DNA strand displacement is another dynamic mechanism to program DNA hybridization, and it emerged as a powerful concept for the design of molecular computing circuits and recently resulted in large circuits with unprecedented complexity (Figure 5C).<sup>[178]</sup> Moreover, hundreds of different DNA molecules can be programmed to self-assemble to complex nanoarchitectures, including 3D structures with distinct shapes and curves.<sup>[18d,179]</sup> For example, a DNA container has been designed that is loaded with antibody fragments and directed to target cells through surface-coupled aptamers to deliver the payload.<sup>[180]</sup> Furthermore, synthetic membrane-spanning DNA-based channels have been used to sense distinct DNA molecules.<sup>[181]</sup> Research into RNA-based nanoarchitectures is an emerging field and is expected to complement DNA designs by providing more complex modular structures (3D motifs) and functional modules, such as ligand-binding motifs (aptamers) and catalytic molecules (ribozymes) that can be used to engineer sophisticated nucleic acid-based nanomachines.<sup>[18a]</sup>



**Figure 5.** Cell-free synthetic biology and genome-wide engineering. A) Toehold switches can be designed to control the expression of a reporter gene (*lacZ*) in a trigger-RNA sequence-specific manner. The addition and subsequent binding of the trigger RNA to the complementary RNA sequence activates *lacZ* expression. This reaction occurs in cell-free extracts containing the components essential for translation. RNA is isolated and amplified from samples expected to contain viral trigger RNA. A simple freeze-dried cell extract containing tailored toehold switches on paper discs can provide a diagnostic colorimetric readout based on the enzymatic activity of LacZ. B) Cell-free metabolic engineering is based on purified enzymes and components that can be mixed in a predefined way to convert distinct substrates into specific products. C) DNA nanotechnology enables the design of biocomputing circuits and nanomachines. The use of computer-assisted design (CAD) software enables complex computing circuits to be assembled which take advantage of programmable hybridization between complementary DNA strands to detect the presence or absence of specific input signals. Output modules consist of quenchers (black dot) or fluorophores (green star). Strong fluorescent signals occur only upon displacement of the DNA strand and subsequent local elimination of the quencher from the fluorophore. DNA nanomachines can be programmed by CAD software to direct the folding of a large DNA scaffold by using short staple strands that are capable of binding to complementary scaffold regions. An example is the nanorobot, a drug-loaded container containing a ligand-responsive lid. Upon detection of a cancer antigen, the lid opens and the drug is released. The illustration of the nanorobot is adapted from Ref. [180] with permission. D) A minimal reconstituted cell division: The positioning of the MinDE protein system produces oscillatory patterns on a lipid bilayer in the presence of the energy source ATP. The figure was adapted from Ref. [186b].

### 5.2. Reconstitution of Minimal Synthetic Systems

Self-replication, self-organization, and adaptation are important features of living systems. Membranes have the capacity to compartmentalize functional systems, control the in- and outflow of molecules, and organize cellular structure.<sup>[17a]</sup> Synthetic mimetics of cell membranes are, for example, giant unilamellar vesicles (GUVs), which can be formed from predefined phospholipid constituents.<sup>[182]</sup> Synthetic vesicles are used as model systems to investigate membrane protein functions, but they also serve as compartments for the bottom-up construction of minimal cellular systems. Such biomimetic systems (e.g. GUVs and others) that contain functional parts or systems have the capacity to produce energy,<sup>[183]</sup> to run a transcriptional oscillator,<sup>[184]</sup> or to perform biochemical reactions and so represent small cell-like bioreactors.<sup>[185]</sup>

An example of reconstituting a cellular phenomenon is based on the MinCDE protein system from *E. coli*, which has been studied in cell-free environments to identify the minimal requirements for maintaining its functionality. The presence of the two purified proteins MinD and MinE, a flat mem-

brane, and ATP was sufficient to initiate the dynamic self-organization of the proteins (see Figure 5D) and result in the formation of surface waves.<sup>[186]</sup> In additional studies, the MinCDE protein system was compartmentalized in cell-like systems, thereby resulting in protein-based pole-to-pole oscillations<sup>[187]</sup> and the coordinated localization of downstream proteins in the middle of the cell-like system.<sup>[188]</sup> Importantly, both observations were dependent on geometric parameters of the cell-like systems. The cell-free study of such cellular phenomena provides important insights into their mechanisms and may also be the basis for the engineering of customized versions in synthetic minimal cells.

Self-replication is a prerequisite of reproduction to propagate copied genetic information from parent to daughter systems. RNA can store information based on its primary sequence, but when forming more complex tertiary structures, it can also perform catalytic reactions. In vitro evolution and selection methods can be used to screen large libraries of RNA molecules to isolate variants that have the capacity to catalyze the replication of RNA molecules in cell-free conditions.<sup>[189]</sup>

## 6. Outlook

Synthetic biology is an evolving technology that has matured from the simple construction of unnatural parts to large-scale synthetic biological systems that have the potential to revolutionize biomedicine and biotechnology. These advances have been possible because of the significant technological progress made in multiple areas, including cheaper DNA synthesis, next-generation sequencing, multiplexed, efficient, and simple genome engineering technologies, the availability of vast amounts of genomic sequences and the development of novel chemical biology tools that enable the efficient coupling of unnatural and natural biology.

Several pioneering projects have been accomplished in different areas of synthetic biology and permit a look into the future. An increasing number of designer cells are being engineered that are capable of producing a variety of drugs (including artemisinin and opiates), and there are smart therapeutic cells that detect and treat disease states in a self-controlled manner (e.g. CART cells and closed-loop gene circuit-engineered cell implants). Moreover, the *in vitro* synthesis of whole genomes and genome-wide intervention techniques have just started to emerge and have already produced *E. coli* strains that can be further engineered to produce proteins with an expanded chemical space. Similarly, artificial nucleic acid variants with new properties have great potential in therapy and diagnostics. Together with advances in web-based DNA design software and plasmid sharing tools (e.g. Benchling and Addgene, respectively) for managing, engineering, and analyzing large DNA libraries, the design and synthesis of tailored DNA-encoded biological systems has been streamlined.

In the future, the combination of cutting-edge technologies including advanced genome editing, assembly of sophisticated gene networks, and computer-aided design will drive synthetic biology to the next level. Designer bacteria, yeast, and mammalian cells equipped with highly complex interconnected synthetic gene circuits and pathways that interface with the environment or cellular metabolism will provide unprecedented sensor, processing, and production capacity to enable new opportunities in biopharmaceutical manufacturing and cell-based therapies. Ultimately, fully synthetic cells designed by combining standardized functional modules in a plug-and-play manner and programmed by molecular, physical, or electronic input will produce specialty chemicals and drugs in bioreactors or diagnose and correct medical conditions when implanted into patients. Thus, synthetic biology is bound to become the prime pillar in the personalized/precision medicine era.

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