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SYNTHETIC BIOLOGY: PUTTING ENGINEERING INTO BIOENGINEERING

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11.1 HISTORY AND PERSPECTIVES OF SYNTHETIC BIOLOGY

The field of synthetic biology has recently received tremendous attention. Nevertheless, to most researchers it remains somewhat elusive what synthetic biology really is. Is it a new discipline? Or is it just a new phrase for old stuff? Is it similar to the contemporary field of systems biology as the phonetic similarity might suggest?

Briefly, no single mature concept of synthetic biology exists yet, which makes a short historic view on the early occurrences of the term and the different proposed conceptual backgrounds for synthetic biology a potentially good point to start. As we will see, there are a number of different strands of origin for synthetic biology. In a further step, we illustrate some perspectives of and requirements for synthetic biology.

11.1.1 History

To the best of our knowledge, the first user of the term "synthetic biology" was Stéphane Leduc (1853–1939) at the Medical School in Nates, France, who had an

Systems Biology and Synthetic Biology Edited by Pengcheng Fu and Sven Panke

interest in defining life and to create lifelike forms from chemicals. In his book "La biologie synthétique" published in 1912 Leduc covered a multitude of experiments with inanimate substances that seem to mimic various animate structures—crystal growth, mineral formations, electrolytic and colloidal solutions that react and develop similarly as cellular structures, tissues, and nuclei. The ultimate aim was to present the reader with new ideas about the nature and definition of life, the physicochemical basis for biological activity, evolution, and morphogenesis. Leduc thought that the appearance of forms resembling plants produced by osmotic effects in concentrated colloidal mixtures of inorganic salts had something to tell us about the emergence of life. Although he did not claim that these forms were actually living, even during his lifetime Leduc became completely marginalized and the passion for this topic died out in the early 1930s with the rise of cell physiology, biochemistry, and genetics.

It took then more than 60 years until the term "synthetic biology" was used for the second time. In 1978, the Nobel Prize in Physiology and Medicine was awarded to Werner Arber, Daniel Nathans, and Hamilton O. Smith for their discovery of restriction enzymes and their application to molecular genetics. In an editorial comment of the journal *Gene*, Waclaw Szybalski and Ann Skalka wrote: "The work on restriction nucleases not only permits us easily to construct recombinant DNA molecules and to analyze individual genes but also has led us into the new era of *synthetic biology* where not only existing genes are described and analyzed but also new gene arrangements can be constructed and evaluated" [1].

Maybe prompted by this comment, "synthetic biology" headed a *Nature* review on a book that discussed recombinant DNA technology in 1979 [2] and a review article published by Barbara Hobom in 1980 in *Medizinische Klinik* that covered the corresponding new possibilities [3]. The subsequent time of public debate on possible accompanying biohazards led to an article on "social responsibility in an age of synthetic biology," published in the journal *Environment* [4]. Finally, in an article published in 1986 again in a German journal (*Verhandlungen der Deutschen Gesellschaft für Innere Medizin*), Gerd Hobom reviewed the recent advances in gene technology and stated that biology had left the status of a purely descriptive scientific discipline and was now heading toward a synthetic discipline—synthetic biology. He compared the new possibilities of gene technology, that is, the possibility to recombine genes from different organisms with the development of organic chemistry, where 150 years before there had been a transition from mere description and analysis of naturally occurring chemical compounds to the directed synthesis of novel chemicals. Correspondingly, he stated that the new technologies could also be viewed as tools to create simple biological systems for further analysis [5].

While the term "synthetic biology" had been primarily used to address the new capability of recombining existing genes so far, the synthesis of new genes came into focus in 1988 at a conference organized by Steven Benner in Interlaken, Switzerland. Benner, a chemist at the University of Florida, titled this conference "Redesigning the molecules of life" after the originally intended title "Redesigning life" was considered too provocative in the light of the ongoing recombinant DNA debates [6,7]. Benner's goals were, and still are, to generate molecules by chemical synthesis that reproduce the complex behavior of living systems, including self-reproduction and Darwinian-like

evolution, thereby contributing to our understanding of the chemistry behind life. At the time of this conference, although the term synthetic biology was not explicitly used for the ongoing endeavors, the notion of synthetic biology in the sense of designing artificial DNA molecules was around. It took another 22 years until this notion of chemically designing molecules for manipulating living systems was labeled synthetic biology: At the annual meeting of the American Chemical Society (ACS) in San Francisco, Benner's colleague Eric T. Kool, professor of chemistry at Stanford University, described his work of designing nonnatural, synthetic molecules that nevertheless function in biological systems as synthetic biology [8].

Besides these chemical research-driven activities, another strand of synthetic biology was initiated around the year 2000, when several groups mainly from the biophysics community published on designing and engineering genetic circuits [9,10]. The driving force of these activities was the idea that new insights into the functioning of circuits could be obtained by their *de novo* reconstruction. Taking this a step further led to an engineering perspective of synthetic biology, aiming at the rational construction of biological parts, devices, or systems that have new and not necessarily natural functionality and can be employed for useful purposes.

These issues featured very prominently in the "The First International Meeting on Synthetic Biology," which took place in June 2004 at the Massachusetts Institute of Technology in Cambridge, USA. We consider this meeting as the inaugural event of the discipline. In addition to the work on designing genetic circuits, research from various other areas such as protein engineering, metabolic engineering, and biological chemistry was presented.

Since then, the term synthetic biology has reaped tremendous popularity, which is reflected by the significant boost in the number of mentioning of the term "synthetic biology" in scientific publications over the recent years (Fig. 11-1). As synthetic biology has gained momentum, various research communities have embraced the term, and most likely, many other disciplines will follow suit.

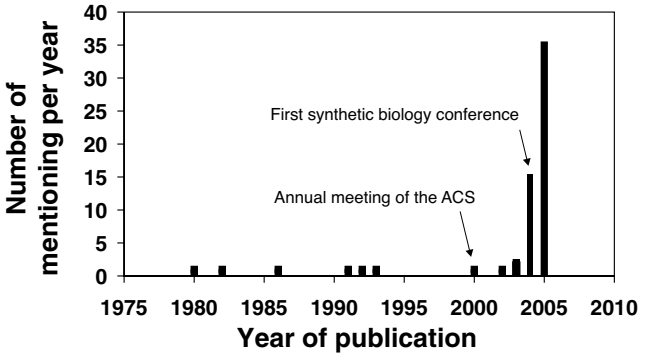

Figure 11-1 Number of mentionings of the term "synthetic biology"" in scientific publications over the recent years.

11.1.2 Perspectives of Synthetic Biology

As seen before, the term synthetic biology was used in different research communities rather independently. Today, basically, as a result of the outlined historic development, one can distinguish between two different perspectives of synthetic biology, the science and the engineering perspective. We will first sketch both the perspectives and then concentrate on the engineering perspective and illustrate this in detail (cf. Table 11-1 and [11]).

The scientific perspective of synthetic biology is mainly discovery and understanding driven. Biologists are interested in learning more about how natural living systems work by rebuilding biological systems and functions (i.e., real physical instances) from scratch according to the current understanding and to test these rebuilt systems or functions, very much in the spirit of ''reverse engineering'' or ''reverse systems biology'' (cf. [12]). Chemists involved in synthetic biology try to synthesize new, nonnatural ''biochemicals,'' such as alternate self-replicating macromolecules, to ultimately study the origin of life. Thus, the chemistry-oriented branch of synthetic biology represents a specific field of chemical research striving to analyze and understand our living world, which is an extension of the concept of ''biomimetic chemistry''.

Synthetic biology can also be viewed from an engineering perspective. Biological systems or their parts are used in processing chemicals, energy, information, and materials. Unfortunately, the engineers' efforts in this area (e.g., in the areas of metabolic or protein engineering) are only decorated with a few success stories, reflecting today's limited ability to engineer biology in a directed and successful manner. In the engineering perspective, synthetic biology aims at overcoming the existing fundamental inabilities by developing foundational technologies to ultimately enable a systematic forward engineering of biology for improved and novel applications. In this perspective,

Table 11-1 Different perspectives of synthetic biology

Synthetic Biology View From the Different Sides ...	Biology	Chemistry	Engineering
Respective goals	Rebuilding represents a vehicle to test our understanding of complex systems.	Creating new biochemicals to study the origin of life.	Designing new biological systems in a forward engineering manner for useful purposes.
Synthetic biology seen as	A research tool	A specific research area	A discipline
Also known as	Reverse engineering	Organic chemistry, biological chemistry	Biological engineering
In the tradition of	Biology	Biomimetric chemistry	Biochemical engineering, metabolic engineering, protein engineering

"synthetic biology" would be synonymous to "biological engineering" and describe another field of engineering next to, for example, mechanical or electrical engineering, with which it would share a common set of methodologies.

Despite these fundamental differences, a common denominator exists in the described areas of synthetic biology: All branches are similar in so far as they deal with the designing and building of biological components, functions, and systems. In each branch, however, the final purpose for doing so is different.

11.1.3 Synthetic Biology from the Engineering Perspective

Biology, as a scientific discipline, has traditionally focused on studying single events or mechanisms in a more or less isolated manner, but in great detail (Fig. 11-2). Examples are the detailed investigation on the mechanism of a specific enzyme reaction or the in-depth analysis of a single gene's function.

This is now complemented by the new field of systems biology, which targets at a system-level understanding of whole biological systems [13]. Armed with detailed mechanistic knowledge on a multitude of isolated phenomena, this new approach aims at a holistic understanding of biological systems with all the interactions between different cellular processes. It is powered by the recognition that biology cannot be understood by looking at its parts alone but requires an understanding of its systemic characteristics and also by the advent of powerful measurement techniques ("omics techniques") that enable this type of research.

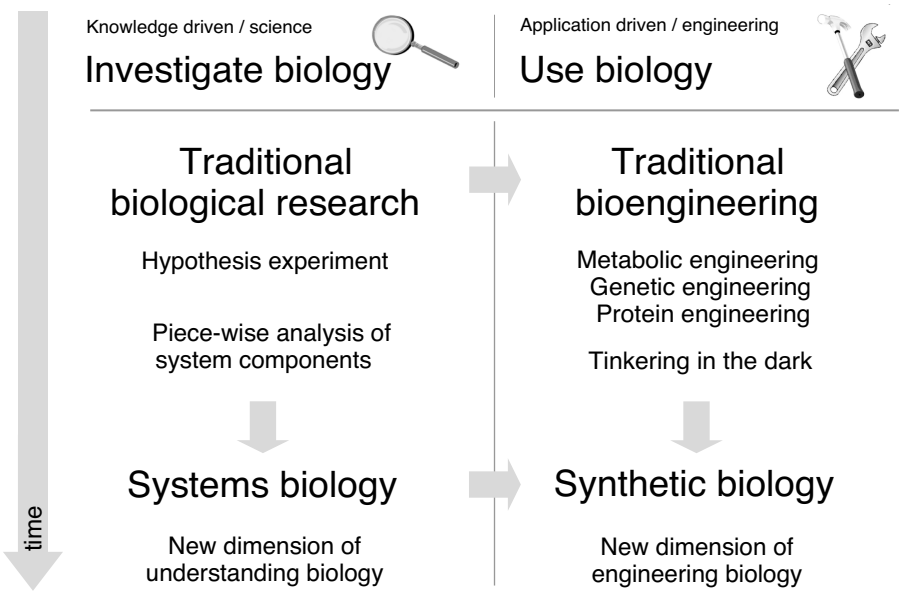

Figure 11-2 An overview of certain aspects of the scientific and applied side of biology at different times.

In all the previous periods of biological research from ancient times to the era of recombinant DNA technology, the knowledge acquired was exploited by engineers for practical applications, from dairy products and beer to metabolic and protein engineering. However, the modifications of the biological material (i.e., strains, enzymes, etc.) to achieve improved properties involved (and still involves) a great degree of uncertainty. Indeed, the desired output of a manipulation is rarely obtained in a straightforward manner, but requires a prolonged trial-and-error period (''tinkering in the dark''). Here, it is important to note that this is in stark contrast to the work in other (nonbiology-related) engineering disciplines, such as mechanical or civil engineering.

Now, that systems biology promises a new quality of understanding and, at least, an intellectual framework to understand biology from first principles, just as good as we understand mechanics or thermodynamics, we can start to think of designing biological systems, and ultimately we will want to do it in a way as we design other functional objects such as cars or bridges. In other words, at least in specific areas biology has matured enough to start thinking of designing biological parts in a forward engineering manner. Such forward engineering design of biological functions or systems we would call synthetic biology. In summary, one could argue that the scientific discipline of systems biology paves the way for the engineering discipline of synthetic biology aiming at the design of new and improved biological functions.

The following thought experiment might be helpful to grasp the difference between systems and synthetic biology:

Assume that a car was something derived from nature that had been optimized by evolution—like a biological cell. Furthermore, assume that our knowledge about this biological car would be very limited. The systems biologist would start investigating the car. He would discover that there is an engine and a gearing system, and that the engine is linked to the gearing system, which causes the wheels to turn, and eventually he would understand how this biological system, the car, works.

In turn, the synthetic biologist would use the knowledge acquired about the gearing system, engine, and so on and would dismantle these parts, would try to optimize (redesign), for example, the engine, to standardize the parts so that they can be used for other cars, but also for other systems, work on the corresponding interfaces, and finally reassemble the parts of the car in a new manner to build something new, for example, a moon rocket.

11.2 WHAT IS REAL ENGINEERING?

In the last section, we have used the term engineering several times and have also mentioned that ''engineering'' (as in metabolic engineering) is not necessarily equal to ''engineering'' (as in mechanical engineering). Looking at a classical engineering project, we will try to derive the characteristic features of a ''true'' engineering work.

11.2.1 An Engineering Example

Imagine the manufacturing of a new car. First, properly skilled mechanical engineers are needed, who were trained to know that an engine, a gearing system, wheels and so

on are required, and how these parts are interconnected. With this knowledge, the engineers make use of computer software (e.g., for computer-aided design), do calculations, and finally come up with a design of a new car. According to their plans, parts (e.g., headlights) are then manufactured. Previously introduced standards (e.g., ISO standards) that are respected during the design process ensure that the different parts will later fit together, even though they might have been produced by different companies. Once manufactured, the produced parts will in most cases first be stored in warehouses until they are used for assembly. Note that design engineers not only develop the plans for the fabrication of the single parts but also elaborate ways for assembling these parts (e.g., in which order) so that finally the designed car becomes a reality as a result of a structured design process.

11.2.2 Key Features of Engineering Endeavors

From this short illustration of a typical engineering project, we can derive several characteristic features of true engineering endeavors whose relevance to biological engineering is worth exploring: (1) forward engineering design on the basis of know-how, (2) abstraction, (3) standardization of components and conditions, and (4) decoupling of system design from system fabrication. Some of the ideas presented in the following were taken from a recent review by Drew Endy [11].

11.2.2.1 Forward Engineering Design on the Basis of Know-How In nonbiology-related areas, engineers can usually draw on a sound knowledge base. Phenomena relevant for design projects in chemical, mechanical, electrical, or civil engineering are in most cases understood from first principles or at least up to a level that makes forward engineering design possible. The sound mastering of thermodynamics and reaction kinetics (chemical engineering), mechanics (mechanical engineering), physics (electrical engineering), or statics (civil engineering) can serve as an example. In each of these areas, the existing in-depth understanding permits computer-based design of new systems by going through iterations between computer models and simulations (but in most cases not including experimentation). By this procedure, extensive testing of new design variants can be performed *in silico*, which in most cases is more time and cost efficient and also much safer than an actual realization and real-life testing. In other words, sound knowledge acts as a basis for real engineering and enables forward engineering design with a predictable outcome.

11.2.2.2 Component and Device Abstraction Engineering endeavors are typically characterized by hierarchies of abstractions. As already indicated in the illustration of the car fabrication, the different parts of the car are set up at different hierarchical levels. The car (overarching top level) contains one specific part—the engine, which at a lower level consists of a number of cylinders, which again can be decomposed into several other parts, such as seal rings, and so on. Generally, there are parts that cannot be decomposed into smaller parts (such as screws); there are parts that consist of several other parts (such as headlights) and finally the whole system (car) that has been built of various parts.

This hierarchical structure has several practical advantages: First, the introduction of system boundaries basically hides information and is thus a way to manage complexity. People, who assemble headlights into the chassis of a car, do not need to know the exact bodywork of the rest of the car. In other words, abstraction is useful as it allows individuals to work independently at each level of the hierarchy. Furthermore, abstraction provides an organization or a methodology of how to combine the parts and consequently supports the engineering of systems with many integrated components.

11.2.2.3 Standardization of Components and Conditions To efficiently make use of an introduced abstraction hierarchy that principally allows for plug-and-play of the different components, the connection and interfaces between the different parts need to be defined, that is, standardization is required. In classical engineering disciplines, standardization is provided by institutions such as the International Organization for Standardization, a federation of national standards bodies, providing standards for almost every sector of business, industry, and technology. Defined standards for components and conditions ensure that connections or interfaces between components fit, even when they are fabricated by different companies. Beyond, as the metrics of tools (e.g., screw drivers) are also subject to standardization, this guarantees that the tools match the according part (e.g., screw).

11.2.2.4 Decoupling of Design from Fabrication Another typical feature of true engineering is the decoupling of the design process from the actual fabrication of novel devices or systems. There are people designing new devices or systems (i.e., developing the plans of how to fabricate them) and other people (construction people, craftsmen) actually realize these plans (i.e., actually building or fabricating the devices or systems according to the specified design). This separation between design and fabrication is realized since both tasks (design and construction) require a distinct set of skills and expertise, which is typically not provided by the same individuals in a mature engineering field.

Nevertheless, this decoupling of design and fabrication requires the design engineers to have a sound knowledge about how things are actually produced and how parts are assembled together. In other words: The design for an object is useless if no way exists to fabricate it. Or, the design for parts of a car is useless if no concept is provided of how these parts can be assembled together (e.g., in which order—compare the planning of an assembly line). Of course, this includes the respecting the importance of standards ranging from a common language needed between the two interacting sets of people (the designers and the craftsmen) to the fact that it is necessary that the craftsmen's tools fit the designed parts.

11.3 VISION FOR SYNTHETIC BIOLOGY

11.3.1 A Little Bit of Science Fiction

To imagine what synthetic biology could become in the future, we just have to replace the car with a biological cell and have to employ the outlined features of classical

Towards a cell-level biofactory

1. Design catalyst strain
2. Construct catalyst strain (synthesis of DNA segments and assembly)
3. Produce catalyst strain (cultivation on rich medium, and then transition to a catalytic machinery)
4. Produce chemical compound with catalyst strain (from inexpensive starting material)

Figure 11-3 Steps toward a cell-based biofactory.

engineering disciplines. Some of the ideas presented in the following were taken from a recent article published in *The Scientist* [14]. Imagine, for example, a cell-level biofactory, easy to produce by cultivation, that replaces a 50-step chemical synthesis route, for example, to a complex oligosaccharide drug molecule (such as the antithrombotic pentasaccharide Arixtra [15]).

The steps from the design to the application of the catalyst are outlined in Figure 11-3. Ultimately, the design of this cellular catalyst would be straightforward, solely computer-based, and would draw on readily available parts that simply would have to be combined in a plug-and-play manner. Then, after the design and the genetic construction of the strain, comprising synthesis of the required genetic segments and assembly in a bacterial strain, the cell-based biofactory would be amplified by cultivation and finally would be used for production of the complex oligosaccharide starting from the inexpensive substrate.

At a first glance, one could gain the impression that this concept is very much in line with the classical approach of metabolic engineering. However, we will see that much more is required than the overexpression or knockout of a few genes. For example, the *de novo* design and construction of new biofunctional systems will involve building of novel proteins, genetic circuits, and metabolic networks.

Most likely, we would start our endeavors of designing this novel catalyst with an organism with a reduced, possibly redesigned genome (serving as a sort of chassis on which we can expand in a rational fashion), to which we would add additional functionality in the form of nucleotide sequences including the required regulatory, gene coding and other functional regions. The organism with the minimized set of protein-coding genes would most probably have only a rudimentary set of metabolic capabilities (to eliminate interference with the inserted pathways), would have lost all elements that contribute to genome dynamics (such as transposons and insertion elements), and would, in general, be reduced to the specific functions that are required for the well-characterized behavior under predefined manufacturing conditions.

To this organism with a minimized genome, we would then add the set of *de novo* synthesized genes that provide the capability to synthesize the desired oligosaccharide starting from a cheap substrate such as glucose. The amount of proteins could be carefully controlled by adjusting the corresponding elements on the DNA, such as promoter and ribosome binding strength. The genes of the pathway might have been assembled from templates from different species and then adapted for the expression in the chosen host or they might be the result of a rational protein engineering effort that has conveyed the desired functionality to a specific protein. As energy and reducing power must be provided for this synthesis, preferably in a carefully stoichiometrically

balanced fashion to prevent the production of side reactions, we additionally would have to include reactions that fulfill these tasks. For this, we would ultimately employ carefully characterized and readily available DNA modules that have been used for these tasks frequently before.

To prevent unnecessary metabolic burdens in early process phases, the conversion of the host cell to the actual catalyst would be inducible and comprehensive—for example, to such an extent, that growth and production could be completely uncoupled but cellular functions could be rescued for maintenance on the pathway. One fundamental prerequisite here would be that we are able to indeed decouple specific cellular functions from the remaining cellular activities. One way to achieve this might be the deliberate introduction of mutually independent functionalities (orthogonality), such as ribosomes that interact with novel ribosome binding sites (see also below) or enzymes that depend on novel coenzymes.

But these mutually independent functionalities would hopefully also extend to the dynamic properties of the designed pathways. Natural enzymes are frequently adapted to the needs of the cell to maintain homeostasis and operate with metabolite concentrations within the μM–mM range. Consequently, for an improved version of the catalyst that produces high product titers, we need to identify the relevant allosteric inhibitions (some of which might be still be unknown since they only become apparent at concentrations higher than the typical intracellular ones) and remove these inhibitions by redesigning the respective enzymes.

The novel properties of this cellular production machine will hopefully ultimately be designed and optimized at the computer. These designed components would then be converted into the respective amino acid sequences and finally translated into a nucleotide sequence, from which the desired functionality can be expressed. Finally, this designed DNA sequence will then be chemically synthesized, assembled with other parts and introduced in the chassis—the organism with the minimized genome.

It is important to note that such cell-based biofactory for fine chemical manufacturing is just an example of many conceivable synthetic biology projects and applications. From the degree of mastership that is required to execute such a project, it becomes clear that the implications are much more far-reaching and can be extended to any other area for which biotechnology has been or will be considered. To be clear, today, every ongoing synthetic biology project only scratches at a project like the one illustrated above.

11.3.2 Potential Fields of Application

The illustration of the cell-based biofactory represents an example where a synthetically devised organism could execute various functions that allow producing a chemical compound, a drug, or even maybe energy in the form of hydrogen from agricultural waste. The recent and envisioned breakthroughs in biology and technology, however, do not only present unprecedented opportunities that could restructure and revolutionize the manufacture of chemicals, pharmaceuticals, and energy, but also may offer unique ways to enable carbon sequestration and environmental remediation. In addition, several medical applications can be envisioned as well as projects

Table 11-2 An overview of potential areas of application for synthetic biology together with illustrating examples

Area	Examples
Production of pharmaceuticals, chemicals, energy	Develop a bacterial or fungal cell that can be programmed to produce complex hydrocarbon precursors (e.g., oils, plastics), to produce hydrogen or ethanol, to convert waste into energy, to convert sunlight into hydrogen
Chemical/biological threat detection and decontamination	Develop a bacterial cell that can be programmed to fix any desirable amount of atmospheric CO_2
	Develop a bacterial cell that swims to the threat and decontaminates it
Medical applications	Develop bacteria that can parasitize cancer cells
	Develop circuits that guards against cancer, which if activated self-deconstructs the cell
Analytics and diagnostics, sensors and actuators	Develop bacteria, fungi, or plants that can be programmed to monitor environmental state, but that never survive mutation, whose DNA are not subject to horizontal gene transfer (both coming and going)
	Develop proteins that can sense any kind of harmful chemical compound (e.g., TNT)

stemming from the field of processing of information. Here, Table 11-2 provides an overview of potential areas of application, together with some illustrating examples.

The examples foreseen for areas not related to chemical synthesis underline that indeed the term ''metabolic engineering'' is too narrow to describe the new discipline of synthetic biology, as this term is always used in the context of chemical production by means of manipulated biological cells. In contrast, it is envisioned that synthetic biology will employ organisms and biological systems more broadly to solve real-world problems, and thus it has an enormous potential for human health, renewable energy, and the environment.

In fact, a few companies have already been founded with the goal of harvesting some of the early benefits in the area of synthetic biology. Possibly, the most prominent companies are the two U.S. companies Synthetic Genomics and Codon Devices, both founded in 2005. Synthetic Genomics wants to develop and commercialize genome reconstruction and synthesis technologies and particularly engages in the area of ethanol and hydrogen production. Codon Devices aim to develop a technology platform that is expected to accurately synthesize kilobase-to-megabase-long DNA sequences. The company's early commercial focus is on providing engineered devices for molecular biology research and biotherapeutics.

11.4 REQUIREMENTS FOR SYNTHETIC BIOLOGY

After portraying our vision of synthetic biology and after suggesting some ideas of what the discipline might be able to deliver in the future, we now examine the actual requirements to make this vision come true.

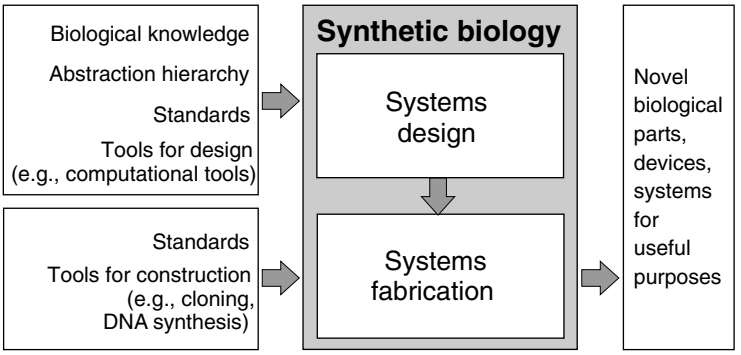

Figure 11-4 Synthetic biology encompasses systems design and fabrication. Each part has its specific prerequisites and inputs. Ultimately, synthetic biology delivers novel biological entities with improved functionality.

Synthetic biology encompasses the redesign of existing, natural biological systems for useful purposes and in the long run the design and construction of new biological parts, devices, and systems. Generally, future synthetic biology endeavors can be subdivided into two distinct divisions: systems design and systems fabrication (cf. Fig. 11-4).

11.4.1 Design

The design division of synthetic biology deals with the forward engineering (re)design of biological parts, devices, or systems. In the following, we will discuss the general requirements for the design as well as the current limitations and problems in the respective areas: (1) knowledge, (2) computational design and (3) standardization, modularity and orthogonality.

11.4.1.1 Knowledge As outlined in one of the previous sections, every established engineering classical discipline (such as mechanical or civil engineering) can draw on a sound body of knowledge, ideally ranging down to the first principles. In biology, we have not yet reached such level of in-depth understanding and consequently, true biological engineering has not been possible until now. The recent advances in the postgenomic research, however, provide hope that sooner or later we will be able to draw on a body of knowledge that allows for such a directed engineering of biology. Here, especially the concerted efforts of systems biology provide novel degree of comprehension, so that systems biology could be considered as a driving force for synthetic biology.

11.4.1.2 Computational Design As a further requisite for synthetic biology, computational tools are necessary that enable the computer-based (re)design of biological parts, devices, or systems and form the synthetic biologist's computer-aided design (CAD) software package equivalent, in analogy to the design tools available in the areas of mechanical or civil engineering. Such a design tool would need

to integrate the detailed available knowledge into a user-friendly program and thus would bring this knowledge out of the realm of research into the realm of engineering. Also in the field of synthetic biology, the designs tools would be based on mathematical models that realistically reproduce biological behavior. Using such software, the design engineer would try to improve the behavior of a system *in silico* by adjusting design parameters—a task that can also be fulfilled by automatic optimization procedures targeting a selected objective function. Simulation capabilities implemented in the design tool would finally allow computational testing of design variants. Still, to obtain such design tools much research work is necessary as first rough mathematical models (e.g., describing gene transcription and translation or kinetics of metabolic or signaling pathways) are only now becoming available [16]. Moreover, to be useful for a forward engineering design, the employed mathematical models need to have predictive power. Beyond, in cases where only small numbers of molecules are involved (such as in gene transcription and translation, where transcription factors and mRNA molecules only occur in very low discrete numbers), the models need to be able to even reproduce the inherent stochasticity of such processes. This is imperative, as it was shown that stochasticity in combination with certain system architectures can—on a stochastic basis for decision making—result in different system states [17]. Thus, a robust design of new devices and systems must be able to exclude such eventualities.

Another important area for further computational design efforts is the field of protein design: Nowadays, *de novo* protein structure prediction from a linear amino acid sequence can only be achieved for small protein domains [18] and quantitative prediction of enzymatic activity and selectivity from 3D protein structures, in general, is not yet feasible—although significant progress has been made in this direction. For example, by selecting an enhanced ''dead-end elimination'' algorithm, an efficient computational procedure could be established to first convert *E. coli*'s periplasmic ribose binding protein into a set of proteins with completely novel substrate binding specificities [19] and later into a protein with triose phosphate isomerase activity [20]. The novel enzyme had a catalytic constant in the order of $0.1\ s^{-1}$, which is quite a remarkable achievement for a computational design. Nevertheless, there remains a long way to go until true forward engineering of proteins ''at discretion'' becomes possible.

Our still limited abilities in protein design only highlight another important limitation in our current synthetic biology designs: the lack of detailed knowledge of many important systems parameters, which synthetic biology shares with systems biology. When trying to implement specific behaviors, it is usually possible to identify parts that qualitatively have the required behavior (positive or negative regulation, composite promoters, etc.). However, to organize these parts into a system with the desired behavior, we also need the right dynamics—in other words, we need specific DNA–protein or protein–protein binding constants, Hill coefficients, protein degradation rates, and so on. However, the quantitative characterization of many systems is by far not comprehensive enough, and even if the corresponding parameters were measured, they might not fit the system we intend to desire. Here is another big field for protein design in synthetic biology: the rational modification of specific binding properties.

Alternatively, one has to resort to evolutionary methods to obtain the required modifications. This was shown by rescuing a nonfunctional inverter gene network in which LacI was supposed to repress the synthesis of CI repressor, but did so only partially, so that CI was constantly synthesized [21]. Possible solutions here were decreasing the leakiness of *cI* repression or reduction of the repressive effect of CI itself. Screening a library of mutated DNA fragments covering the RBS of the *cI* gene and the gene itself delivered clones with a variety of mutations that, in general, seemed to weaken the repression effect of CI, such as reducing the dimerization of CI molecules or reducing DNA–protein interactions. In other words, directed evolution was an excellent tool to adapt the system parameters such that the inverter characteristic could be rescued.

11.4.1.3 Standardization, Modularity, and Orthogonality Another requisite for the design in a true engineering sense is the availability of standards. Parts, devices, or systems stored in a database (or warehouse) need to have standardized interfaces so that a design engineer can make use of these as modules for his design in a plug-and-play manner. A first synthetic biology warehouse, the MIT Registry of Standard Biological Parts, has been established at the Massachusetts Institute of Technology, Cambridge, United States of America (http://parts.mit.edu/). It uses the standardized vector format of ''idempotent vectors'' that lends itself easily to assemble and allows interoperability of assembled sequences (https://dspace.mit.edu/bitstream/1721.1/21168/1/biobricks.pdf). Alternatively, the NOMAD technology has been suggested [22]. Here, vectors are designed in such a way that a DNA insertion into an assembly vector recreates exactly the same restriction site architecture of the assembly vector alone by exploiting restriction sites with compatible but nonrecleavable ends or by exploiting type II restriction enzymes. Both techniques allow multiple rounds of insertion on either side of an insert. However, despite its success, initiatives such as the MIT Registry have to be backed up in the future by more sophisticated design tools and a large capacity to validate and document such standard parts.

Next, it is not clear whether we already measure the most useful quantitites for engineering and, if we can agree on the set of quantitites, how to measure them so that measurements can be reproduced and contributed by multiple labs. Even such an apparently simple concept like promoter strength is poorly defined. Frequently, it is reported in terms of protein activities. This, however, is an aggregate quantity that integrates the number of messages produced per time from a promoter, the (again aggregated) efficiency of initiation of translation, the efficiency of the translation itself including codon usage effects, the amount of protein that has correctly folded into a functional form and the current steady-state of protein production and degradation. Consequently, the corresponding experiments across labs are usually difficult to compare. Alternatively, the promoter strength could be very narrowly defined as ''PoPS'' (polymerases per second) that would quantitatively describe the number of RNA polymerase molecules that pass a specific point on the DNA per time (http://parts.mit.edu/r/parts/htdocs/AbstractionHierarchy/index.cgi). Such a quantity would lend itself easily to comparison of many promoters, as it is much more narrowly defined. However, it is currently unclear how to measure such a parameter directly.

This idea of standardization of parts is inevitably linked to the concept of modularity and functional self-containment of parts. After all, it will be almost impossible and highly undesirable to recreate the interdependency that is characteristic of today's living systems. Rather, from an engineering perspective, it is much more desirable to draw on a limited number of well-characterized and optimized parts and devices that do not interact with each other besides the interactions that have been introduced on purpose. This provokes, of course, the question whether such modularity—composability "at discretion"—is possible in biology.

Current evidence is that it is, at least for many instances. Even though the current failure rate when assembling modular parts on DNA level—promoters, ribosome binding sites, coding sequences, terminators, and so on—for example, from the MIT registry is still significant, this reflects more of a deficiency in implementing all available knowledge into the design process than of a fundamental problem. The composability of genetic elements is, after all, the underlying dogma of recombinant DNA-technology.

Recent work on RNA molecules has extended the concept of modularity to gene regulation. For example, it is possible to design modular RNA aptamer domains for small molecules that can be coupled to antisense effector domains to regulate translation in response to the presence or absence of small molecule effectors [23]. Aptamer domains responsive to different small molecules have been used successfully with the same antisense domain and vice versa, demonstrating the modularity of the concept. Taking to the extreme, this means that there is a new series of modular tools available that can interfere in a programmable and responsive manner with gene expression on RNA level of many different genes.

However, the modularity concept also appears to work on protein level: In particular, the domain architecture of many regulatory proteins plays here very much in favor of such approaches. One specific example is the design of polydactyl zinc finger DNA binding proteins [24]. Combinations of zinc finger domains provide the sequence specificity of the DNA binding domain (DBD) by recognizing essentially a subset of three or four nucleotides per zinc finger domain. Such proteins display modularity in two ways: typically, the DBD is functionally independent from the effector domain and zinc finger domains are functionally relatively independent of each other—so that by selecting a set of specific zinc fingers *in silico*, one can specify arbitrary sequence specificity into a novel DBD, which can then be coupled to a novel effector domain. This concept, though not yet truly universal, has already delivered some spectacular successes in designing DNA-binding domains that recognized up to 18 nucleotides [25–28].

Similar functional reprogramming could also be achieved on the level of protein–protein interactions with signaling proteins. For example, the domains of the eukaryotic neuronal N-WASP protein, involved in actin polymerization, are very amenable to recombination, including with domains from other proteins. These recombinations lead, for example, to proteins that execute novel logical behavior [29].

Alternatively, nature provides examples where signaling proteins have left the task of providing specificity within signaling pathways to scaffold proteins. These recruit a kinase and the kinase's substrate and assemble them in close proximity for

phosphorylation [30]. Reorganizing such scaffolds along common kinases allows recombining signaling pathways, so that osmotolerance could be converted to a function of mating pathway induction.

A final point that will be essential for successful design is its orthogonality (mutual independence). When implemented, the design has to be executed by the cell in the intended fashion, which implies that the interactions of the design with the cell occur only at the anticipated points. Given the high degree of interdependency of cellular functions and one common reaction space for all the different interactions (the cytoplasm), this is an absolutely nontrivial task. For example, it will be vital to eliminate cross talk of gene regulatory elements such as regulatory proteins, particularly when considering the design of large artificial networks. A promising approach is presented here by the concept of engineered riboregulators [31], where *cis*-acting repressing parts of the mRNA and trans-acting activating RNA molecules combine to regulate gene expression in a fashion that could be widely extended to many genes. Importantly, when four sets of such crRNA–taRNA combinations were tested for cross talk, none could be detected, advocating well for the exploitation of this technology in large artificial networks.

An alternative approach would be to reserve subsets of specific functions in the cell only for the execution of a design. For example, rather than feeding mRNAs of genes that are part of the design into the common cellular ribosome pool, proper engineering of the mRNA–ribosome pair can reserve a subset of ribosomes specifically for the translation of one mRNA [32] and thus isolate the translation of the target mRNA from the rest.

Taking this concept a step further, it should be possible to introduce not only new specificity with existing molecular species, but also to introduce new molecular species. Again, this might allow whole new sets of unique interactions that exist quasi ''in parallel" to traditional cellular functions. The topic of alternative chemical structures with self-replicative properties is explored further in Chapter 13 of this book by Holliger and Loakes, so we are not treating it here.

11.4.2 Fabrication

The fabrication division of synthetic biology is responsible for the actual realization of the design engineers' plans, so that finally a new biological part, device, or system turns into reality. Once the design engineer has delineated a novel functional module and has converted (or, in other words, coded) this design into a sequence of nucleotide bases, it is necessary to physically produce this strand of DNA, possibly to assemble it with other already existing oligonucleotide segments and finally to introduce it in an organism (ideally with a minimal genome), which will then express the implemented functions.

So far, our ability to extensively modify chromosomal DNA was restricted by the possibilities of the traditional (and laborious) molecular biology techniques, such as traditional cutting and pasting of DNA, site directed mutagenesis, PCR and error-prone versions of it, and so on. Actually, we rather modified or combined existing natural DNA sequences than constructed DNA from scratch. However, this repertoire

of competencies appears much too limited and time-consuming for the envisioned novel functions.

11.4.2.1 DNA Synthesis Recently, however, tremendous improvements in the speed, accuracy, and price of *de novo* chemical synthesis of DNA have been made, so that these limitations will be eliminated soon (see also Chapter 12). Significant efforts are currently being made in the development of DNA synthesis technology, so that there is reasonable hope that existing technical challenges will be rapidly overcome, and consequently the designers can outsource the DNA preparation task of the fabrication division and concentrate on the actual design task. Bulk DNA synthesis capacity appears to have doubled approximately every 18 months for the last ten years; the commercial price of synthesis of long fragments of DNA (>500 bp) has decreased by a factor of ~2 over the past years [33]. Right now, we can witness the change from classical DNA synthesis technology to novel forms, such as reactors based on microfluidic concepts and photochemical methods. At present, DNA *de novo* synthesis is performed by assembling overlapping short (25–70 bp long), chemically synthesized oligonucleotides into longer DNA fragments in a PCR-based assembly process [34] and has already led to the complete reconstruction of some smaller phage genomes such as the polio virus [35,36]. These efforts are typically accompanied by enzymatic efforts to reduce the error rate [36,37]. But many of, at least, the chemical steps involved can now be reproduced in miniaturized forms in microfluidics chips, where exploiting the small scale should lead to not only a reduction in the materials costs, but also in the opportunity to provide optimized reaction conditions and thus reduced error frequencies [38]. Taking the concept even further, oligonucleotide synthesis can also be miniaturized on photoprogrammable chips. Coupled with error detection by hybridization, exceptionally low error rates in the order of one mistake in every 1400 bp are possible [39].

11.4.2.2 Chassis and Cloning of Giant DNA Finally, once we have synthesized novel strands of DNA, we need to integrate them into an organism. This splits into two aspects—the organism and the actual introduction.

We have already discussed the desire for reduced genomes in model organisms. Taking this to the extreme, growth in the presence of a rich but synthetic and defined medium requires as few as 206 genes, basically comprising the DNA replication, transcriptional, and translational machinery, rudimentary DNA repair functions, protein processing and degradation, cell division, and rudimentary metabolic and energy functions [40]. Toward this theoretical goal, one can either substantially reduce the relatively large genomes of established model systems and exploit the abundance of molecular biology tools for these model organisms, or reduce very small genomes of other organisms in exchange of the requirement to develop novel molecular biology tools.

With respect to the latter, nonpathogenic *Mesoplasma florum* with very attractive cultivation properties and a genome size of 793 kb is currently being established as a chassis. Its genomic sequence has become recently available and molecular biology methods have been developed (http://www.broad.mit.edu/annotation/genome/

mesoplasma_florum.2/Info.html). A similar approach is followed with *Mycoplasma genitalium*, which was already carefully investigated by transposon mutagenesis for nonessential genes [41].

Regarding the former, a prominent example is *Escherichia coli* whose genome has been reduced in various projects by 6 percent [42], 8 percent [43], or 15 percent [44], respectively, without any noticeable effect on the investigated physiological properties and by 30 percent resulting in defects in cell replication [45]. *Bacillus subtilis*' genome has been reduced by 8 percent, again with only minor effects on physiology [46], confirming the observation that under controlled laboratory conditions a substantial part of a bacterium's genome is indeed dispensable.

The complementation of such minimized genomes will inevitably involve the handling of giant DNA. This requires novel methods from storing via faithfully amplifying to insertion in stable fashion into an organism. First steps in this direction have been made recently with the megacloning technique that allowed insertion of a 3.5 Mb *Synecocystis* genome (a photosynthetic bacterium) into the 4.2-megabase genome of *Bacillus subtilis* [47].

11.5 DESIGN AND APPLICATION

There are two areas in which the ideas of synthetic biology, in our view, have already been implemented to a substantial extent—the design of artificial genetic networks and the design of novel production pathways for chemicals. The first topic is intensively covered in Chapter 15 of this book by Greber and Fussenegger, so we will concentrate on the latter subject.

In the production of novel pathways, the benefits of *de novo* DNA design are particularly apparent. Here, suitable designs allow a significant acceleration of the construction process, for example, when, codon usage is from the very beginning optimized for each novel gene and novel DNA elements are suitably structured, for example, by flanking restriction sites, so that the adaptation of the DNA element to novel insights is very simple. This has played a major role in expressing polyketide synthases in *E. coli*, but also in recombining their domains them in such a way that novel polyketides could be produced [48,49].

Another project that catches very much the spirit of synthetic biology is the attempt to construct from scratch a cheap terpenoid production pathway in *E. coli* leading to artemisinic acid, a precursor to the antimalaria drug artemisinin. This goal requires essentially the building of an entire new pathway in a suitable production organism, which in this case is *E. coli* or *Saccharomyces cerevisiae*. A pathway from acetyl-CoA to amorphadiene was created in *E. coli* by splicing the genes of the mevalonate pathway of *S. cerevisiae* into artificial operons and *de novo* synthesizing the amorphadiene synthase gene from the plant *Artemisa annua* [50]. The remaining step from amorphadiene to artemisinic acid required an *A. annua* cytochrome P450 monooxygenase that catalyzes the remaining three oxidation steps and could so far only be functionally expressed in *S. cerevisiae*, so the pathway from the *S. cerevisae* metabolite farnesylpyrophosphate to amorpadiene and then to artemisinic acid was

reconstituted in an *S. cervisiae* mutant engineered for farnesylpyrophosphate overproduction [51].

Although the design of novel biological systems is only beginning, all ingredients of the engineering approach are visible: the role of *de novo* DNA synthesis/fabrication, the design of well-behaved parts on DNA and protein level, the organization of parts into the next functional level of devices and the corresponding abstractions, and the attempt to introduce standardization, even though for the time being only on parts level. With the design of ever more complex systems, the need to emphasize these elements will undoubtedly increase.

11.6 SAFETY AND SECURITY ASPECTS

The reports on the resynthesis of the genomes of the polio virus [35] and the 1918 influenza virus [52] graphically illustrate that large scale *de novo* DNA synthesis might also be used for activities that raise concerns about the safety of synthetic biology, which also have been picked up by the community of synthetic biology researchers (http://openwetware.org/wiki/Synthetic_Biology:SB2.0/Biosecurity_resolutions). If biology is becoming indeed engineerable and we acquire indeed the capabilities to manufacture even more complex systems according to our specifications, then the question that arises is how we are going to manage the safety and security aspects of this potential technological revolution successfully. The answer can be given on two levels—organizational and technical.

From a European perspective—which is the perspective of the two authors of this chapter—the organizational issues appear to be well taken care of. At the moment, synthetic biology does not contain fundamentally new scientific issues that require a reevaluation of the current safety or security standards. Rather, it tries to exploit selected existing concepts to accelerate the progress in the application of biological sciences. Furthermore, even though the agenda of synthetic biology is ambitious and promising, it is for the time being exactly that—an agenda, not a reality. So even if the goal might be the design of complex systems, our current capabilities are much more modest. In addition, the applications that one might have in mind for synthetic biology—for example, a more efficient production of chemicals—will typically lead to strains that are much less fit to survive in natural environments than their none-ngineered counterparts. With these arguments in mind, our view is that there are the rules that apply to genetically modified organisms and just as well apply to the field of synthetic biology. Such experimental work is typically regulated in considerable detail and ethics commissions to evaluate researches that might touch upon the questions of fundamental ethical importance are in place. Where synthetic biology has links to technology that is already working reliably—for example, large-scale *de novo* DNA synthesis, see the cases above—regulations (such as analysis of ordered large DNA sequences) are in place that should prevent the abuse of these technologies in those areas where these regulations can be effectively enforced. Beyond that, it is important to note that even the capacity to produce a viral genome within tolerated error margins does not produce viruses that could be applied. For the time being, such efforts would

face the same problems that all contemporary biological weapons face (storage, distribution, application) and which makes them difficult to manage.

The improvement of safety or security on technical grounds—the question whether synthetic biology could provide techniques that render the field inherently safer—is so far less obvious. Although some suggestions have been made as to how "synthetic biology-engineered systems" could be prevented from interacting with the natural environment—for example, relying on unnatural amino acids that are not available in the environment, introducing unnatural codon amino acid assignments and the corresponding codon sequence in genes, so that important genes could only be translated in correspondingly synthesized organisms—these strategies have to be tested first to confirm that the introduction of such alternate "codes of life" into the environment does not have unexpected consequences. But this example should suffice to illustrate that if there are concerns that synthetic biology might pose a novel safety or security risk, it is also likely that the accelerated development capabilities that would go along with the successful progress of the field would deliver clues on how to address such issues.

In summary, if synthetic biology indeed turns out to be the revolutionary approach that we envision it to be, there might be safety and security questions that we will need to address. For the time being and the foreseeable future, the potential dangers appear to be well within the grasp of existing safety regulations.

11.7 CONCLUSION

Engineering requires a sound knowledge base and exploits a number of distinguishing features such as forward engineering design, abstraction and standardization of components and conditions, and the decoupling of system design from system fabrication. While systems biology hopefully will allow the consolidation of the knowledge base to a sufficient extent, the implementation of these engineering-specific methodological elements into the application of biological systems is in our view the most powerful aspect of the new discipline of synthetic biology. Adoption of these elements would lead to a much accelerated design process that, at some point in the not-so-distant future, will generate biological designs with a very high chance of success and predictability. Crucial elements in the implication of these elements are on the design side suitable computer-based design tools, the successful establishment of standards, the success of the concepts of modularity of parts and orthogonality. On the fabrication side, further progress in the accuracy and the efficiency of large-scale *de novo* DNA synthesis and assembly and the providing of suitably engineered chassis will be the key.

11.8 TEACHING MATERIAL LINKS

- http://www.syntheticbiology.org—synthetic biology community homepage
- http://en.wikipedia.org/wiki/Synthetic_biology—Wikipedia, the free encyclopedia, about synthetic biology

- http://parts.mit.edu/—Registry of Standard Biological parts
- http://www.igem.org/—iGEM (International Genetically Engineered Machine competition) is an international arena where student teams compete to design and assemble engineered machines using advanced genetic components and technologies.

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