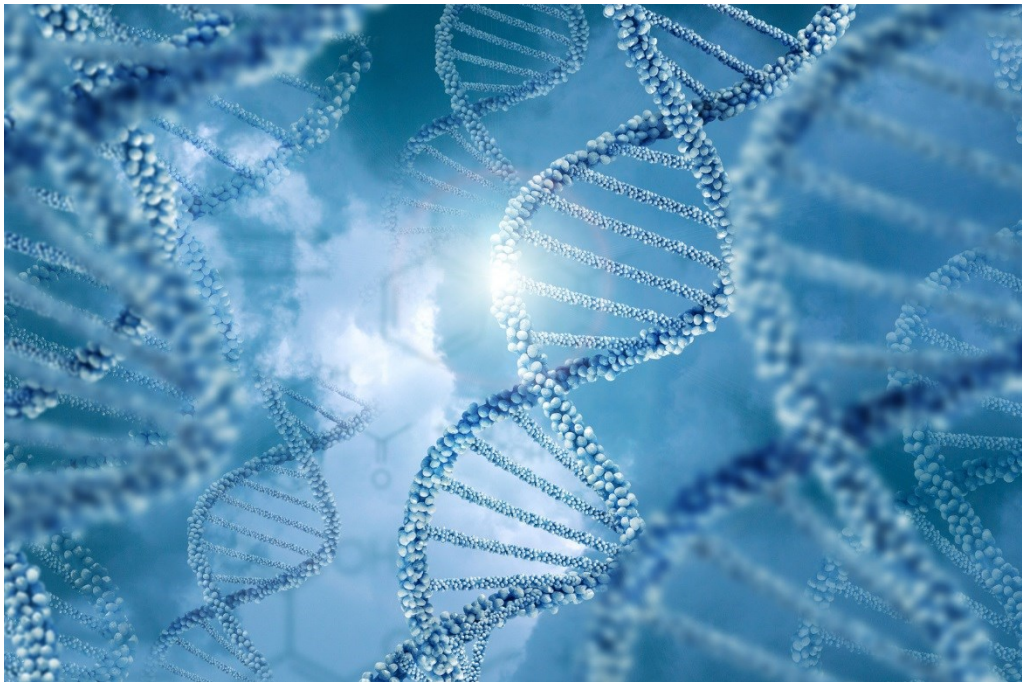
Synthetic Biology



2nd Interim report

of the

German Central Committee on Biological Safety

June 2018

ZKBS
Zentrale Kommission
für die Biologische Sicherheit

Az. 46012

Contents

Summary

In this report, Synthetic Biology is considered a scientific concept in which engineering practice is applied to the construction of novel biological systems and cells at the genetic, biochemical, and physiological level. Synthetic Biology goes far beyond classical biology as it no longer aims at the description and analysis of organisms, but at the design of living entities for novel applications. Such entities process information, alter chemicals, generate materials and structures, produce energy, and maintain and regulate specific biochemical pathways. With the help of Synthetic Biology, researchers wish to produce novel pharmaceuticals, vaccines, or food additives. Synthetic Biology can help relieve pressure from natural resources, improve human health or provide alternatives to fossil-based fuels. Finally, Synthetic Biology allows a basic research approach to study what living is all about.

Since the beginning of the 21st century, the number of scientific publications related to Synthetic Biology has raised from about 500 p.a. to 4000 p.a. in 2017. The progress so far has been summarized in this report along with a discussion on safety considerations for the five major fields that are generally agreed to be part of Synthetic Biology: synthesis of **artificial genes and genomes**, **metabolic engineering** incl. bio-factories, design of **genetic signalling circuits**, **cells with minimal genomes** and **protocells**, and **xenobiology**.

As most of the research approaches in Synthetic Biology generate genetically modified organisms (GMOs), their potential risk can be assessed with existing methodologies as outlined in the German Genetic Engineering Act, the European Directives 2001/18/EC and 2009/41/EC and the Cartagena Protocol on Biosafety to the Convention on Biological Diversity. The insertion of **synthesized genes or genomes** causing genetic modifications that cannot occur naturally by mating and/or natural recombination creates a GMO. The same applies to the introduction of new or alternative **metabolic pathways** into a cells' genome. The installation of **genetic signalling circuits** that sense a specific input and react by a distinct output creates a GMO, as well as the downsizing of an organisms' genome to produce a **minimal cell** only possessing the essential genes needed to survive. Another approach with a similar goal is to create cells from chemical components together with a minimum of genetic information, the so-called **protocells**. Research concerning protocells is still at the beginning. If replicating protocells will be achieved, they would have no natural counterpart that could serve as a basis for a risk assessment relying on the known risk potential of naturally occurring donor and recipient organisms. Concerning **xenobiology**, researchers try to establish bio-orthogonal systems that do not, or to a lesser extent, interact with natural organisms. Orthogonality can be reached by introducing xenonucleic acids or by

expansion of the genetic code, e.g. allowing the insertion of non-canonical amino acids into polypeptides. Since such approaches are based on the genomic introduction of novel tRNAs or aminoacyl-tRNA synthetase genes, these organisms are considered GMOs.

In summary, the research approaches currently pursued in Synthetic Biology in Germany as well as worldwide involve no specific risks for biological safety other than those already being assessed for “conventional” genetic engineering by applying the GenTG and other international regulations.

Zusammenfassung

In diesem Bericht wird die Synthetische Biologie als ein wissenschaftliches Konzept betrachtet, in dessen Rahmen diverse, u. a. ingenieurwissenschaftliche Methoden dafür verwendet werden, auf genetischer, biochemischer und physiologischer Ebene neue biologische Systeme und Zellen zu konstruieren. Die Synthetische Biologie geht dabei über die klassische Biologie hinaus, indem Organismen nicht mehr nur analysiert und charakterisiert werden, sondern lebende Einheiten für neue Anwendungen konstruiert werden. Solche Einheiten verarbeiten Informationen, modifizieren Chemikalien, erzeugen Materialien und Strukturen, produzieren Energie und unterhalten und regulieren spezifische biochemische Stoffwechselwege. Mit Hilfe der Synthetischen Biologie versuchen Forscher, neue Pharmazeutika, Impfstoffe oder Lebensmittelzusatzstoffe zu produzieren. Die Synthetische Biologie kann dabei helfen, natürliche Ressourcen zu entlasten, die menschliche Gesundheit zu verbessern und Alternativen zu fossilen Brennstoffen zu liefern. Auch für die Grundlagenforschung ist die Synthetische Biologie wertvoll, da sie neue Wege der Erforschung des Ursprungs und der Basis des Lebens eröffnet.

Seit Beginn des 21. Jahrhunderts ist die Anzahl der wissenschaftlichen Publikationen, die sich mit Synthetischer Biologie beschäftigen, von etwa 500 pro Jahr auf 4000 pro Jahr im Jahr 2017 angestiegen. Die aktuellen Entwicklungen auf dem Gebiet der Synthetischen Biologie werden in diesem zweiten Bericht der ZKBS zusammengefasst und die Sicherheitsaspekte der fünf Hauptforschungsfelder diskutiert, die generell als Teil der Synthetischen Biologie angesehen werden: Die Synthese **künstlicher Gene und Genome**, die Erstellung **maßgeschneiderter Stoffwechselwege** inklusive Bio-Fabriken, die Konzeption **genetischer Schaltkreise**, das Erzeugen von **Zellen mit Minimalgenomen und von Protozellen** sowie die **Xenobiologie**.

Da die meisten Forschungsansätze in der Synthetischen Biologie gentechnisch veränderte Organismen (GVO) generieren, kann deren potenzielles Risiko mit den bereits existierenden Methoden bewertet werden. Diese finden sich im deutschen Gentechnikgesetz (GenTG), den europäischen Richtlinien 2001/18/EC und 2009/41/EC sowie dem Cartagena-Protokoll über die biologische Sicherheit zum Übereinkommen über die biologische Vielfalt. Das Einfügen **synthetisierter, künstlicher Gene und Genome** führt zu genetischen Veränderungen, die nicht auf natürlichem Weg durch Kreuzen und/oder natürliche Rekombination entstehen können, und generiert einen GVO. Dasselbe trifft auf das Einfügen **neuer oder alternativer Stoffwechselwege** in das Genom einer Zelle zu. Das Einbringen **genetischer Signalwege** in Zellen, sodass diese ein spezifisches Signal wahrnehmen und

mit einer definierten Antwort reagieren können, erzeugt ebenfalls einen GVO. Dies gilt auch für das Verkleinern des Genoms eines Organismus mit dem Ziel eine **Minimalzelle** zu erzeugen, die nur noch die für das Überleben essenziellen Gene aufweist. Ein ähnliches Ziel, aber mit anderer Herangehensweise, verfolgt die Herstellung sogenannter Protozellen, die aus chemischen Bestandteilen und einem Minimum genetischer Information aufgebaut werden. Die Forschung zu Protozellen befindet sich noch in den Anfängen. Sollte es eines Tages gelingen, replizierende Protozellen herzustellen, würde für diese kein natürliches Vorbild existieren. Dementsprechend kann die auf einem Vergleich mit natürlich vorkommenden Spender- und Empfängerorganismen basierende Risikobewertung nicht angewendet werden. In der **Xenobiologie** versuchen Forscher bio-orthogonale Systeme zu etablieren, die nicht bzw. nur in einem geringen Ausmaß, mit natürlichen Organismen interagieren. Orthogonalität kann durch das Einbringen von Xenonukleinsäuren erreicht werden oder durch eine Erweiterung des genetischen Codes, die z. B. den Einbau nicht-kanonischer Aminosäuren in Polypeptide erlaubt. Da diese Ansätze darauf basieren, Gene für neue tRNAs oder Aminoacyl-tRNA-Synthetasen in das Genom zu inserieren, handelt es sich bei den entstehenden Organismen ebenfalls um GVO.

Zusammenfassend birgt die derzeitige Forschung zur Synthetischen Biologie sowohl in Deutschland als auch weltweit keine anderen Risiken für die biologische Sicherheit, als solche, die bereits mithilfe des GenTG und anderer internationaler Regulierungen für „konventionelle“ gentechnische Veränderungen bewertet werden.

1 Introduction

1.1 What is Synthetic Biology

For millennia, humans have been selectively breeding plants and animals with desirable characteristics. The decipherment of the genetic code and its modification as well as the discovery of restriction enzymes in the 1970s, the occurrence of DNA sequencing, DNA synthesis, and DNA transfer protocols finally provided the basis for genetic engineering. Scientists were now able to transfer genetic information associated with useful characteristics from one organism to another as well as to create new modules of DNA from scratch designing organisms with new properties (reviewed in Cameron *et al.*, 2014). Synthetic Biology is going even further by "merging engineering design practice into the construction of biology systems and cells at the genetic level" (Freemont, 2015). The conceptual approach thus applies engineering principles to biology and can employ tools from molecular biology as well as from mathematics, physics, (bio)informatics, chemistry and engineering. While these tools pave the way for new developments in Synthetic Biology, a tool per se cannot be equated with Synthetic Biology. The resulting development, however, can be ranked among Synthetic Biology, if it follows the concept of engineering biology.

The number of publications on Synthetic Biology started to rise during the early 2000 years and has been increasing ever since (fig. 1).

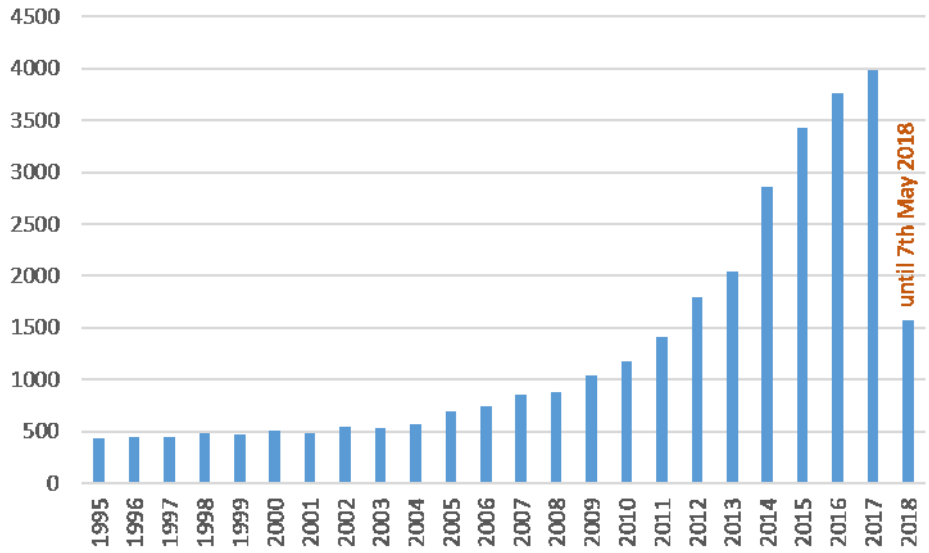


Figure 1: Publications concerning Synthetic Biology
Number of publications listed in NCBI PubMed under the keyword "Synthetic Biology" per year.

Starting with ideas such as the “repressilator” or the “toggle-switch” that meant the partial synthesis of organisms (see chap 2.2), researchers in Synthetic Biology also aim at creating completely artificial organisms. Synthetic Biology benefits from the continuous and rapid improvement and the invention of new tools allowing for significantly more extensive experimentation (fig. 2).

The ultimate goal in Synthetic Biology, beside basic natural sciences research following Richard Phillips Feynman’s statement *„What I cannot create, I do not understand“*[1], is to obtain biological systems with multiple customized applications: Systems include such, which process information, altered chemicals, generate materials and structures, produce energy, and maintain and regulate specific processes. A large focus is on novel pharmaceuticals and vaccines as well as on relieving pressure on natural resources. Some applications have made it to the market (see the “living” inventory of the Synthetic Biology Project of the Woodrow Wilson Center[2]). There is also a diverse community with a dedicated meeting series (SB.X[3]), the International Genetically Engineered Machine (iGEM)[4] competition for students, the “Warwick Integrative Synthetic Biology Centre (WISB)”[5] and in Germany the recently founded “German Association of Synthetic Biology (GASB)”[6], the “Zentrum für Synthetische Mikrobiologie” at the Philipps University Marburg (SYNMIKRO)[7] and the MaxSynBio network[8], which compiles research groups from nine Max Planck Institutes across Germany, as well as the Department of Theology of the Friedrich Alexander University Erlangen-Nürnberg.

[1] http://archives-dc.library.caltech.edu/islandora/object/ct1%3A483

[2] www.synbioproject.org

[3] http://sb7.info/

[4] http://igem.org/Main_Page

[5] https://www.wisb-uow.co.uk/

[6] https://www.synthetischebiologie.org/

[7] www.synmikro.com

[8] https://www.maxsynbio.mpg.de/

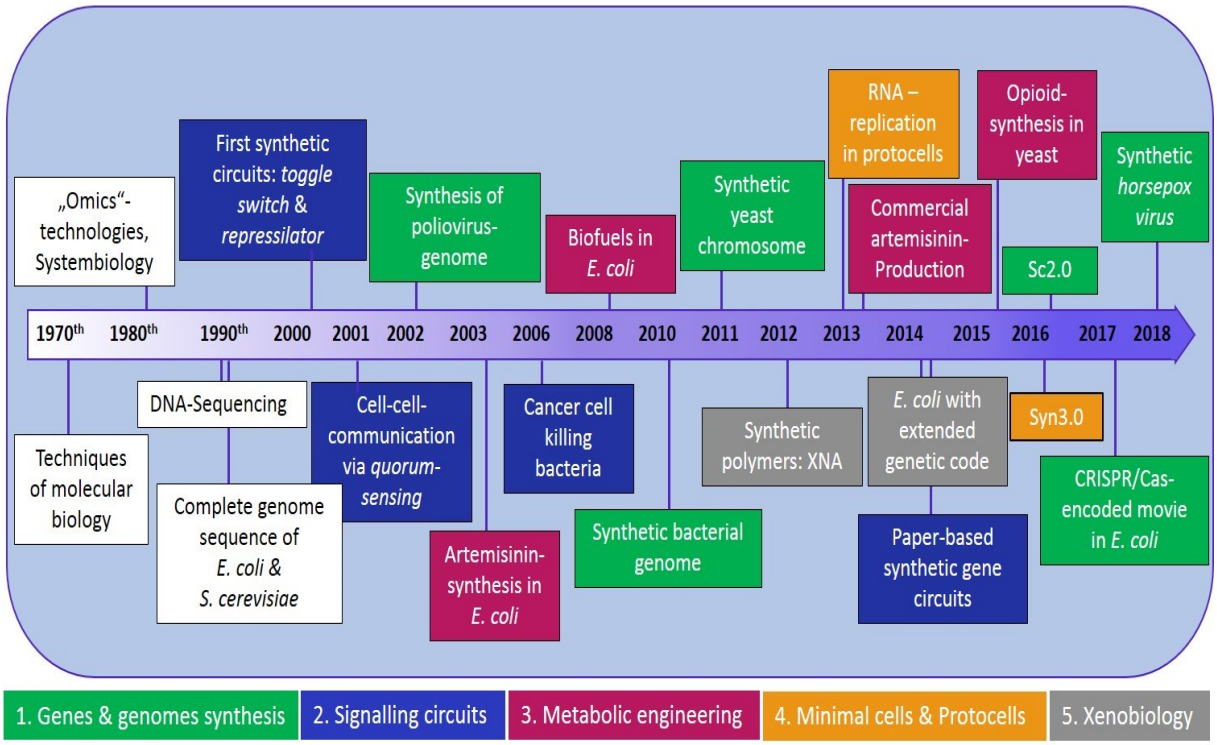


Figure 2: Timeline of the key developments in Synthetic Biology
The development of basic techniques of molecular biology and genomics paved the way for the evolution of Synthetic Biology. After engineering simple modules, research got more and more complex with diverse potential applications, for example in the health sector. The different colors represent the five research fields of Synthetic Biology.

Although a large number of publications on Synthetic Biology exists, there is not a generally accepted definition of Synthetic Biology yet, and no specific regulation with respect to biological safety exists for Synthetic Biology in Germany or Europe. However, the organisms produced with the help of Synthetic Biology are currently considered genetically modified organisms (GMO), for which extensive regulations are already in place. These comprise the European Directives 2009/41/EC and 2001/18/EC on the contained use and on the deliberate release of GMOs that have been implemented in the Genetic Engineering Act (Gentechnikgesetz, GenTG) or the Cartagena Protocol on Biosafety to the Convention on Biological Diversity[9], a multilateral treaty.

[9] https://bch.cbd.int/protocol

1.2 Aim of the report

In this second report on Synthetic Biology, the Central Committee on Biological Safety (ZKBS) summarizes recent activities and the most important developments in Synthetic Biology research worldwide and conducts an assessment on whether these activities and their products pose a threat for biological safety.

The different subfields of Synthetic Biology are analyzed separately with a special focus on essentially two questions:

- Does the respective subfield pose potential risks to biosafety?
- Are the current risk assessment methods for GMOs in Germany/the European Union applicable or are new specific regulations for one or more of the subfields needed?

The report is addressed to the German Federal Ministry of Food and Agriculture as well as to other risk assessors and interested stakeholders. It assesses Synthetic Biology in the first place on the basis of the German GenTG, which is an implementation of the European Directives 2001/18/EC and 2009/41/EC. The conclusions drawn are thus likely to be valid for the European regulations as well.

With this report as a basis, an ongoing, continuously monitoring of advancements in Synthetic Biology will be undertaken. Relevant key papers will be presented on a regular basis on the homepage of the ZKBS[10].

[10] www.zkbs-online.de

2 Research approaches in Synthetic Biology

Regardless of the lack of a universally accepted definition, most stakeholders in the field of Synthetic Biology generally agree on which research subfields are part of Synthetic Biology (tab. 1).

1.	**Synthesis of genes and genomes**
	Encompasses the design and synthesis of artificial genes and synthetic chromosomes up to whole genomes,
	e.g. optimization and synthesis of microorganisms as **vaccines** e.g. design of optimized **chassis organisms** for biotechnological applications and basic research
2.	**Design of genetic signalling circuits**
	In analogy to computer science, circuits with components from different organisms and signalling systems are created in living systems. Upon a predictable interaction a defined *input* leads to a specific *output,*
	e.g. **biological sensors** that respond to environmental stimuli or metabolites in the human or animal body for medical applications e.g. **artificial regulation of gene expression patterns** to be used in microbial biofactories to substantially increase the yields of (synthetically) produced compounds
3.	**Metabolic engineering**
	A variety of genes is introduced into an organism to produce a desired metabolic product,
	e.g. **bio-factories** that produce biofuels or pharmaceutical components e.g. construction of **artificial metabolic pathways** to trap compounds like CO_2 for environmental protection as well as concurrently producing valuable components
4.	**Minimal cells: Genome reduction and production of protocells**
	Simplification of biological systems that only possess essential genes for survival. A special focus is on the generation of a protocell, the simplest artificial chemical model of a living cell,
	e.g. **simplified model organisms** that help to understand the basics of cell function and the emergence of life e.g. easy-to-handle **chassis organisms**
5.	**Xenobiology**
	Aims at the creation of orthogonal systems by altering the genetic code and/or by incorporating non-natural amino acids into proteins,
	e.g. organisms that act as **bio-containment** e.g. proteins with **new features**

Table 1: Overview of subfields in Synthetic Biology

In the following, the ongoing activities of each subfield are summarized and assessed. Synthetic Biology uses a number of tools from different disciplines. The use of a specific tool, however, does not automatically classify the resulting product as Synthetic Biology. As an example, research on **gene drives** is not considered part of Synthetic Biology. A functional synthetic gene drive contains at least one foreign gene, the endonuclease that cuts the DNA at a defined sequence and that can insert a second gene, the cargo gene. The resulting organism is comparable to GMO as defined in the German and European laws on genetic engineering as well as in the Cartagena Protocol on Biosafety to the Convention on Biological Diversity. In Germany, gene drives are assessed on a case-by-case basis by the ZKBS (see position statement with file ref. 45310.0111[11]).

2.1 Synthesis of genes and genomes

Recent advances in technical aspects of **genome editing technologies** and **DNA synthesis** in combination with a massive reduction of costs have dramatically expanded the ability to engineer cells and modify genomes in a directed and combinatorial manner (reviewed in Kim, 2016 and Esvelt & Wang, 2013). Synthetic Biology uses these techniques for the rational design of genes and whole genomes. The use of these tools is not equivalent to Synthetic Biology, as the intended modification can vary between the introduction of point mutations and the creation of entirely synthesized genomes. The concept of Synthetic Biology would, however, not be applicable to genome editing methods in general.

A widely-used tool for multiplexed genome-wide modification is the multiplexed automated genomic engineering (MAGE) developed by Wang *et al.* (2009). MAGE allows for a simultaneous insertion, deletion, or mutation in multiple loci (reviewed in Singh & Braddic, 2015). Another recently developed technique, replicon excision for enhanced genome engineering through programmed recombination (REXER), uses the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system to excise DNA and enables the programmable replacement of genomic DNA in *Escherichia coli* with long (> 100 kb) synthetic DNA. REXER allows for a stepwise whole-genome replacement in *E. coli* in approximately 14 steps (Wang *et al.*, 2016).

[11] https://www.zkbs-online.de/ZKBS/DE/03_Fokusthemen/Gene-Drive-Systeme/Gene-Drive-Systeme_node.html

Recently, Lau *et al.* (2017) massively recoded the genome of *Salmonella* Typhimurium by direct iterative recombineering. The technique uses 10 – 25 kb synthetic DNA constructs that are amplified by a rolling circle mechanism, assembled in yeast and used for iterative recombination in *S.* Typhimurium. As a result, the authors obtained a *Salmonella* strain with 1557 synonymous leucine codon replacements across 176 genes.

Genetic engineering can also be used to maximize the expression of an *in vitro* synthesized gene by using codon-optimization, modifications of the CpG content and/or by introducing other small changes like the removal of repetitive sequences (Parret *et al.*, 2016). In contrast, the de-optimization of a genome (or gene) tries to change specific codons that are used above average in a given species into synonymous rare codons. In this way, attenuated polio- and influenza viruses or *Streptococcus pneumonia* have been designed as potential vaccine candidates (Coleman *et al.*, 2008; Mueller *et al.*, 2010; Yang *et al.*, 2013; Coleman *et al.*, 2011). Genome synthesis has also been used for the fast production of influenza vaccines via reverse genetics, which allowed for the fast production of a vaccine strain against an emerging pandemic virus (Dormitzer *et al.*, 2014), and for the rapid production of a bluetongue virus vaccine (Nunes *et al.*, 2014).

Blackburn *et al.* (2015) demonstrated that the synthesis of genes encoding proteins with novel functions can be coupled directly to high-throughput expression and microfluidic protein analysis (via MITOMI, mechanically induced trapping of molecular interactions). This speeds up protein engineering by completely circumventing molecular cloning and cell-based steps.

Recently, Boles *et al.* (2017) reported on a “digital-to-biological converter”, a tabletop device comparable in its functioning to 3D-printer that receives digitally transmitted DNA sequences and converts them into biopolymers such as DNA, RNA and proteins (yielding e.g. genes, viral genomes or antibodies). Plesa *et al.* (2018) designed “DropSynth”, allowing the low-cost multiplexed synthesis of genes by using a library of barcoded beads that pull down oligonucleotides necessary for the assembly of a desired gene, which are then processed and assembled in water-in-oil emulsions.

DNA assembly is key to constructing gene expression systems, whole chromosomes, or genomes (reviewed in Casini *et al.*, 2015; Hughes & Ellington, 2017). DNA assembly started with restriction enzyme-based cloning techniques like BioBricks and Golden Gate-cloning. Later, simpler and more standardized techniques that reduced the limitations on sequence design were developed. These are scarless restriction enzyme-free cloning and assembly techniques like Gibson assembly, sequence and ligation-independent cloning (SLIC), ligation

cycling reaction (LCR), paper-clip assembly, *in vivo* recombination in yeast and circular polymerase extension cloning (CPEC). High-throughput DNA-assembly methods are on the rise and assembly in micro volumes minimizes the use of costly reagents and enables multiplexed reactions and automation. Patrick *et al.* (2015) presented an easy way to reduce reagent volumes by printing microfluidic devices with a 3D-printer.

Very recently, a new nanopore technology for direct sequencing of “ultra-long” strands (up to 882 kb) of DNA has resulted in the most complete human genome ever assembled with a single technology, closing twelve gaps in the human reference genome (Jain *et al.*, 2018).

The first complete **synthetic genome** synthesized without the use of a template was the 7.5 kb genome of poliovirus (Cello *et al.*, 2002). The first “artificial cell”, JCVIsyn1.0, has been described in 2010, when the 1080 kb genome of *Mycoplasma mycoides* was synthesized and transplanted into a bacterial recipient cell (Gibson *et al.*, 2010). Very recently, Noyce *et al.* (2018) generated a 212 kb infectious synthetic chimeric horsepox virus (scHPXV) by large-scale gene synthesis.

The **international consortium Sc 2.0** aims at synthesizing all 16 chromosomes of the yeast *Saccharomyces cerevisiae* and creating a yeast cell controlled by these artificial chromosomes (reviewed in Maxmen, 2017; Richardson *et al.*, 2017). While the first entire artificial chromosome (chromosome III, 272 kb) was published in 2014 (Annaluru *et al.*, 2014), six chromosomes have been synthesized until now (Richardson *et al.*, 2017). The yeast genome is not just re-built synthetically, but will comprise about 1.1 million changes in order to improve it for basic research and to optimize it for biotechnological applications. The synthetic yeast Sc 2.0 will lack nearly 8 % of the wildtype genome. Non-coding sequences like introns and retrotransposons as well as tRNAs are removed with the tRNAs being regrouped on a 17th neo-chromosome (Richardson *et al.*, 2017). Recombinase sites flanking every gene allow for the novel SCRaMbLE (*Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution*) system of inducible evolution (Shen *et al.*, 2016). Furthermore, all amber-stop-codons are replaced for a future reassignment of *amber* to a non-natural amino acid (see also chap. 2.5).

Another approach to eukaryotic genome synthesis are **mammalian artificial chromosomes** (MAC) (reviewed in Martella *et al.*, 2016), a useful “add-on chromosomal element” for example in gene therapy that has been optimized for the transfer to cultured human cells (Brown *et al.*, 2016a). The latest project in eukaryotic genome synthesis is the **human genome project (HGP)-write** (outlined in Boeke *et al.*, 2016), whose objective was to synthesize all 23 human chromosomes (comprising 3 000 000 kb). Because of ethical

concerns, the project first decided to focus on techniques for genome synthesis rather than concentrating on the human genome. Very recently, another focus shift was announced: the generation of a virus-resistant human cell line, a more technically attainable near-term goal[12]. Such a cell line would facilitate the easy production of certain vaccines, antibodies and other biological drugs without the risk of viral contamination.

Projects on biotechnology-based **genetic rescue** of endangered and extinct species have also used the new genome editing and genome synthesis techniques. An example is the "Woolly Mammoth Revival" aiming to create a hybrid woolly mammoth-Asian elephant embryo as early as 2019 using CRISPR/Cas9[13].

A fascinating new use of the CRISPR technique is the generation of a **synthetic memory** by storing gained information permanently into the genome. This can help to discover what cells experience in their native environment and to understand and diagnose diseases. The first approaches were undertaken with the bistable toggle switch (reviewed in Ho & Bennett, 2018; see also chap. 2.2), until DNA recombinases came into the focus of interest and several regulatory principles were involved to control the "memory". Farzadfard *et al.* (2014) demonstrated a technique for genomically encoded analog memory in living *E. coli* populations based on dynamic genome editing with bacterial reverse transcriptases. As a further development, the same lab presented the mammalian synthetic cellular recorders integrating biological events (mSCRIBE), an analog memory system that enables the recording of cellular events within human cell populations in the form of DNA mutations. The genomic memory in mammalian cells is created by a self-targeting guide RNA (stgRNA) that harbors a protospacer adjacent motif (PAM) in its sequence. The sgtRNA repeatedly targets Cas9 against its own locus causing mutations by error-prone repair resulting in a continuous self-evolving Cas9-stgRNA system (Perli *et al.*, 2016)). By biologically linking the activity of this system to regulatory events of interest, mSCRIBE could be used to study gene regulation events and/or the environmental influences a cell population or organism has been exposed to. Kalhor *et al.* (2016) demonstrated the use in recording a cell's history, enabling e.g. the reconstruction of the lineage of the cells that compose an animal's body.

The use of fewer cells for a sensitive recording was achieved by using DNA base editors and high-copy number plasmids carrying the recorder DNA. This new memory device, called CRISPR-mediated analog multi-event recording apparatus (CAMERA), can record a variety

[12] https://www.nature.com/articles/d41586-018-05043-x

[13] http://reviverestore.org/what-we-do/extinction-continuum/

of environmental, biological and chemical signals like the exposure to viruses and light (Tang & Liu, 2018). A recombinase-based state machine records inputs from a cells' outside by either inverting or excising DNA in so-called DNA registers harboring overlapping and orthogonal recombinase recognition sites. A distinct DNA state for every identity and order inputs can be created (Roquet *et al.*, 2016).

A molecular recording by acquisition of CRISPR spacers was demonstrated by Shipman *et al.* (2016). They showed that the Cas1 and Cas2 proteins from *E. coli* type I-E are sufficient for the integration of 33 bp synthetic oligos (= protospacer) into CRISPR-arrays and that the integration order of protospacers can be reconstituted by sequencing. The system was used to store a short movie by encoding the grey values of image pixels as base sequence inserted into the genomes of a population of living *E. coli* (Shipman *et al.* 2017). Other storage systems like the DNA Fountain that approaches the information capacity per nucleotide have stored a full computer operating system, a movie and other files in cells (Erlich & Zielinski, 2017). Furthermore, audio recordings of "Smoke on the Water" from Deep Purple and Miles Davis' "Tutu" performed at the Montreux Jazz Festival have been stored in and retrieved from DNA[14].

Assessment of the ZKBS

The progress made in genome editing, DNA synthesis and assembly technologies has made the generation of extensively modified genomes considerably easier and even allows the synthesis of whole viral and bacterial genomes or (smaller) chromosomes, which can be pieced together to yield a eukaryotic genome. Yet, the *de novo* design of genomes is not possible; genomes produced *in vitro* are strongly based on natural models, allowing assessment of their risk potential by comparing them with the "donor organism" of the nucleotide sequence (see also position statement of the ZKBS on the risk assessment of *M. mycoides* JCVIsyn1.0, file ref. 6790-05-01-94 of September 2010).

The synthesis of genes or genomes *in vitro* is not within the scope of the GenTG as long as these nucleic acid segments are not introduced into the genome of a living organism.

The introduction of newly synthesized and modified genomes into living organisms is covered by the GenTG, as long as these modifications cannot occur naturally by mating and/or natural recombination.

[14] https://twistbioscience.com/company/press/dna-data-storage-montreux-jazz

2.2 Design of genetic signalling circuits

The introduction of an artificial signalling circuit can produce cells with new biological behaviors, dynamic gene expression and logic control. This is achieved by introducing regulatory components from different organisms that are combined with each other and often orthogonal to establish independent functions (reviewed in MacDonald & Daens, 2016). To simplify and standardize those circuits, freely combinable modules like BioBricks can be used[15].

A reliable control of gene expression is a critical step in gene circuits. Gene expression systems whose regulation is based on small-molecule strategies (e.g. the lactose-operon with the repressor LacI or the tetracycline-resistance operon with the repressor TetR*)* are among the many parts that researchers in synthetic biology use to engineer genetic circuits resembling classical electrical engineering circuits (McDonald & Deans, 2016). Those circuits can act as (toggle) switches (Gardner *et al.*, 2000) or clocks (Elowitz & Leibler, 2000) or result in cells that demonstrate programmable Boolean logic functions acting as computing devices (Miyamoto *et al.*, 2013). The first synthetic genetic oscillator, the “repressilator”, was a circuit in *E. coli* that consisted of a triple negative-feedback loop of sequential repressor-promoter pairs. Activation of the circuit resulted in the ordered, periodic oscillation of repressor protein expression (Elowitz & Leibler, 2000). This circuit was improved recently resulting in robust and long-lasting oscillations (Potvin-Trottier, 2016).

Multi-input decision-making systems increase the specificity of a regulatory circuit and are of particular importance for therapeutic synthetic networks (see below). Guinn & Bleris (2014) developed the first implementation of a biologic decoder in human cells, which is capable of converting two inputs into four outputs. In another approach, multiple light and chemical inputs were processed to generate gradual outputs over two orders of magnitude (Liu *et al.*, 2017a). This system could be used, for example, to express a therapeutic gene dependent on a specific temporal and spatial situation.

Quorum sensing can be used to induce and control dynamic genetic circuits and to bridge the communication from intracellular to population-level (reviewed in Bittihn *et al.*, 2018). Based on quorum sensing, Chen *et al.* (2015) created a synthetic consortium of cooperating *E. coli* bacteria. They built a gene circuit spanning two distinct *E. coli* populations, comprising an “activator” strain and a “repressor” strain that communicate through a pair of intercellular

[15] http://parts.igem.org/Main_Page

signalling molecules and achieve synchronous oscillations. Prindle *et al.* (2011) have tried to reduce the noisy cellular environment by constructing a microfluidic assay in which each bacterial colony communicates via quorum sensing, while the numerous colonies communicate with each other via long-distance gas-phase redox signalling (H_2O_2). This leads to a synchronized oscillation of the colony "biopixels" and is successfully used in a macroscopic biosensor to detect arsenic. Another option for long-distance communication is via electrical signalling (Humphries *et al.*, 2017). The electronic control of gene expression in bacteria via an electrode-driven system has achieved reversible and specific gene control and can be applied to cell mobility or cell-to-cell communication (Tschirhart *et al.*, 2017).

A synthetic **intercellular communication system based on mammalian cells** has been used for creating a nose-inspired cell consortium that is programmable by gaseous fragrances (Müller *et al.*, 2017). Two sensor-sender cells convert the fragrance intensity into diffusible cell-to-cell signalling compounds that are detected by the receiver-digitizer cell type. The latter processes the signals with digital AND, OR and NOR logic and additionally harbors a signal amplifier module to improve signal-to-noise ratio.

Gene expression can also be controlled using **optogenetic circuits** that respond to light and enable a spatial and temporal resolution of gene expression (Olson *et al.*, 2014). In 2015, Gomez *et al.* generated a light-controlled viral gene delivery prototype, whose nuclear translocation is facilitated by light-switchable proteins in the viral capsid that react to red light. Another light-induced system developed by Nihongaki *et al.* (2015) uses CRISPR/Cas9 to photo-activate endogenous gene expression with multiple guideRNAs in a spatiotemporal fashion. Moreover, optogenetic circuits provide a valuable tool to control precisely engineered metabolic pathways and to enhance the biosynthesis of valuable products. Fermentation in *S. cerevisiae* was coupled to light enabling to tune enzyme expression by periodic light pulses (Zhao *et al.* 2018).

Protein expression in cells can also be fine-tuned by **riboregulators** (McDonald & Daens, 2016) and **riboswitches** (reviewed in Ausländer & Fussenegger, 2017 and McKeague *et al.*, 2016), RNA-based regulatory elements that use the RNA's capability to form complex secondary structures and to bind to chemical structures or proteins. While a riboswitch is a regulatory segment of an mRNA that binds a messenger molecule resulting in translation, a riboregulator forms a complementary stem loop structure to prevent translation by restricting access to a ribosome-binding site (RBS).

Protein expression can be post-translationally controlled by **regulating the proteolysis**, for example, by fusing a so-called degron to the protein of interest that only initiates the

degradation when an inactivating peptide is cleaved off. This approach has been applied to create polyproteins containing multiple repressors, whose cleavage led to multiple outputs (Fernandez-Rodriguez & Voigt, 2016).

Lastly, **synthetic transcription factors** have expanded the toolkit with modules to control the expression of genes and dynamically regulate genetic networks. Binding of these synthetic transcription factors is often achieved by zinc finger (ZF) motifs, the transcription activator-like effector (TALE) proteins, or more recently motifs from the CRISPR/Cas9 system (reviewed in Heiderscheit *et al.*, 2018). In 2016, Brödel *et al.* reported on the creation of orthogonal synthetic transcription factors that can flexibly act as either activators, repressors, dual activator-repressors or dual repressor-repressors, thus integrating multiple inputs into promoter logic.

Synthetic gene circuits can be applied in diverse areas. A promising approach are **biological sensors** that detect and respond to environmental stimuli in a predefined way. For example, Daszczuk *et al.* (2014) created a *Bacillus subtilis* strain that is able to detect meat spoilage and to subsequently indicate this with green fluorescence. Other applications are gloves whose fingertips fluoresce upon contact with inducers, e.g. a certain chemical (Liu *et al.*, 2017b) or a sensor that detects and eliminates extracellular mercury contamination (Tay *et al.*, 2017).

Genetic circuits are also applied for **medical diagnostics** and can provide real-time surveillance of diseases to understand their progression and to improve therapy (Slomovic *et al.*, 2015; Saeidi *et al.*, 2011). Kotula & Silver (2014) engineered *E. coli* K12 as a living sensor in the gut. Upon a trigger, a memory element derived from the bistable cI/Cro region of phage lambda, was induced. The cI/Cro memory element exists in either of two distinct states (cI or Cro), and when induced stably retains the Cro state thus detecting and reporting on a distinct health state. To report on the level of the micronutrient zinc, a metabolically engineered *E. coli* strain that produces different pigments in response to different extracellular zinc levels, was constructed (Watstein *et al.*, 2015). Recently, Tastanova and colleagues (2018) developed a Synthetic Biology-based cellular biomedical tattoo for the detection of hypercalcemia associated with cancer. In response to persistently increased blood Ca^{2+} a melanin-generated color change occurs on the skin indicating that the patient should undergo a medical checkup.

Genetic circuits have the potential to generate **new therapeutics** to improve classical medical approaches. Both phages (Barbu *et al.*, 2016) and bacteria (Alvarez & Fernandes, 2017) were genetically engineered to deliver a therapeutic molecule to patients and to treat

(infectious) diseases. *E. coli* cells were modified to sense and kill a pathogenic *Pseudomonas aeruginosa* strain. The bacteria sense the secreted auto-inducer of *P. aeruginosa* and respond with the production of a toxin directed against *P. aeruginosa* (Saeidi *et al.*, 2011). A similar system was generated for killing *Vibrio cholerae* (Jayaraman *et al.*, 2017). A modified version of this system encodes an anti-biofilm enzyme in a probiotic *E. coli* strain that can be used prophylactically and therapeutically against *P. aeruginosa* during gut infections (Hwang *et al.*, 2017).

Engineered bacteria can also be used to **treat cancer** (reviewed in Chien *et al.*, 2017). Hepatic colorectal metastases were reduced in size when treated with a combination of chemotherapy and administration of modified *Salmonella* Typhimurium strain. The bacteria harbored a circuit that resulted in synchronous lysis at a predefined population threshold value and in the release of a genetically encoded therapeutically cargo (Din *et al.*, 2016). A significant shortcoming in many current cancer treatments, however, is the inability to distinguish and eliminate cancerous cells from the surrounding healthy tissue. Living sensors that can discriminate between different cell states could solve this issue. Xie *et al.* (2011) designed a multi-input biosensor that detects the expression profile of different microRNAs (miRNAs), identifies cancerous cells and reacts with the induction of pro-apoptotic genes.

Engineered **mammalian gene circuits** are another option for the targeted treatment of disease. Preferentially, cells with these therapeutic circuits are encapsulated within biomaterials and implanted into the host to isolate the cells from the host tissue preventing immunogenic side effects (reviewed in Haellman & Fussenegger, 2016). Reprogrammed mammalian cells have been successfully validated in animal models of several metabolic disorders including widespread diseases like diabetes (Xie *et al.*, 2016) and insulin resistance (Ye *et al.*, 2017) as well as hyperglycemia and hypertension (reviewed in Teixeira & Fussenegger, 2017).

Diabetes could be treated with glucose-sensing cells. These cells react upon glycolysis-mediated calcium entry via an excitation-transcription system controlling the expression of insulin or the insulin release-stimulating protein glucagon-like peptide 1 (Xie *et al.*, 2016). In a different approach, insulin resistance was addressed with cells activated by high insulin levels that trigger the expression of the therapeutic transgene adiponectin. The system was shown to reverse the insulin-resistance syndrome in different mouse models (Ye *et al.*, 2017).

While the behavior of implanted **optogenetically controllable designer cells** is often controlled by percutaneous illumination, Folcher *et al.* (2014) have shown cell control by a

combination of optogenetics and cybernetics. In their model, the transgene expression was induced by light pulses from a light-emitting diode that is stimulated through mental state-specific brainwaves and a brain-computer interface. In another example, Shao *et al.* (2017) engineered cells that are regulated wirelessly with the help of a smartphone in order to enable semiautomatic glucose homeostasis in diabetic mice.

Human cells have also been used as a **molecular computation** platform with nine different cell populations performing distinct biocomputing operations that were assembled into 3D cultures. This cell consortium executed bio-computing calculations with nearly unlimited parallel-processing capacity (Ausländer *et al.*, 2018).

There is also an effort to use **RNA-only** based circuits in therapeutic applications in order to avoid potentially harmful genomic integrations. Wroblewska *et al*. (2015) designed an RNA-only gene circuit using RNA-binding proteins that can be wired in a plug-and-play fashion to create networks of higher complexity. The system is based on *in vitro*-produced, modified mRNA molecules and works in mammalian cells. RNA-only nanodevices were developed to exhibit ribocomputational operations in living *E. coli* cells (Green *et al.*, 2017).

A new concept is the use of synthetic **diagnostic gene circuits in cell-free systems**. The application of gene circuits on paper allows storage at room temperature and easy re-activation by rehydration. This was applied for the detection of Ebola virus mRNA using toehold riboregulators that induce a color change via gene expression (Pardee *et al.*, 2014). A similar diagnostic test based on the CRISPR/Cas9 system and a toehold switch was developed for Zika virus (Pardee *et al.*, 2016a).

To accelerate circuit design and make it accessible to non-experts as well, the program Cello[16] was developed (Nielsen *et al.*, 2016). Upon submission of information such as DNA sequences for the sensors, information on species, physical implementation, genetic and logic constraints, Cello designs the desired genetic circuit. The program was used to design 60 test circuits in *E. coli* and achieved 92 % correct output states across all circuits.

Assessment of the ZKBS

The creation of genetic circuits involves the (new) combination of accurately defined, usually well characterized DNA segments of different organisms that are introduced into

[16] cellocad.org

the genome of a host organism. These are often model organisms that have long been known in research using them as so-called biological safety measures. Overall, in these studies genetically modified organisms are created that are covered by the scope of the GenTG.

2.3 Metabolic engineering

Microorganisms naturally produce a plethora of substances that are interesting for biotechnological applications. The recent advances in gene and genome synthesis as well as bioinformatics allow for the development of better industrial strains or new interesting substances. Metabolic engineering usually belongs to classical biotechnology. Synthetic Biology, however, with its conceptual approach adapts the metabolic engineering by adding modularization and standardization to realize rational engineering on a broader scale (reviewed in Qi *et al.* 2015; Nielsen *et al.*, 2014).

One of the first milestones of Synthetic Biology has been the production of the antimalarial drug **artemisinin** in *S. cerevisiae* (Martin *et al.*, 2003; Ro *et al.*, 2006). The sesquiterpene naturally extracted from the plant *Artemisia annua* was produced by overexpressing genes from *E. coli, S. cerevisiae, Staphylococcus aureus* and *A. annua* to obtain the intermediate product artemisinic acid, which is then chemically transformed into artemisinin. However, this process was not cost-effective in commercial production (Peplow 2016; Kung *et al.*, 2018). An alternative could be the production of artemisinin in the high-biomass crop tobacco. The insertion of all artemisinic acid biosynthetic genes into the plant was achieved using combinatorial supertransformation of transplastomic recipient lines (Fuentes *et al.*, 2016).

Engineered plants can be used as “protein factories”[17]. The experimental anti-Ebola drug MZapp™, a mix of three antibodies, is usually harvested from an engineered *Nicotiana benthamiana* plant. A current approach promises increasing yields via the production and secretion in *Nicotiana tabacum*[18]. Furthermore, plants are engineered to change their seed oil composition in order to generate novel oils that are more suitable as feed or fuel (reviewed in Haslam *et al.*, 2016).

[17] www.phyllotech.com

[18] https://medium.com/@NSF/bioengineered-plants-help-defend-against-ebola-and-other-deadly-diseases-3f0065acf36f

Biofuels in general and other materials, which usually are generated from mineral oil, are prominent examples for metabolic engineering (Phelan *et al.*, 2015). *E. coli* K12 strains are used to produce isobutanol, fatty acid-based "biodiesel" and gasoline (reviewed in Cameron *et al.*, 2014) as well as biopolymers like poly-3-hydroxybutyrate that shall substitute mineral oil-derived plastics (Kelwick *et al.*, 2015). Similar applications such as the production of butandiol in industrial scale have been commercially marketed[19].

Other products that are or will soon be commercially produced include products from the pharmaceutical sector as well as valuable "fine chemicals" like food, fragrance and cosmetic ingredients. Amyris[20] produces a range of petroleum-sourced products in yeast, e.g. the emollient squalene, which can be a substitute for shark oil. Evolva uses baker's yeast to produce ingredients for the food and beverage industry, personal care and consumer health sectors like stevia sweeteners, nootkatone (the flavour of grapefruit, widely used as insecticide), vanillin and resveratrol (an antioxidant derived from grapes, where it naturally serves as an antifungal compound)[21].

S. cerevisiae has been used for the synthesis of opioids, traditionally produced from opium poppies, by installing a complex pathway involving more than 20 genes coding for enzymes from plants, mammals, bacteria and the yeast itself (Galanie *et al.*, 2015). Another alkaloid, the potential anticancer compound noscapine, has been synthesized in *S. cerevisiae* by using more than 30 genes from plants, bacteria, mammals and yeast (Li *et al.*, 2018). Furthermore, the antibiotic penicillin was synthesized by introducing the complex biosynthesis pathway of a fungus in *S. cerevisiae* (Awan *et al.*, 2017).

Metabolic engineering is also used for **bioremediation** or toxin degradation. The carbonaceous compound of the extremely toxic chemical warfare agent sarin can be catabolized by an engineered *E. coli*. The isopropanol generated is then degraded using the acetone carboxylase complex from *Xanthobacter autotrophicus* (Brown *et al.*, 2016b).

The use of **alternative substrates like methane or CO_2 for microbial growth** has been shown for the cyanobacterium *Synechococcus elongates* PCC 7942. The cyanobacterium was metabolically engineered to produce isoprenoids like amorpha-4,11-diene or squalene from CO_2 via photosynthetic conversion and can serve as a bio-solar cell factory (Choi *et al.*, 2016). A cycle for carbon dioxide fixation was reconstituted by assembling 17 enzymes from

[19] http://www.novamont.com/eng/read-press-release/mater-biotech/

[20] https://amyris.com

[21] http://www.evolva.com/ingredients/

nine different organisms of all domains of life (including bacteria, viruses, archaea, plants and humans) (Schwander *et al.*, 2016). This so-called CETCH 5.4 cycle uses enoyl-CoA esters for carbon fixation, a group of efficient carbon-fixating enzymes that was not selected for autotrophic CO_2 fixation during evolution. The new pathway therefore goes beyond improving or reshuffling existing pathways and adds a seventh, synthetic alternative to the six naturally evolved CO_2 fixation cycles.

Metabolic engineering often has to deal with problems like slow diffusion rates, competing pathways or the secretion of intermediates. In order to increase production yields, metabolic pathways can be relocated into already existing or synthetic organelles, or artificial (protein-) scaffolds and can be used to arrange the enzymes of a single metabolic pathway in complexes (reviewed in Pröschel *et al.*, 2015; Reifenrath *et al.*, 2016).

Exciting applications that go beyond the production of a desired compound use *E. coli* strains that are capable of electrical conduction or that secrete heterologous proteins into mammalian cells. Chen *et al.* (2014) engineered *E. coli* to produce amyloid-based fibrils that can bind gold nanoparticles and quantum dots resulting in a biofilm with the function of electrical conduction. Such biofilms could be externally controlled as electrical switches. Reeves *et al.* (2015) constructed a non-pathogenic *E. coli* strain that harbors the *Shigella flexneri* type 3 secretion system under control of constitutive or inducible promoters that express, secrete, and deliver heterologous proteins into mammalian cells.

Along with the continuous advance in genetic engineering techniques, **computer-aided design tools** are increasingly involved in metabolic engineering (reviewed in Garcia-Ruiz *et al.*, 2018).

Assessment of the ZKBS

As already stated in the first report on Synthetic Biology the technical progress has considerably extended the possibilities of designing tailored metabolic pathways and has even allowed for the design of entire synthetic pathways. To improve existing or to develop novel metabolic pathways, genes are modified and genetic determinants of different organisms are introduced in the genome of an already existing organism. The generation as well as the handling of those organisms are covered by the GenTG.

2.4 Minimal cells: Genome reduction and production of protocells

Research on **minimal cells** can serve two purposes: it can help fundamental research to identify the minimal set of genes and understand basic cellular processes. On the other hand, the knowledge on minimal cells will be used to develop a chassis organism, which is self-replicating, but has the simplest structure and genome possible. Such an organism will be easy to engineer and could serve for a wide variety of applications, e.g. in industrial production processes (reviewed in Acevedo-Rocha *et al.*, 2013).

To minimize an organisms' genome, its essential genes are identified and a minimal cell is constructed by one of two approaches: **top down** or **bottom up**. In the **top down** approach, an existing genome is downsized by stepwise deletion until only the essential genes required for the survival of the organism are left. In the **bottom up** approach the genome comprising the essential genes is synthesized chemically followed by implantation into a surrogate cytoplasm (reviewed in Glass *et al.*, 2017). Typically, used techniques involve CRIPSR/Cas9 and/or recombinant DNA techniques (Martinez-Garcia & de Lorenzo, 2016).

Top down approach: Creation of minimal cells by step-wise genome reduction

The set of universally essential genes is not defined yet, minimal genomes of different organisms investigated so far show a huge variety depending on the organism's ecological niche and the experimental approach applied (Acevedo-Rocha *et al.*, 2013). A frequently-cited comparative genomic study proposed a set of 206 protein-coding genes in a nutrient/chemically-rich environment for endosymbionts already possessing a reduced genome such as *Mycoplasma genitalium* or *Buchnera aphidicola* as well as the biotechnologically relevant species *E. coli* and *B. subtilis* (Gil *et al.*, 2004).

Based on interests in fundamental research, *E. coli* and *Mycoplasma* species have been the most prominent organisms for genome reductions in the last years. Numerous experiments hypothesize that the genome of *E. coli* can be reduced to about 300 genes (out of ~4500 genes in total)[22] (de Lorenzo, 2016; Juhas *et al.*, 2014; Acevedo-Rocha *et al.*, 2013).

For *M. mycoides*, the genome size has been reduced to about 50 % to create ***M. mycoides* JCVI-syn3.0** (Hutchison *et al.*, 2016). The genome reduction was performed based on JCVIsyn1.0 (see chapter 2.1) through a cycle of rational design, full-genome synthesis, genome transplantation, and testing. The final minimal genome (531 kb, 473 genes) is

[22] http://ecoliwiki.net/colipedia/index.php/Essential_genes

smaller than that of any independently replicating bacterium found in nature. Interestingly, the function of ~ 30% (149) of the retained genes is hitherto unknown.

Besides *Mycoplasma* and *E. coli* species, biotechnologically relevant strains have been streamlined and optimized for a better performance: The **Minibacillus project**[23] aims to reduce the genome of *B. subtillis* in a top-down approach and has generated a strain with a 36 % reduced genome (Reuß *et al.*, 2016 and 2017). The strain *Pseudomonas putida* KT2440 was subject to a series of genome deletions (~ 4 %) that erased e.g. phages, flagella genes and a suite of instability determinants that lead to strains with improved industrially relevant traits with an up to 40 % higher yield of recombinant protein than in the initial laboratory strain (Lieder *et al.*, 2015). Recently, the genome of *Corynebacterium glutamicum* was reduced by 13.4 %, while still showing wildtype-like growth behavior (Baumgart *et al.*, 2018).

The international EU-project MycoSynVac[24] aims at engineering *Mycoplasma pneumoniae* as a universal chassis for animal vaccination.

Bottom-up approach: Creation of protocells

In a bottom-up approach, researchers use chemical components to elucidate the origin of life and to create protocell chassis for the application as biotechnological production platforms (reviewed in Caschera & Noireaux, 2014).

A **functional protocell** would be a chemical system capable of self-assembly and self-reproduction. It needs encapsulation, metabolism, growth and reproduction, and should store information in a way that allows for replication and evolution (Caschera & Noireaux, 2014).

Early protocell research has achieved the synthesis of poly-A RNA, RNA template replication, polymerase chain reaction, protein expression and the *de novo* synthesis of lipids inside protocells (reviewed in Jia *et al.*, 2017). Nowadays the building blocks of protocells and their functionalities are becoming more and more diverse and protocell creation is facilitated by the use of microfluidic systems (Elani, 2017).

Artificial membranes are often made up of phospholipid bilayers that are able to encapsulate cell-free transcription-translation (TX-TL) systems (Stano *et al.*, 2013) and are functionalized by integral membrane proteins produced in cell-free systems (Soga *et al.*,

[23] http://www.minibacillus.org/project

[24] http://www.mycosynvac.eu/

2014). Another classical membrane building block are fatty acids and related single-chain lipids that assemble into micelles or lamellar vesicles and show vesicle division when a shearing force or a photochemical impulse is applied (Budin *et al.*, 2012). Vesicles have also been formed from triazole-linked phospholipids in a biomimetic coupling reaction in presence of a copper catalyst. The size of these vesicles was controlled via light intensity in a spatiotemporal fashion (Konetski *et al.*, 2016). Membranes based on amidophospholipids have been produced using chemical or histidine ligation (Brea *et al.* 2014 and 2017) and giant vesicles (GV) were created from phospholipids. The GVs can be divided and form daughter cells. A step to self-proliferation has been taken by fusing these daughter cells with so-called conveyer GVs that restored the substrates depleted throughout division (Kurihara *et al.*, 2015). Examples for non-vesicle protocells are coarcevates made up of a crowded matrix of polysaccharids/polypeptides that have been shown to be capable of cell-free gene expression (Tang *et al.*, 2015), or polymersomes made up of polymers that were able to incorporate the protein expression machinery and to express a bacterial membrane protein (Martino *et al.*, 2012).

The **division of protocells** needs additional membrane compounds and several groups have therefore studied the enzyme-catalyzed formation of new phospholipids inside protocells. The *de novo* synthesis of phospholipids has been approached by synthesizing two membrane proteins in a cell-free system encapsulated in liposomes (Kuruma *et al.*, 2009). Scott *et al.* (2016) have reconstituted phospholipid biosynthesis in liposomes by expressing eight *E. coli* enzymes with the PURE (protein synthesis using recombinant elements) system, which contains all necessary translation factors of *E. coli* in a purified form (Shimizu *et al.*, 2001). Taylor *et al.* (2017) have presented an oil-in-water droplet comprising an amphiphilic imine dissolved in chloroform that catalyzes its own repeated division and thus shows autonomous division. Other approaches have used proteins of the cell division apparatus for protocell division. Most prokaryotic cells depend on the protein FtsZ for division, which assembles into the Z ring at the cell center and acts as a scaffold for the division apparatus. A membrane-targeted FtsZ protein was inserted in vesicles and was shown to self-assemble into constricting Z ring-like structures (Osawa *et al.*, 2008). The direct expression of not only FtsZ but also its interaction partners, the membrane anchoring proteins FtsA and ZipA, inside a protocell also resulted in a morphological change of the vesicle (Furusato *et al.*, 2018). Another approach to artificial cell division is the application of the Min proteins (MinC, MinD and MinE) of *E. coli*. MinD and MinE oscillate between the cell poles, followed by MinC that directs FtsZ to the middle of the cell (reviewed in Zieske *et al.*, 2016). These oscillations have been reconstituted in microdroplets interfaced by lipid

monolayers. When co-reconstituted with a membrane targeted FtsZ-protein, Min and FtsZ proteins were antagonistically localized in the droplets (Zieske *et al.*, 2016).

The **compartmentalization of membrane vesicles** has been addressed by Elani *et al.* (2015), who used vesicles with two compartments separated by a lipid bilayer for distinct biological processes such as the *in vitro* synthesis of green and red fluorescent protein. Karzbrun *et al.* (2014) used a silicon chip to construct artificial DNA compartments capable of protein synthesis that could interact with each other and be supplied with nutrients through thin capillaries.

Protocells functionalization can be achieved by inserting different membrane proteins such as a voltage-dependent anion channel (Tang *et al.*, 2015). Artificial DNA nanopores inserted into lipid bilayers cluster locally and can form membrane protrusions or act as cytoskeletal components by stabilizing autonomously formed lipid nanotubes (Birkholz *et al.*, 2018). The shape of a protocell can also be modelled by encapsulating an actomyosin network into lipid vesicles making them capable of morphological adaptations such as blebbing (Loiseau *et al.*, 2016). The same group also developed a communication system between protocells based on membrane properties (Tang *et al.*, 2018). They built a lipid vesicle as a transmitter cell that upon a small molecule signal expressed a porin protein leading to an efflux of glucose and subsequent substrate signalling. The receiver cell, a proteinosome composed of a glucose oxidase-membrane encapsulating a horseradish peroxidase, reacted with the enzymatic processing of a red fluorescent read-out.

Metabolism in dynamic protocells can be regulated by selectively fusing negatively and positively charged vesicles (Caschera *et al.*, 2011) such as the fusogenic proteoliposomes described by Ishmukhametov *et al*. (2016) that were used to reconstruct a minimal electron transport chain capable of ATP synthesis. In an approach to construct photosynthetic protocells, the photosynthetic reaction center has been reconstituted in the membrane of giant unilamellar vesicles and has been shown to produce a proton gradient upon red-light illumination (Altamura *et al.*, 2017).

Protocells can have different **applications**. They have been shown to replicate viral genomes and synthesize the viral particles, for example of bacteriophage T7 (Shin *et al.*, 2012), encephalomyocarditis virus (Kobayashi *et al.*, 2012) or the Φ29 bacteriophage (van Nies *et al.*, 2018). They have also been used to influence the behavior of natural cells. In a first approach, Lentini *et al.* (2014) used a phospholipid vesicle containing a DNA riboswitch, a transcription-translation machinery and IPTG (a molecular mimic of allolactose that triggers

transcription of the *lac* operon) to influence the behavior of *E. coli* cells. Upon a trigger, the protocells' riboswitch activated translation of the pore protein α-hemolysin and led to the diffusion of IPTG from the artificial cells. IPTG taken up by *E. coli* can then activate the lac operon and trigger expression of *lacZ*, *lacY* and *lacA*, which was measured by qRT-PCR. Later, the group built protocells that can detect *Vibrio fischeri*, *Vibrio harveyi*, *E. coli* and *Pseudomonas aeruginosa* and communicate with the cells via quorum sensing molecules (Lentini *et al.*, 2017).

Protocells have also been used for interactions in a prey-bait fashion using two cellular communities of protocells. Coacervate micro-droplets that contained proteases were mixed with protein-polymer microcapsules containing a payload such as DNA, dextran or nanoparticles. The proteases of the coacervate droplets lysed the proteinosomes and transferred the payload into themselves (Qiao *et al.*, 2017).

While many different approaches to one of the necessary functions have been done, cell-sized compartments harboring all features needed to fulfil the criteria for a functional protocell have not been achieved so far.

Assessment of the ZKBS

In general, the reduced genome of a minimal organism results in a reduced capability to adapt to the environment, correlating with a reduction in fitness and, if applicable, in pathogenicity. Most of these organisms can only survive under defined conditions and do not pose an increased risk to biological safety.

The risk potential of minimal organisms created through targeted downsizing of their genome can be well estimated by comparing these organisms with the parent organisms. This is in line with the GenTG, whose scope covers organisms whose genome has been modified "in a way that does not occur naturally by crossing or natural recombination" (Art. 3 Paragraph 3 GenTG). An example for such an organism is *Mycoplasma mycoides* JCVI-syn3.0. The ZKBS assigned the bacterium to risk group 2. *M. mycoides* JCVI-syn3.0 thus belongs to the same risk group as wild type *M. mycoides* since a potential attenuation achieved by the minimization of the genome was not shown yet (ZKBS, 2017, file ref. 6790-05-01-0094).

The risk assessment according to the GenTG typically relies on the comparison of the GMO with donor and recipient organisms used for the creation of the GMO. The risk of

protocells designed from scratch without taking a natural model as a basis cannot be assessed based on the known risk potential of the donor/recipient organism. Accordingly, the GenTG does not apply to protocells. Self-replicating protocells would require their own assessment criteria and, if necessary, safety measures.
Until today, separate constituents/components of protocells are being investigated (such as a functional cytoskeleton or cell division systems). An autonomously replicating protocell that cannot be compared to a natural organism has not been achieved yet. At present, protocell research is not considered to be associated with any risks to biological safety.

2.5 Xenobiology

Xenobiology is a field in Synthetic Biology that aims at creating **orthogonal organisms** that cannot share information with natural organisms (genetic firewall) and/or that need supplementation with synthetic nutrients (trophic containment). The focus of research is either on the design of alternative nucleic acids (xenonucleic acids, XNAs) made of new base pairs, specific sugars, and modified backbones or on changing the genetic code to incorporate non-canonical amino acids (ncAAs) into proteins (Acevedo-Rocha & Budisa, 2016).

Xenonucleic acids have been created to prevent genetic crosstalk between natural species and genetically modified organisms. These XNAs have a chemical backbone that differs from deoxyribose and ribose and does not interfere with DNA/RNA biosynthesis. Possible XNA-backbones are made of anhydrohexitol (HNA), threose (TNA), glycerol (GNA), arabinose (ANA), cyclohexene (CeNA), 2'-fluoro-arabinose (FANA) and locked nucleic acids (LNA) (Herdewijn & Marlière, 2009; Pinheiro *et al.*, 2012). XNAs have also been shown to be capable of evolution as they can be enzymatically synthetized and reverse transcribed with polymerase mutants derived from the *Thermococcus gorgonarius* polymerase TgoT (Pinheiro *et al.*, 2012).

An **application for XNAs** are aptamers, oligonucleotides that can bind to a specific DNA target and could be used in diagnostics and therapeutics. For example, Matsunaga *et al.* (2015) have incorporated a third artificial base pair into a DNA aptamer, which showed increased stability and a sustainable inhibition of interferon-γ activity.

A form of XNAs are DNAs that contain **non-natural nucleotides** that differ from the four canonical nucleotides (A, T, C and G). Malyshev *et al.* (2014) have designed a plasmid with the **non-natural base pair** d5SICS-dNaM that was successfully replicated in *E. coli*. An additional CRISPR/Cas9-based system that recognizes and cuts plasmids that have lost the non-natural base pair, guarantees the stable propagation of the plasmid (Zhang *et al.*, 2017). Another example for a non-natural base pair is that between Z-P (6-amino-5-nitro-2(1H)-pyridone and 2-amino-imidazo[1,2-a]-1,3,5-triazin-4(8H)one) that when inserted into DNA-duplexes forms helical DNA structures (Georgiadis *et al.*, 2015). GACTZP-containing oligonucleotides were added to a library of random sequences that were selected to bind to liver tumor cells. The GACTZP oligonucleotides were found to bind best to the tumor cells and therefore can add functionality to oligonucleotide libraries (Zhang *et al.*, 2015). Eremeeva *et al.* (2017) have studied the possibility to use a DNA with four non-canonical base pairs termed DZA. The DZA was amplified by the cellular machinery and was shown to be protected from cleavage by restriction endonucleases.

Recoding of the genetic code is used to enable the **incorporation of non-canonical amino acids** (ncAA) into proteins in order to create new properties (reviewed in Acevedo-Rocha & Budisa 2016). An insertion of an ncAA has been achieved already in the 1990s (Kowal & Oliver, 1997) and is now an increasingly observed trend. Different techniques can be used to assign a specific DNA codon to an ncAA. Many groups have practiced the **suppression of a stop codon** (amber - UAG, opal - UGA or ochre - UAA) for the incorporation of ncAA. To liberate a stop codon and incorporate the ncAA, an orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pair has to be introduced into the cell (reviewed in Krishnakumar & Ling, 2014; Acevedo-Rocha & Budisa, 2016). An example for stop codon recoding is the insertion of 3-iodotyrosine at the amber stop codon in *E. coli* (Hammerling *et al.*, 2014). These recoded bacteria were used to propagate the bacteriophage T7, which evolved during propagation to integrate the non-natural amino acid in its proteins and even showed a beneficial mutation in the gene for T7 type II holin. Lajoie *et al.* (2013) changed all 321 amber codons of *E. coli* strain MG1655 to the ochre codon and deleted release factor 1 (RF1) that usually terminates translation of the amber codon. The amber codon was then introduced into essential genes and assigned to the ncAAs p-azido-L-phenylalanine (pAzF) or biphenyl-L-alanine (bipA), resulting in auxotrophic strains showing only few escape mutants (Rovner *et al.*, 2015; Mandell *et al.*, 2015). In another example, a crude lysate from the UAG-deficient strain was used for cell-free protein synthesis to incorporate ncAAs at 40 UAG codons in an elastin-like polypeptide (Martin *et al.*, 2018).

Sense codon recoding, which requires the change of the anticodon of the tRNA, is another option for incorporating non-canonical amino acids. In this approach, a rarely used sense codon is used. Sense codon recoding can be easily done in mycoplasmas. These bacteria lack some of the tRNAs recognizing arginine and rare arginine codons can therefore be recognized by a modified pyrrolysine tRNA/aaRS system. However, if a natural tRNA exists, recoding a sense codon will create competition. Such competition might call for the depletion of the natural tRNA (Krishnakumar & Ling, 2014). Pezo *et al.* (2013) have recoded the tryptophan codon UGG to histidine in a recombinant transketolase gene in *E. coli* and propagated the bacteria for more than 2500 generations. In a current approach, the leucine codons TTA and TTG were replaced with synonymous codons (CTA and CTG) in the *S.* Typhimurium LT2 strain. This led to the replacement of 1557 codons in 176 genes that could be used for reassignment (Lau *et al.*, 2017).

Ostrov *et al.* (2016) have used the codon redundancy to construct an *E. coli* with only 57 codons instead of 64, aiming to produce a biocontained bacterium for industrial applications. They replaced rarely used serine-, arginine- and leucine-codons as well as the amber stop codon with synonymous alternatives, resulting in the replacement of 62,214 codons across all protein-coding genes.

Another method to **expand the genetic code** and to incorporate ncAA more efficiently is to use a quadruplet codon strategy. An orthogonal ribosome has been evolved to decode quadruplets as well as the amber stop codon and with the help of synthetic quadruplet-decoding tRNAs can incorporate numerous ncAAs into a protein (Neumann *et al.*, 2010; Wang *et al.*, 2014).

Other ideas for **creating orthogonality** are artificial ribosomes or chirally different systems. Ribo-T is a functional ribosome based on an rRNA hybrid covalently linked into a single entity that allows the growth of *E. coli* (Orelle *et al.*, 2015). A mirror-imaged polymerase that amplifies chirally mirror-imaged L-DNA was used to assemble the first mirror-imaged gene (Pech *et al.*, 2017).

Assessment of the ZKBS

Xenobiology aims at designing bio-orthogonal systems that do not or only to a lesser extent interact with natural biological organisms. Organisms possessing XNAs are considered as GMO in accordance with the GenTG as these organisms contain a novel

combination of their genetic material, i.e. the XNA.

The introduction of XNAs or the change or expansion of the genetic code in cells in order to incorporate ncAAs (involving the expression of novel tRNAs or aminoacyl tRNA synthetases) creates organisms whose genome have been modified in a way that could not occur naturally by mating and/or natural recombination. These organisms therefore fall under the scope of the GenTG. These modifications, as well as the introduction of orthogonal/artificial ribosomes or chirally different systems are not expected to be associated with any additional risks to biological safety.

The approaches in xenobiology are rather associated with an increase in biological safety by limiting the expression of the proteins to defined conditions in the laboratory (e.g. the supplementation of a specific ncAA) or to specific organisms equipped for this purpose (e.g. with the appropriate tRNA-/aminoacyl tRNA synthetase system).

In conclusion, the current approaches pursued in Synthetic Biology are mainly covered by the GenTG, the exception being modifications made to the genome that could occur naturally, DNA synthesis, and individual subfields of artificial cell research, such as the investigation of bacterial cell division systems that take place *in vitro*, i. e. outside living systems. These experiments involve no specific risk potential, since they do not employ viable organisms. At present, the production of self-replicating biological systems is not yet possible. For such novel living systems that do not have a natural model, no generally accepted assessment criteria exist or the assessment criteria set forth in the GenTG are not applicable. The progress in this area is assessed with a case-by-case approach and might require an extended risk assessment in the future.

3 Research trends and social discussion

As pointed out in the preceding text, Synthetic Biology is a varied and diverse research field. Trends in research include standardization, automation, and computational modelling, making Synthetic Biology easier and more predictable.

Standardization is using BioBricks[25], standardized genetic modules, or the Synthetic Biology Open Language (SBOL), an open standard for the representation of and communication on *in silico* biological designs.[26] **Automation** refers to software tools for designing, building, testing, and analyzing biological systems. An overview of the existing software tools is given in Appleton *et al.* (2018), while Myers *et al.* (2017) describe a standard-enabled workflow for Synthetic Biology.

The web-based Wet Lab Accelerator (Bates *et al.*, 2016) even allows scientists to execute **robotic wet lab protocols** without requiring any background in scripting. The experiments are performed in cloud labs that offer access to automation platforms and other lab tools.

Potential ethical, legal and social implications of Synthetic Biology and its future applications as they can affect human health have been addressed and there are ongoing discussions about the potentials and biosafety/biosecurity risks (reviewed in Douglas & Stemerding, 2015; Voigt, 2017).

[25] https://biobricks.org/

[26] http://sbolstandard.org/

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