Chapter 6
Do I Understand What I Can Create?

Biosafety Issues in Synthetic Biology

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Contents

Abstract Synthetic biology offers many new opportunities for the future. The increasing complexities in engineering biological systems, however, also puts a burden on our abilities to judge the risks involved. Synthetic biologists frequently cite genius physicist Richard Feynman "What I cannot create I do not understand". This leitmotiv, however, does not necessarily imply that "What I can create, I do understand", since the ability to create is essential but not sufficient to full understanding. The difference between having enough knowledge to create a new bio-system and having enough knowledge to fully grasp all possible interactions and its complete set of behavioural characteristics, is exactly what makes the difference for a sustainable and safe development. This knowledge gap can be closed by applying adequate and up-to-date biosafety risk assessment tools, which -in their majority – have yet to be developed for the major subfields of synthetic biology (DNA-based biological circuits, minimal genomes, protocells and unnatural biochemical systems). Avoiding risk is one part, the other one should be to make biotechnology even safer. This aim could be achieved by introducing concepts from systems engineering, especially from safety engineering, to syntheic biology. Some of these concepts are

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presented and discussed here, such as Event Tree and Fault Tree Analysis. Finally the impact of the de-skilling agenda in synthetic biology – allowing more and more people to engineer biology – needs to be monitored, to avoid amateur biologists causing harm to themselves, their neighborhood and the environment.

6.1 Introduction

Fast becoming one of the most dynamic new science and engineering fields, synthetic biology has the potential to impact many areas of society. Synthetic biologists may use artificial molecules to reproduce emergent behaviour from natural biology, with the goal of creating artificial life or seeking interchangeable biological parts to assemble them into devices and systems that function in a manner not found in nature (Benner and Sismour 2005, Endy 2005, Heinemann and Panke 2006, Luisi 2007, Serrano 2007). Approaches from synthetic biology, in particular the synthesis of complex, biological systems, have the capacity to change the way we approach certain key technologies and applications in biomedicine (e.g. in-vivo synthesis of pharmaceuticals, vectors for therapy), biochemistry (e.g. extension of the genetic code, non-natural proteins, bio-orthogonal reporters), environment (e.g. bioremediation, GMO biosafety), energy (bio-hydrogen production), defense against biological weapons, or materials science (e.g. for information technology, biosensors) (European Commission 2005). Its potential benefits, such as the development of low-cost drugs or the production of chemicals and energy by engineered bacteria are enormous (Ro et al. 2006, Keasling 2008).

There is, however, also the possibility of causing intentional or accidental harm to humans, agriculture or the environment. While deliberate damage is dealt with under the heading biosecurity, the potential unintended consequences have to be considered under the term biosafety. The difference between the English terms safety and security is hardly manifested in other languages (see Table 6.1). In the future, other more comprehensive terms could be used such as bioprotection or biopreparedness (see e.g. FAO 2002).

6.1.1 Biosafety vs Biosecurity

According to the WHO (2004) biosafety is the prevention of *unintentional* exposure to pathogens and toxins, or their accidental release, whereas biosecurity is the prevention of loss, theft, misuse, diversion or *intentional* release of pathogens and toxins.

In the past novel (bio-)technologies have often raised the suspicion that they might not only be useful but also cause potential unexpected and unwanted effects. Scientists and engineers have worked to avoid altogether or at least minimize unintended consequences in order to make the technology useful and safe. The motivation of many scientists to look into biosafety issues in synthetic biology is

Table 6.1 Conflation of safety and security is common in non-English languages

English	German	French	Spanish	Russian	Chinese[1]
security	Sicherheit	sécurité	seguridad	безопасность	安全
safety	Sicherheit	sûretè	seguridad	безопасность	安全

re-inforced by the negative public reactions towards GMOs in Europe (Serrano 2007). In Europe – probably in contrast to the US – the general public, the media, civil society organizations and most scientists could be concerned about safety issues of synthetic biology (Schmidt 2006, de Vriend 2006, Kelle 2007, Kronberger 2008[2]). Although it is possible that scientific assessment and subsequent management of biosafety issues is most likely not sufficient to see public acceptance for each and every technique and application, it is still necessary to conduct biosafety risk assessment as a basis for further decision making.

6.1.2 The Different Flavors of Synthetic Biology

As a pre-requisite to further biosafety work we have to be clear about the novel issues that accompany synthetic biology, and try to distinguish as clearly as possible the issues that arise in synthetic biology from those associated with other life science activities. The best way to start is to have a clear definition or at least a working definition of synthetic biology. Several definitions exist on synthetic biology, however, the one that has received the most attention describes synthetic biology as "the design and construction of new biological parts, devices, and systems, and the re-design of existing, natural biological systems for useful purposes."[3] This definition clearly reflects the MIT approach to synthetic biology and the idea to develop a registry of standard biological parts that can be assembled to devices and systems at will. Although the MIT agenda has certainly sparked the development of the whole field, e.g. by organizing the first international Synthetic Biology Conference in Boston in 2004, or by supporting the Biobricks Foundations that runs the annual iGEM competition, it however tends to omit other important areas in synthetic biology, especially when it comes to the design of non-existing and/or unnatural biological systems (see Table 6.2 for an overview). Carefully screening the literature and talking to several dozen synthetic biologists the conclusion can be drawn that synthetic biology includes the following subfields:

[1] However, according to biosecurity experts in China, *shengwu anquan* means biosafety and *shengwu anbao* means biosecurity (Qiang 2007)

[2] Results of focus groups in Austria carried out in September 2008, personal communication by Nicole Kronberger.

[3] See: http://syntheticbiology.org/Who_we_are.html accessed at November 6, 2008

Table 6.2 Characteristics of the main science and engineering areas commonly found under the heading synthetic biology (Benner and Sismour 2005, Glass et al. 2006, Heinemann and Panke 2006, Luisi 2007, O'Malley et al. 2008)

	Brief description of the four subfields in synthetic biology			
	DNA-based bio-circuits	Minimal genome	Protocells	Chemical synthetic biology
Aims	Designing genetic circuits, e.g. from standardised biological parts, devices and systems	Finding the smallest possible genome that can "run" a cell, to be used as a chassis, reduced complexity	To construct viable approximations of cells; to understand biology and the origin of life	Using atypical biochemical systems for biological processes, creating a parallel world
Method	Design and fabricate; applying engineering principles using Standard parts and abstraction hierachies	Bioinformatics-based engineering	Theoretical modeling and experimental construction	Changing structurally conservative molecules such as the DNA
Techniques	Design of genetic circuits on the blackboard, inserting the circuits in living cells	Deletion of genes and/or synthesis of entire genome and transplanting the genome in a cytoplasm	Chemical production of cellular containers, insertion of metabolic components	Searching for alternative chemical systems with similar biological functions
Examples	"AND" gate, "OR" gate; genetic oscillator repressilator; Artemisinin Metabolism, "Bactoblood"	DNA-Synthesis and transplantation of *Mycoplasma genitalium*	Containers such as micelles and vesicles are filled up with genetic and metabolic components	DNA with different set of base pairs, nucleotides with different sugar molecules

(1) Engineering DNA based biological circuits, by using e.g. standard biological parts;
(2) Finding the minimal genome;
(3) Constructing protocells, in other words, living cells from scratch; and
(4) Chemical synthetic biology, creating orthogonal biological systems based on a biochemistry not invented by evolution.

Some other research fields also tend to be included, although they have a more supportive role to the four fields mentioned above, helping to reach the goal of engineering biological systems. Among the two most important supporting technologies we find are: (1) ever more cost-efficient DNA synthesis; and (2) a growing number of computational biology tools.

DNA synthesis, carried out by specialized DNA synthesis companies, allows outsourcing for researchers and thus reducing cost and time needed to acquire a specific DNA gene sequence. Advances in synthesis technology also lead to increased accuracy and reliability, and decreasing cost of DNA constructs. The complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome (about 580 kb), published by Gibson et al. (2008) clearly shows the technological potential and what might be possible in the not so distant future. Bioinformatics on the other hand catalyzes SB research by providing tools for simulation and in-silico testing of biological systems. This includes for examples attempts to calculate genetic circuits by automated design (Jaramillo 2008), or software to design and later predict stability of so-called never-born-proteins (Evangelista et al. 2007).

On some occasions more advanced forms of synthetic biology are named too, namely synthetic tissue engineering and synthetic ecosystems (engineered ecosystems on the basis of SB engineered organisms).

This chapter will mainly focus on the novel biosafety aspects in relation to the four subfields mentioned above, as these are seen as the most relevant ones for the time being.

6.2 Biosafety Issues

Starting from this working definition and naming the most relevant areas in synthetic biology, we can now provide a preliminary list of biosafety challenges that may arise at various levels and at various times in the development of the field. Relatively few papers discussing biosafety have been published so far (see e.g. Church 2005, Tucker and Zilinskas 2006, Fleming 2006, Garfinkel et al. 2007, risk assessment has also been discussed by the NSABB[4]) although frequent calls to address safety issues in synthetic biology have been voiced at conferences, meetings etc. by scientists

[4]See: NSABB (2007) Roundtable on Synthetic Biology. October 11, 2007. National Science Advisory Board for Biosecurity. http://www.biosecurityboard.gov/Annotated%20Agenda% 20Website.pdf

and non-scientists, as well as research funding agencies (e.g. European Commission 2005). Given the small number of publications on this subject so far, this analysis is mainly based on interviews with 20 key European synthetic biology scientists and research carried out as part of the SYNBIOSAFE project.[5] Three main areas have been identified that seem to contain relevant biosafety issues in synthetic biology:

(i) improving risk assessment,
(ii) establishing biosafety engineering and
(iii) diffusion to amateur biologists.

The three issues will be discussed according to the relevant synthetic biology subfields as shown in Table 6.2.

6.2.1 Risk Assessment

Proper risk assessments methods are needed to be able to assess the risks involved in any biotech activity in order to decide whether or not a new technique or application is safe enough for the laboratory (Biosafety Level 1 to 4), or for commercialization in the area of medical diagnostics and therapy, pharmaceuticals, food, feed, agriculture, fuel, industrial applications, and bioremediation, requiring the release of novel organism or products thereof.

It is clear that the last decades have brought a lot of insights into safety issues of Genetically Modified Organisms (GMOs) and this knowledge forms the basis for current risk assessment and biosafety considerations today. When these risk assessment methods where developed, the currently foreseen SB approach was probably considered as rather utopic. Therefore we need to ask if the current GMO risk assessment practice is good enough to cover all developments under the label "synthetic biology" in the upcoming years. The following examples seem to warrant a review and adaptation of current risk assessment practices:

(i) DNA-based biological circuits consisting of many DNA "parts";
(ii) Surviveability of novel minimal organisms – used as platform/chassis for DNA based biocircuits – in different environments;
(iii) Exotic biological systems based on an alternative biochemical structure

6.2.1.1 DNA-based Biocircuits

Among the most recent statements on the state of the art of risk assessment of GMOs was the meeting paper for the Fourth Meeting of the Conference of the Parties serving as Meeting of the Parties to the Cartagena Protocol on Biosafety, that took

[5] See: www.synbiosafe.eu

place in Bonn, in May 2008 (CBD 2008). In Chapter III.17 it says *"Further it was agreed that all risk assessments of living modified organisms should be conducted on a case-by-case basis as the impacts depend upon the trait inserted, the recipient organism, and the environment into which it is released."* This description reveals that developments in SB could lead to significant gaps, despite the risk assessment framework presently in place for GMOs. One of the differences between genetic engineering and SB is that instead of single parts, whole systems can be transferred, potentially using hundreds or thousands of traits (genes/parts) from different donor organisms (see Fig. 6.1). Emergent effects in the creation of synthetic genetic circuits could cause problems in the design process and create new uncertainties, so it is important to analyse whether the established risk assessment practice is capable of dealing with these multiple hybrids. The answer is that it cannot deal with such biocircuit systems. Instead of "just" having to assess how the new genetic element behaves in the new cell in a particular environment, now it is necessary to assess also the interactions among the many genetic parts themselves, that were inserted into the cell. These interactions will have no comparable counterpart in nature, making it more difficult to predict the cell's full behavioural range with a high degree of certainty.

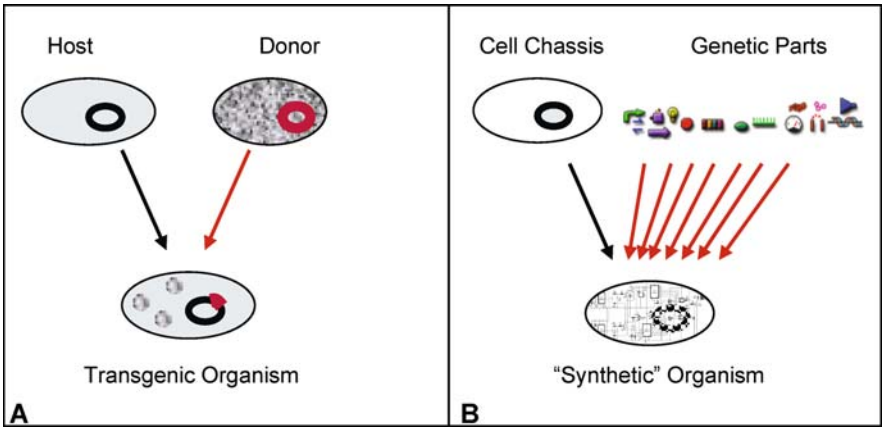

Fig. 6.1 Schematic description of the differences between transgenic organisms derived from genetic engineering (**A**) and potential future "synthetic" organisms derived by assembling genetic parts into circuits and implanting them into a minimal genome, a so-called cell chassis (**B**). Current risk assessment practices may well work for (**A**) but not for (**B**)

Several new challenges arise from such systems, if we assume that the biological system has been designed and inserted into a host (or chassis).

Predictability: Can behavioural characteristics of the new network be predicted to a degree of certainty that allows a reasonable estimation of risk factors?

Evolutionary forces: What happens to the network if one or several parts change their function or stop working as intended? How will the whole network change its characteristics?

Robustness: How can the genetic/functional robustness be measured? What would be a meaningful and suitable "unit" for robustness in bio-circuits? Do different forms of applications require different levels of robustness (i.e., cells in an industrial fermenter vs cells in human body e.g. for insulin control)?

Reliability: How reliable is the biological circuit? How can reliability be measured? And what are meaningful units?

Hazard: Could there be an unplanned event or series of events resulting in death, injury, occupational illness, damage to or loss of equipment or property, or damage to the environment?

Limits of the analogy to electronic circuits: How robust are orthogonal bio-circuits designed to avoid crosstalk between functional elements of its circuit?

Thinking into the future, the following questions could arise.

Engineering complexity: How to deal with new bio circuits that involve deliberately engineered complex behaviours such as non-linearity, path depended behaviour, randomisation, or chaotic characteristics? Will it be possible to program a cell that can reprogram itself?

A biological toolbox such as the MIT based Registry of Standard Biological Parts[6] using parts, devices and systems, almost automatically raises these kinds of safety questions (Schmidt 2008).

Parts: There might be a need to think about safety standards when dealing with these parts: Some parts will be more of a safety problem than others so different safety categories should be used for parts. The simplest example would be a part that encodes for proteins that interfere negatively with human physiology. The safety categorization of parts would best be based on the conventional BSL 1 to 4 levels.

Devices and systems: A gene circuit could exhibit different safety characteristics than the parts it is based upon. Thus different safety categories should also be used for devices and systems.

Cell chassis enhancement: Parts that extend the environmental range of a cell chassis, by increasing for example the tolerance of relevant biotic and abiotic conditions, should be considered in a special safety category.

Biosafety clearinghouse: How can a safety issue be reported that was discovered in a certain bio-circuit and that was not foreseen (emergent) so other people can learn from that experience?

Provision: How can safety and security aspects be integrated into the design process so the design software automatically informs the designer in case the newly designed circuit exhibits certain safety problems?

[6] See: http://partsregistry.org/Main_Page

So far the datasheets on registered biobricks parts hardly contain explicit information on safety. Only the reliability of simple parts has been included so far, distinguishing genetic reliability[7] and performance reliability[8] that describe the number of generations it takes to cripple 50% of the circuits in the cells (Canton et al. 2008). Although this is clearly a first step towards a more comprehensive safety characterization of biological circuits, there is still a long way to go before the safety characterizations may eventually be the basis of a proper risk assessment process deciding whether or not such a biocircuit is safe enough for commercialization or release into the environment.

6.2.1.2 Minimal Genome

Organisms with a highly reduced set of genes and physiological functions will by definition be restricted to a very narrow ecological niche. Therefore the minimal organism with a minimal genome is per-se a safe organism as it can only inhabit particular environments and will not be able to exist outside of these. To proof this limited viability it would, however, be useful to carry out a number of trials deploying the minimal cell in environments that differ from its original optimal environment in order to acquire some real experimental data on the range of suitable environments for the minimal organism. Based on these trials better predictions could be made about its real environmental host range (see Oye and Yeddanapudi 2008).

Further evaluations will be necessary for minimal organisms that have novel biological circuits (such as parts, devices, systems) implanted. These "synthetic organisms" (see Fig. 6.1) cannot be considered to be minimal organisms, and care has to be taken in case the implanted biological circuit helps to enlarge the environmental niche of the cell, either deliberately or without this intention.

6.2.1.3 Protocells

The search for the minimal genome looks top–down for a minimal version of life by reducing an existing genome until it cannot any longer sustain living processes. The protocell approach however, attempts to create life from the bottom–up, by assembling relevant and necessary biological subunits in a way that "life" emerges out of it. So far only partial success has been achieved with this approach reflecting the many difficulties accompanying this endeavour. (e.g. Szostak et al. 2001). But regardless of whether protocells actually fulfill all requirements necessary to be considered "alive", they can still be of interest here. As such cells show some but not all of the characteristics of life (compartimentalisation, growth, metabolism, evolution, reproduction, replication, autopoesis, response to stimuli), they can be considered as "limping cells" (Luisi 2006 personal communication).

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[7] Genetic reliability: The number of culture doublings before a mutant device represents at least 50% of the population.

[8] Performance reliability, The number of culture doublings before 50% of the population is unable to correctly respond to an input.

Natural forms of limping cells that rely on other cells (and sometimes vice–versa) for suvival, can be seen in mandatory endosymbionts such as organelles (chloroplast, mitochondria), or mandatory exosymbionts such as *Nanoarchaeum equitans* (Waters et al. 2003, Keeling 2004). Although not a cell in the classical sense, the extremely large Mimivirus, that can even be infected by a so-called virophag, could be an interesting point of reference (Raoult and Forterre 2008, La Scola et al. 2008). Other more dubious forms of life on the brink of life were allegedly found in recent years, such as nanobes or nanobacteria, but with an unclear scientific basis (see e.g. Urbano and Urbano 2007).

It could be that a protocell is first realized as a mandatory symbiont to natural forms of life before it is able to survive all by itself. Should that happen, then the host range needs to be identified to avoid unlikely but not impossible "infections" by protocells, especially if they are very different from natural cells.

Although there is currently little evidence that protocells will cause major safety risks, developments in that field need to be watched in case a breakthrough in creating "life from scratch" is going to happen anytime soon.

6.2.1.4 Chemical Synthetic Biology

Scientists working on the origin of life have frequently asked the question why life as we know it has evolved the way it is and not differently. Based on the idea that life could have evolved differently, scientists now try to design and create life forms – or at least biological systems – based on unnatural biochemical structures. The focus of their efforts has been to come up with alternative biomolecules to sustain living processes. Areas of research include for example the chemical modification of DNA, polymerases, amino acids and proteins. One area of research is the identification of amino acid sequences (proteins) that have a stable architecture but do not occur in nature. As there is only a tiny fraction of theoretical possible proteins actually occurring naturally, with many more possible but not yet born proteins, so-called "never-born-proteins" that could provide a lot of useful novel functions for molecular biology (Luisi et al. 2006, Luisi 2007, Seelig and Szostak 2007).

Changing the translational mechanism (from mRNA to proteins via tRNA and the ribosome) is another focus of interest. For example, a mutant *Escherichia coli* tRNA synthetase was evolved to selectively merge its tRNA with an unnatural amino acid. This tRNA could sitespecifically incorporate the unnatural amino acid into a protein in mammalian cells (Liu et al. 2007).

Another area of work consists of modifying DNA by replacing its chemical building blocks, especially the sugar molecules and the base pairs. The attempts to come up with an unnatural nucleic acid consisting of a different backbone molecules resulted in novel informational biopolymers such as: Threose Nucleic Acid (TNA), Glycol Nucleic Acid (GNA), Hexitol Nucleic Acid (HNA), Locked Nucleic Acid[9]

[9]The LNA is a nucleic acid analogue containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA mimicking sugar conformation.

(LNA), or PNA: Peptide Nucleic Acid. (Chaput et al. 2003, Zhang et al. 2005, Vandermeeren et al. 2000, Ng and Bergstrom 2005, Schoning et al. 2000, Kaur 2006, Orgel 2000, Vester and Wengel 2004).

Replacing or enlarging the genetic alphabet with unnatural base pairs resulted for example in a genetic code with 6 instead of 4 base pairs (Sismour et al. 2004, Yang et al. 2006) and of up to 60 potential base pairs tested for possible incorporation in the DNA (Leconte et al. 2008).

These unnatural nucleic acids cannot be recognized by natural polymerases, and one of the challenges is to find/create novel types of polymerases that will be able to read the unnatural constructs. At least on one occasion a mutated variant of the HIV-Reverse Transcriptase was found to be able to PCR-amplify an oligonucleotide containing a third type base pair. Only two amino acids must be substituted in this natural polymerase optimized for the four standard nucleotides to create one that supports repeated PCR cycles for the amplification of an expanded genetic system. It is without doubt surprising to find a useful polymerase to be so close in 'sequence space' to that of the wild type polymerase. (Sismour et al. 2004)

Currently no living organisms based on such an unnatural nucleic acid exists and there is little evidence for anything like it to occur anytime soon. But the combination of an extended genetic code and an adequate novel polymerase could certainly lead to the next step towards implementing an artificial genetic system, for example in *E. coli.* (Sismour et al. 2004) Although it is unclear when – if at all – such unnatural organisms will be created, we should still ask how we could assess the potential risk that these alien organisms could present.

An utopic worst-case scenario would be for example the arrival of a novel type of virus based on a different nucleic acid and using an unnatural reverse transcriptase.

Another worst-case scenario would be an organism based on an enlarged genetic alphabet that can avoid natural predators at all, enabling almost unrestricted spread.

6.2.2 Biosafety Engineering

Synthetic biology is said to change biotechnology into a true computable, controllable and predictable engineering discipline. Some people have even proposed the term "intentional biology" instead of synthetic biology in order to underline the engineering approach, to get rid of all the unintended consequences in biological systems (Carlson 2001). Biosafety in fact deals with these unintended consequences, or rather, to put it more precisely it deals with avoiding these unintended consequences. Thus synthetic biology could be understood as the ultimate biosafety tool. So far so good, the only downside is that it is still a long way to go before we come even close to controling all biological processes in an engineered system. It is even likely that we will never be able to reach this goal completely, due to the stochastic and probabilistic character of the underlying biochemical processes. Nonetheless synthetic biology holds the potential to make biology not only easier but also safer to engineer.

Safety engineering is already an established subset of systems engineering in many engineering disciplines (e.g. mechanical engineering, aviation, space flight, electronics, software). (System) safety engineering is an engineering discipline that employs specialized professional knowledge and skills in applying scientific and engineering principles, criteria, and techniques to identify and eliminate hazards, in order to reduce the associated risks (DoD 2000). Safety engineering assures that a system behaves as needed even when parts of it fail. This is more than needed in synthetic biology due to the evolutionary patterns of all biological systems. If synthetic biology is going to become the new systems engineering of biology, then it needs to establish an equivalent subset in safety engineering: biosafety engineering.

A lot can be learned from state of the art safety engineering, e.g. how to design a fault-tolerant system, a fail-safe system or (in an ideal world) an inherently safe system. A fault-tolerant system, for example, continues to operate even with non-functional parts, though its performance may be reduced. Such systems normally have some kind of redundancy incorporated, increasing its robustness towards random failure of parts or group of parts.

The analogy to other fields of engineering, however, also has its limits. No other field (e.g. mechanical engineering, aviation, electronics; maybe with the exception of software and computer viruses) has to deal with self-replicating entities. This will continuously put an extra burden to biosafety engineers.

Following are some example of the measures biosafety engineers could take to improve the safety of a new biological construct.

6.2.2.1 DNA-based Biocircuits

Biosafety engineering could be practiced by designing robust genetic circuits that account for possible failure of single parts or subsystems, but still keep working or at least don't cause any harm to human health or the environment. Safety engineering has many techniques to design safer circuits (systems).

There is an inductive approach (Event Tree Analysis) and a deductive approach (Fault Tree Analysis) (NASA 2002, NUREG 1991). Both methods are normally used in assessing the safety of engineering systems (e.g. aircraft, space travel, mechanical engineering, nuclear energy) based on Standard parts and true engineering designs. With true engineering principles now being applied to biology, these analysis methods should also make good sense for synthetic biology.

The inductive approach looks at any kind of event in the systems and projects its effect on the whole system. In a genetic network, for example, a basic event could be a mutation in one of the genetic parts, that causes the part to become dysfunctional. The Event Tree Analysis (ETA) would look at the way the whole system is going to be affected by the failed part. It will answer the questions: Will the system still be able to fulfill its tasks? Will it behave in a different way, and if yes in which way? Or will it shut down completely? Based on this analysis additional safety systems could be installed, such as redundant sub-circuits.

The Fault Tree Analysis (FTA), on the other hand, looks at defined unwanted failures of the systems and then traces backward to the necessary and sufficient causes. For example, a genetic circuit should not fail in a way that leads to the

overproduction of a particular protein that is regulated by the network. The FTA can show which basic events could cause such an overproduction, and thus help to improve the circuit to avoid this unwanted failure, for example in designing the circuit in a way that all basic events would cause the expression of the protein to diminish but never to increase.

The ETA and the FTA could also be used to design not only more robust organisms but also less robust ones. This could be of interest if an environmental release is possible or even required. Design of less competitive organisms by designing an in-built weakness would assure that the organism cannot survive outside its designated target environment. Synthetic biology could also increase the possibilities of controlling the organisms by e.g. incorporating basic metabolic pathways that require essential biochemicals that cannot be synthesized by the organism but have to be supplied from an external human source (auxotrophy). Lack of this external source would lead to the death of the organism.

These are just two examples of what could be done to increase the safety of a biological circuit using ETA and FTA in synthetic biology. The full range of possibilities to include safety considerations in designing biological circuits has not yet been explored in great detail but is required to make synthetic biology a safe undertaking.

6.2.2.2 Minimal Genome

An organism with a minimal genome is already an achievement for biosafety engineering. First of all this organism would be the first to be fully understood and analysed. Because it is "minimal" there are no redundant systems, everything is essential and therefore the cell is extremely vulnerable to mutations. An organism with a minimal genome would not be able to compete against wild type organisms in the environment, as it has no defense mechanisms.

Dealing with the risk of unwanted effects in case of environmental release, the minimal organism is therefore *theoretically* an inherently safe organism.

Future experiments have to show if the theory also meets reality. Upon finding the minimal genome, the following tests are recommended:

- proof the inability of the minimal organism to survive *anywhere* else than under defined laboratory conditions,
- check how long it takes the minimal organism – under perfect laboratory conditions – to evolve to a non-minimal organism (e.g. through horizontal gene-flow from other organisms) that is able to survive in an environment different from the one it was originally designed for.[10]

A minimal genome requires a minimal environment that supplies all essential factors for the minimal organism to survive (e.g. availability of essential chemical precursors, energy, food, temperature, lack of predators). The invariable link

[10]Uptake of genes from other organisms has led to the evolution of another kind of "minimal organism", *Desulforudis audaxviator* that forms a single-species ecosystem almost 3 km below the surface of the earth (Chivian et al. 2008).

between the minimal genome to its perfect environment leads to the conclusion that each set of environmental conditions can have a different minimal genome.

An additional safety engineering effort could be made by designing a particular (synthetic) environment, that is different from any natural environment by a number of factors. The minimal genome that fits into this environment will have an even lower chance of surviving outside its synthetic environment.

6.2.2.3 Protocells

Self-reproduction is a typical feature of living organism that defy standard safety engineering principles. Machines just don't reproduce by themselves. So in the attempt to create life from scratch, why not try to create a biological construct that lacks reproduction? It could be assembled from pieces but without the technical gift of self-reproduction. The initial population could only become smaller and these limping cells could be treated like wet machines.

6.2.2.4 Chemical Synthetic Biology

Efforts made to produce the parallel life forms discussed above (Chapter 6.2.1.4) can also be used to make biological systems safer. One day it could be possible to construct an informational polymer that works like DNA but has a different chemical structure (e.g. other backbone molecules, other base pairs) and can be recognized by its specific polymerase and sustain an organism. These organisms will be like nothing biologists have described so far, and will challenge their taxonomic description. This future biochemical construct would act "like" natural life but would be made out of a different chemical toolbox, that would impede information exchange (gene flow) between natural organisms (based on DNA, 4 pase pairs and 20 amino acids) and these new synthetic organism (see Fig. 6.2). The orthogonal chemical systems would act as a biological containment, prohibiting gene flow between natural and

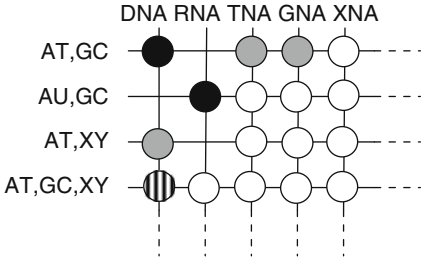

Fig. 6.2 Different orthogonal combinations of unnatural nucleic acid. The columns stand for nucleic acids with different sugar backbones and the rows stand for different base pair combinations. ● Natural genetic code as source for living organism. ◍ Laboratory created unnatural genetic code with functional polymerase. ◒ Laboratory created unnatural genetic code. ○ Other theoretically possible unnatural genetic code

synthetic organisms. In a further step such orthogonality could even be used between synthetic organisms with different biochemical structures.

6.2.3 Diffusion to Amateur Biologists

One of the main aims of synthetic biology is to make biology easier to engineer. Major efforts in synthetic biology are made to develop a toolbox to design biological systems without having to go through a massive research and technology process. With this "deskilling" agenda, synthetic biology might finally unleash the full potential of biotechnology and spark a wave of innovation, as more and more people have access to the necessary skills and toolboxes to engineer biology (Schmidt 2008).

The biosafety risks that accompany the de-skilling of synthetic biology are almost exclusively found under the section DNA-based biocircuits.

6.2.3.1 DNA-based Biocircuits

Efforts made by the Biobricks Foundation with the Registry of Standard Biological Parts and the supporting annual iGEM competition, clearly point towards a future where it should become easer to engineer biology and to design and construct organisms á la carte.[11] In case the utopian vision of assembling organisms from Standard parts would come true, a couple of safety concerns have to be considered.

Laboratory newcomers: Many people working in synthetic biology do not have a professional training in biology, but are chemists, engineers, physicists or computer scientists. Those curricula do not routinely include formal biosafety training, and the amount of newcomers untrained in biosafety rules increases. Therefore it is essential to include biosafety training as part of the interdisciplinary education in synthetic biology.

Do-it-yourself-biology: Motivated by the registry of Standard parts and the annual iGEM competition there is a growing community of amateur biologists or "biohackers".[12] Although the number of active biohackers might be quite limited, it doesn't take a lot to become one and a few rather low-tech do-it-yourself biology documents are already available on the web. A scenario where amateur biologists would design and construct their own pet bugs in their garage would certainly put the health of the amateur, the community around him or her and the environment under unprecedented risk. This scenario has not gone totally unnoticed in the biohacker community and some have started to show at least some interest in safety issues, asking e.g. "how to use a pressure-cooker as an autoclave" or thinking to obtain some lab safety videos. Another area where a de-skilling of biotechnology could be a problem is the illicit bioeconomy. The illicit bioeconomy involves

[11] It has to be noted that many biologists and biotechnologists doubt that one day living organisms will be as easily assembled from bio-parts as electronics circuits from electronic parts. Many iGEM projects fail, and it is still not easy to construct new biological networks.

[12] See: DIY bio, a group based in Boston, MA, USA, trying to establish a biohacker community.

the production of illegal substances (drugs). In contrast to the amateur biologists who try to do things with a low budget, the illicit bioeconomy and its players are known to have a very high budget. It is easily imaginable that drug cartels set up (semi-) profesional laboratories using an easily available biological toolbox to design microorganisms to produce not the plant product artimisinin acid but a plant derived semi-synthetic cocain or heroin (See Schmidt 2008 for more information).

6.3 Conclusions

Working with biological material, biologists need to operate under certain biosafety regulations that aim to prevent any harm to human health, animals or the environment. In genetic engineering adequate biosafety regulations have helped to keep biotechnology safe. When advances in biotechnology take place, however, it is necessary to revisit the current biosafety regulations and its risk assessment tools to check if they are still adequate. Synthetic biology challenges the state-of-the-art biosafety framework in several aspects:

New *methods in risk assessment*: SB requires new methods of risk assessment to decide whether a new SB technique or application is safe enough, avoiding any damage to human health, animals and the environment. The following cases warrant a review and adaptation of current risk assessment practices:

(i) DNA-based biocircuits consisting of a large number of DNA "parts"
(ii) The survivability of novel minimal organisms – used as platform/ chassis for DNA based biocircuits – should be tested for different environments; and
(iii) The effect of exotic biological systems, based on unnatural biochemical structures or genetic code, on natural life forms.

Safety engineering: An important task of a safety discussion is to explore how SB itself may contribute towards overcoming existing and possible future biosafety problems by contributing to the design of safe synthetic biosystems. As biology becomes more and more an engineering discipline, the experiences from systems engineering, in particular safety engineering (including e.g. Event Tree Analysis and Fault Tree Analysis) should be adapted to the specific needs of (synthetic) biology. Examples of how safety engineering could be implemented in synthetic biology are:

(i) Designing less competitive organisms by changing metabolic pathways;
(ii) Replacing metabolic pathways with others that have an in-built dependency on external biochemicals;
(iii) Providing a minimal genome that can be used as an inherently safe chassis;

(iv) Designing protocells that include some but not all features of life, in particular focusing on a protocell that cannot reproduce, but has all other characteristics of life;
(v) Using unnatural biological systems to avoid e.g. gene flow to and from natural species.

Diffusion of SB to amateur biologists: Careful attention must be paid to the way SB skills diffuse (e.g. DIY biology, amateurs, biohackers). The consequences of further deskilling biotechnology are not clear and should be investigated. In particular:

(i) Care must be taken to ensure that everyone, especially newcomers to biology, use the resources of SB safely and has sufficient awareness of and training in relevant techniques and approaches;
(ii) Proper mechanisms (e.g. laws, codes of conduct, voluntary measures, access restrictions to key materials, institutional embedding and mandatory reporting to Institutional Biosafety Committees IBCs) need to be in place to avoid biohackers causing harm.

As the field of synthetic biology matures the issues mentioned here will become more and more relevant. The biosafety challenges will not go away by themselves, but we must work to find an adequate response to them. Hopefully the suggestions made her can serve as a guideline for upcoming biosafety initiatives in synthetic biology. It is time to act.

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References

Anonymous (2003) Hacking the Genome, 2600 The Hacker Quarterly, 20(4):6–9 (Author is stated as Professor L.)

Benner SA, Sismour AM (2005) Synthetic Biology, Nat Rev Gen, 6:533–543

Canton B, Labno A, Endy D (2008) Refinement and Standardization of Synthetic Biological Parts and Devices, Nature Biotech, 26(7):787–793

Carlson R (2001) Biological Technology in 2050, Published as "Open Source Biology and Its Impact on Industry," IEEE Spectrum, Available via: http://synthesis.cc/BiolTech2050.pdf Accessed 12 December, 2008

CBD (2008) Risk Assessment and Risk Management (Articles 15 and 16), Conference of the Parties to the Convention on Biological Diversity Serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety, Fourth Meeting Bonn, 12–16 May, 2008, Available via: http://www.cbd.int/doc/meetings/bs/mop-04/official/mop-04-10-en.pdf Accessed 12 December, 2008

Chaput JC, Ichida JK, Szostak JW (2003) DNA Polymerase-Mediated DNA Synthesis on a TNA Template, J Am Chem Soc, 125, 856–857

Chivian et al. (2008) Environmental Genomics Reveals a Single-species Ecosystem Deep Within Earth, Science, 322:275–278

de Vriend H (2006) Constructing Life, Early Social Reflections on the Emerging Field of SB, Den Haag, Rathenau Instituut, Available via: www.rathenau.nl/downloadfile.asp?ID=1101 Accessed 12 December, 2008

DoD (2000) Department of Defense Standard Practice for System Safety, Available via: http://safetycenter.navy.mil/instructions/osh/milstd882d.pdf Accessed 16 December, 2008

EC (2008) Commission Recommendation of 07/02/2008 on a Code of Conduct for Responsible Nanosciences and Nanotechnologies Research, European Commission, Available via: http://ec.europa.eu/nanotechnology/pdf/nanocode-recpe0894cen.pdf Accessed 12 December, 2008

Endy D (2005) Foundations for Engineering Biology, Nature, 438(7067):449–453

Evangelista G, Minervini G, Luisi PL, Polticelli F (2007) Randomblast a Tool to Generate Random "Never Born Protein" Sequences, Bio-algorithms and Med-systems, 3(5), 27–31

FAO (2002) Report of the Expert Consultation on Biosecurity in Food and Agriculture,10–13 September, 2002, FAO, Rome, Italy, Available via: http://energycommerce.house.gov/cmtemtgs/110-oi-hrg.100407.Rhodes-Testimony.pdfftp://ftp.fao.org/ag/agn/agns/meetings/tcbangkok/tcbrm033en.pdf Accessed 1 December, 2008

Fleming DO (2006) "Risk Assessment of Synthetic Genomics: A Biosafety and Biosecurity Perspective", In: Garfinkel MS, Endy D, Epstein GL, Friedman RM (eds.), *Working Papers for Synthetic Genomics: Risks and Benefits for Science and Society*, pp. 105–164, 2007.

Garfinkel MS, Endy D, Epstein GL, Friedmann RM (2007) Synthetic Genomics: Options for Governance, Available via: http://www.jcvi.org/cms/fileadmin/site/research/projects/synthetic-genomics-report/synthetic-genomics-report.pdf Accessed 12 December, 2008

Gibson DG, Benders GA, Andrews-Pfannkoch C, Denisova EA, Baden-Tillson H, Zaveri J, Stockwell TB, Brownley A, Thomas DW, Algire MA, Merryman C, Young L, Noskov VN, Glass JI, Venter CG, Hutchison III CG, Smith HO (2008) Complete Chemical Synthesis, Assembly, and Cloning of a Mycoplasma genitalium Genome, Science, 319(5867):1215–1220

Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA, Smith HO, Venter JC (2006) Essential Genes of a Minimal Bacterium, PNAS, 103(2), 425–430

Heinemann M, Panke S (2006) Synthetic Biology-putting Engineering into Biology, Bioinformatics, 22(22):2790–2799

Jaramillo A (2008) Genetic Circuits by Automated Design, Presentation at the SB 4.0 conference in Hong Kong, 10–12 October, 2008

Kaur H, Arora A, Wengel J, Maiti S (2006) Thermodynamic, Counterion, and Hydration Effects for the Incorporation of Locked Nucleic Acid Nucleotides into DNA Duplexes, Biochemistry, 45 (23):7347–7355, doi:10.1021/bi060307w.

Keasling JD (2008) Synthetic Biology for Synthetic Chemistry, ACS Chem Biol, 3(1):64–76

Keeling PJ (2004) Diversity and Evolutionary History of Plastids and Their Hosts, Am J Bot, 91(10):1481–1493

Kelle A (2007) Synthetic Biology and Biosecurity Awareness in Europe, Vienna, IDC, Available via: http://www.synbiosafe.eu/uploads///pdf/Synbiosafe-BiosecurityawarenessinEuropeKelle.pdf Accessed 12 December, 2008

La Scola B, Desnues C, Pagnier I, Robert C, Barrassi L, Fournous G, Merchat M, Suzan-Monti M, Forterre P, Koonin E, Raoult D (2008) The Virophage As A Unique Parasite of the Giant Mimivirus, Nature, 455:100–105

Leconte, AM, Hwang GT, Matsuda S, Capek P, Hari Y, Romesberg FE (2008) Discovery, Characterisation, and Optimisation of An Unnatural Base Pair for Expansion of the Genetic Alphabet, J Am Chem Soc, Published on the web D1/25/2008.

Liu W, Brock A, Chen S, Chen S, Schultz PG (2007) Genetic Incorporation of Unnatural Amino Acids into Proteins in Mammalian Cells, Nat Methods, 4(3): 239–244

Luisi PL (2007) Chemical Aspects of Synthetic Biology, Chem Biodivers, 4(4):603–621

Luisi PL, Chiarabelli C, Stano P (2006) From Never Born Proteins to Minimal Living Cells: Two Projects in Synthetic Biology, Orig Life Evol Biosph, 36:605–616

NASA (2002) Probabilistic Risk Assessment Procedures Guide for NASA Managers and Practitioners, Available via: http://www.hq.nasa.gov/office/codeq/doctree/praguide.pdf Accessed 12 December, 2008

Ng PS, Bergstrom DE (2005) Alternative Nucleic Acid Analogues for Programmable Assembly: Hybridization of LNA to PNA, Nano Lett, 5(1): 107–111

NSABB (2007) Roundtable on Synthetic Biology, 11 October, 2007, National Science Advisory Board for Biosecurity, Available via: http://www.biosecurityboard.gov/Annotated20Agenda20Website.pdf Accessed 12 December, 2008

NUREG 0492 (1991) Fault Tree Handbook – Reliability and Risk Analysis, Norman J McCormick, Academic Press, New York.

O'Malley M, Powell A, Davies JF, Calvert J (2008) Knowledge-making Distinctions in Synthetic Biology, BioEssays, 30(1):57

Orgel L (2000) A Simpler Nucleic Acid, Science, 290(5495):1306–1307.

Oye K, Yeddanapudi N (2008) Synthetic Biology Chassis Design and Demonstration for Safety and Security, A Preliminary Blueprint for Research and Policy, Poster presented at the SB 4.0 Conference in Hong Kong, China, 10–12 October, 2008

Qiang W (2007) "Efforts to Strengthen Biosafety and Biosecurity in China", Chapter 6 in Smithson AE (ed.), *Beijing on Biohazards: Chinese Experts on Bioweapons Nonproliferation Issues*, James Martin Center for Nonproliferation Studies, Monterey Institute of International Studies, Monterey

Raoult D, Forterre P (2008) Redefining Viruses: Lessons from Mimivirus, Nat Rev Microbiol, 6:315–319

Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MCY, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the Antimalarial Drug Precursor Artemisinic Acid in Engineered Yeast, Nature, 440: 940–943.

Schmidt M (2006) Public Will Fear Biological Accidents, Not Just Attacks, Nature, 441(7097):1048

Schmidt M (2008) Diffusion of Synthetic Biology: A Challenge to Biosafety. Systems and Synthetic Biology, 2(1–2):1–6

Schoning K, Scholz P, Guntha S, Wu X, Krishnamurthy R, Eschenmoser A (November 2000) Chemical Etiology of Nucleic Acid Structure: The Alpha-Threofuranosyl-(3'–>2') Oligonucleotide System, Science, doi:10.1126/science.290.5495.1347. PMID 11082060.

Seelig B, Szostak JW (2007) Selection and Evolution of Enzymes from a Partially Randomized Non-catalytic Scaffold, Nature, 448(7155):828–831

Serrano L (2007) Synthetic Biology: Promises and Challenges, Mol Syst Biol, 3:158

Sismour AM, Lutz S, Park J-H, Lutz MJ, Boyer PL, Hughes SH, Benner SA (2004) PCR Amplification of DNA Containing Non-standard Base Pairs by Variants of Reverse Transcriptase from Human Immunodeficiency Virus-1, Nucl Acids Res, 32, 728–735

SYNBIOSAFE (2008) Compilation of All SYNBIOSAFE E-conference Contributions, Available via: http://www.synbiosafe.eu/uploads/pdf/Synbiosafee-conferenceallcontributions.pdf Accessed 12 December, 2008

Szostak JW, Bartel DP, Luisi PL (2001) Synthesizing Life, Nature, 409:387–390

Tucker JB, Zilinskas RA (2006) The Promise and Perils of Synthetic Biology, The New Atlantis, Available via: http://www.thenewatlantis.com/archive/12/tuckerzilinskas.htm Accessed 12 December, 2008

Urbano P, Urbano F (2007) Nanobacteria: Facts or Fancies? PLoS Pathog, 3(5):e55. doi:10.1371/journal.ppat.0030055

Vandermeeren M, Préveral S, Janssens S, Geysen J, Saison-Behmoaras E, Van Aerschot A, Herdewijn P (2000) Biological Activity of Hexitol Nucleic Acids Targeted at Ha-ras and Intracellular Adhesion Molecule-1 mRNA, Biochem Pharmacol, 59:655–663

Vester B, Wengel J (2004) LNA (Locked Nucleic Acid): High-Affinity Targeting of Complementary RNA and DNA, Biochemistry, 43 (42), 13,233–13,241

Waters E, Hohn MJ, Ahel I, Graham DE, Adams MD, Barnstead M, Beeson KY, Bibbs L, Bolanos R, Keller M, Kretz K, Lin X, Mathur E, Ni J, Podar M, Richardson T, Sutton GG, Simon M, Söll D, Stetter KO, Short JM, Noordewier M (2003) The Genome of Nanoarchaeum Equitans: Insights into Early Archaeal Evolution and Derived Parasitism, PNAS, 100 (22):12,984–12,988, doi:10.1073/pnas.1735403100.

World Health Organization (2004) *Laboratory Biosafety Manual*, Third Edition, Geneva.

Yang Z, Hutter D, Sheng P, Sismour AM, Benner SA (2006) Artificially Expanded Genetic Information System: A New Base Pair with an Alternative Hydrogen Bonding Pattern, Nucleic Acids Res, 34(21), 6095–6101

Zhang L, Peritz A, Meggers E (2005) A Simple Glycol Nucleic Acid, J Am Chem Soc, 127: 4174–4175