Chapter 2
Synthetic Biology: Diverse Layers of Live

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Abstract Prerequisite for any evaluation of synthetic biology is the precise description of its scientific rationale and its biological objects. Here, we develop a layer model that helps to categorize subfields of synthetic biology along their operative procedures and based on the biological status of the organisms generated by synthetic biology. The layer model classifies synthetic and semisynthetic organisms and cells according to their genetic connectivity and to their potential interaction with natural organisms derived by evolution. We use the model to characterize three distinct approaches within synthetic biology: engineering biology, xenobiology and protocell research. While the latter approach generates organisms that hardly could be termed living, xenobiology aims at orthogonal living systems that are disconnected from nature. Synthetic engineering biology could be considered as extreme form of gene technology since all resulting organisms share the universal genetic code with the natural living beings and are based on the same molecular and biochemical principles. Such biological description can be used to determine both the degree of familiarity and the level of uncertainty associated with synthetic organisms and may thus facilitate to judge potential risks of synthetic biology.

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2.1 Introduction

Any appraisal of synthetic biology should distinguish between the narrative of its naming and its conceptual and experimental development. Synthetic biology has to be seen in the light of the different scientific traditions that have contributed to its formation, in particular gene technology, organic chemistry, chemical engineering and computer science. The term "Synthetic Biology" has been first used by the French chemist Stéphane Leduc in 1912 in his treatise "La Biologie Synthéthique" (Leduc 1912). In his experiments he studied the growth of chemical salt crystals mimicking the appearance of biological processes in his "Jardins Chimiques". He compared the osmotic growth of these forms to that of living beings and argued that in studying these formations one can learn about basic processes of life. His contemporaries, however, criticized that his growing crystals can be regarded only as an imitation of life but not as synthetic life (Campos 2009). The term "Synthetic Biology" was reinvented in the 1970 by the Polish-American geneticist W. Szybalski to describe the newly developed techniques of recombinant DNA technology: "The work on restriction nucleases not only permits us easily to construct recombinant DNA molecules and to analyze individual genes, but also has led us into the new era of synthetic biology where not only existing genes are described and analyzed but also new gene arrangements can be constructed and evaluated" (Szybalski and Skalka 1978). These historical records could suggest a connection between genetic engineering and the naming of contemporary synthetic biology. Though Luis Campos could demonstrate that the inventors of modern synthetic biology did neither know about Leducs early publications nor the further use of this term (Campos 2009). During the preparation of the first international conference on synthetic biology the organizers even discussed the alternative name "Intentional Biology" for this emerging branch of biology. This designation should emphasize the purposeful and applied nature of an engineering biology. The name "Intentional Biology" was, however, promptly abandoned since it could imply that other fields of biology would be "unintentional". The term "Synthetic Biology" was then picked to stress the analogy with "Synthetic Chemistry" (Campos 2009). Against this background, the notion of a synthetic biology refers to the synthetic approach that revolutionized organic chemistry in the 19th century. It certainly should also evoke the huge economic impact of synthetic organic chemistry on the development of the European dye and pharmaceutical industry (Yeh and Lim 2007).

Clearly, synthetic biology would not have been conceivable without the previous and successful development of genetic engineering. It was the elucidation of the universal nature of the genetic code that paved the way for experimental transfer of genes between unrelated organisms (Jackson et al. 1972; Cohen et al. 1972, 1973). These experiments are considered as genuine experimental breakthrough and mark the starting point of modern biotechnology. As early as in 1977, Genentech, one of the first biotech companies, reported the production of a human protein manufactured in bacteria: Somatostatin, a human growth hormone-releasing inhibitory factor. Since then, genetic engineering has been used in a vast number of applications, from the production of useful molecules, such as biopharmaceuticals, the

sustainable generation of renewable fuels, to the design of transgenic plants that are resistant against pathogens and herbivores. Characteristic for this classic period of gene technology was the transfer of single genes or of small clusters of genes into recipient organisms, predominantly the bacterium *Escherichia coli*. Alternatively, single genes were mutated in bacteria, fungi, animals or plants to abolish or change their function. The basic concept of genetic engineering, however, did not aim primarily at reconstructing or improving nature as such. Rather, particular genetic elements were recombined or targeted mutations were introduced into genes to improve certain properties or to enhance productivity. Nevertheless, the experimental foundations of current synthetic biology resulted from that approach when the first conceptual ideas for a radical improvement of nature were developed (Szybalski and Skalka 1978). But realization of these early concepts had to wait for major new developments in the fields of systems biology and bioinformatics that together with the rapid technological progress in DNA analysis and synthesis finally allowed sequencing of whole genomes and large-scale gene synthesis.

Although gene technology and synthetic biology share many common roots, the latter has to be viewed in the light of a larger diversity of scientific traditions such as synthetic organic chemistry and, as described in the following chapters, also in the light of modern engineering and bioinformatics. In addition, the research agenda of synthetic biology clearly surpasses that of gene technology. Most important is the change of focus from manipulation to construction. It is not single genes or gene clusters any more that are mutated or recombined, but complete biological systems that are reconstructed from scratch or redesigned as a whole (Schwille 2011; Bohannon 2011). Synthetic biology even comes along with its own artistic visions (Reardon 2011).

Another important difference between gene technology and synthetic biology is the depth of intervention exerted on the organisms, both in relation to quantity and quality. In general, organisms created by synthetic biology are much more distant to any natural beings than a genetically modified organism which still can be recognized as variant of a natural species. With its methodical approach to rely more on systemic and engineering principles, synthetic biology resembles more applied sciences than basic sciences. Furthermore, synthetic biology departs from classical biological thinking and includes elements of playfulness such as the iGEM competition with its creation of bacteria that smell of bananas and wintergreen, are able to count or to blink. Nevertheless, there is no sharp boundary between classic gene technology and synthetic biology, since many applications of synthetic biology can still be regarded as gene technology. Furthermore, also gene technology is developing and sometimes reaches levels of intervention similar to that of synthetic biology. New technologies, such as CRISPR/Cas9-mediated genome editing will in future even more obscure the conceptual differences and hamper a clear distinction between these two disciplines (Cameron et al. 2014). For many critics of synthetic biology, synthetic biology is anyway nothing else than an extension of gene technology and has been called ‘extreme genetic engineering’ (ETC group 2007). For now, however, it may suffice to state that synthetic biology in its core area and with its conceptual and methodical impetus clearly surpasses classic gene technology.

According to its own definition, synthetic biology deals both with the design of new biological parts, devices and systems and/or the redesign of existing, natural biological systems for useful purposes (see definition on http://syntheticbiology.org). To do so engineering principles like modularization and standardization will be implemented that allow the design and description of standard biological parts. These parts can then be used to equip minimal cells with defined functionalities. The resulting synthetic cells have no direct counterpart in nature but can be used for chemical, pharmaceutical, medical and biotechnological purposes. Another aspect of synthetic biology is the generation of protocells derived from simple and defined molecules. Here, the major goal is the reconstruction of living systems from first principles. In recent years, a third approach has become more and more important aiming at the integration of non-natural (xenobiological) compounds into complex living systems. Due to the different chemical makeup of their genetic material, such cells cannot exchange any genetic information with natural organisms. A similar isolation can also be reached by changing the genetic code through reassigning the translation of triplet codons to other amino acids. In both cases the resulting organisms cannot interact genetically with the environment and thus are called orthogonal. Importantly, orthogonality of synthetic cells provides a high level of biosafety (Marlière 2009; Schmidt 2010; Mandell et al. 2015). Furthermore, due to their chemical alterations, xenobiological approaches have the potential to greatly expand the diversity of potential life forms.

Interestingly, these divergent fields of synthetic biology differ not only in their basic experimental approaches but alike root in different academic disciplines. Many stakeholders of synthetic biology have been drawn into this field from other scientific or engineering disciplines. Therefore, they have been trained in diverse fields and this has shaped their views on synthetic biology. This becomes apparent not only in their view on synthetic biology and in their conceptualizations of life but also in their attitudes of risk and biosafety management.

2.2 The Main Research Agendas of Synthetic Biology

Here, we will describe the basic concepts of the major subfields of synthetic biology. In general, three main areas of synthetic biology can be described as

- **Engineering Biology**, aiming at the transformation of biology into an engineering discipline by introducing standardized modules, parts and devices with well-described characteristics that can be used to construct novel biological systems or to redesign existing living systems.
- **Orthogonal Biology**, trying to create cells that are unable to exchange genetic information with natural organism either by integrating non-natural molecular compounds (xeno-life) or by reassignment of the natural genetic code (recoded life).
- **Protocell Research,** aiming at recapitulation of prebiotic evolution by building simple cellular vesicles that fulfil at least some criteria of living systems.

2.2.1 The Engineering Branch of Synthetic Biology

Traditional molecular biology and gene technology at large both depend on the natural genetic material as it can be found in existing living organisms. The advent of modern DNA synthesis and next generation sequencing technologies makes it possible to read and write DNA in large scale and with high precision. This has not only greatly enlarged the repertoire of available natural genes and enzymes, but also allows to design novel polypeptides without any precedent in nature. The opportunity to use DNA and protein molecules just like any other technically malleable material has attracted engineers from different fields into Synthetic Biology. From an engineer's point of view, a living cell can be considered as highly integrated complex system consisting of biochemical modules, functional devices and higher order signalling systems that works like any other hierarchical technical system (Andrianantoandro et al. 2006). All biological functions of a living cell, like environmental sensing, motility, metabolism, information processing etc. are realized by molecular complexes that fulfil specific functions. Thus, the whole cell looks like a technical apparatus well-designed to exert a complex and purposeful function. However, all simplistic approaches to reconstruct or redesign natural cells have been hampered by the high level of complexity of living cells. Many of the functional modules display intricate interdependencies that are not simply due to their obvious function. Natural organisms are the product of evolution and thus underlie constraints both from their phylogenetic origin and ontogenetic development. In addition, the process of molecular evolution by random mutation and selection entails the organisms with historical contingency. This may explain why some synthetic biologists have a delicate relationship to evolution. They often describe the complexity of living entities that result from their evolutionary origin as unnecessary (Trafton 2011) or complain that "… the design of natural biological systems are not optimized by evolution for the purposes of human understanding and engineering" (Endy 2005). On the other hand, natural evolution is a very efficient optimization strategy and synthetic biologists have also applied the technique of accelerated evolution to reprogram bacterial cells (Wang et al. 2009).

One of the main initial tasks of synthetic biology was the transformation of biology into an engineering technology. Accordingly, one of the major foundational efforts of synthetic biology dealt with refactoring biological systems to make them better suited for engineering purposes (Chan et al. 2005; Voigt 2011). As a direct way to this aim the introduction of basic engineering principles such as modularization and standardization was proposed. The most visible outcome of this approach was the construction of standardized "biobricks" organized in a registry of standard biological parts (Knight 2003; Canton et al. 2008). Biobricks are designed to allow fast, efficient and rational design of living systems by human engineers.

A second aspect of modularization and standardization is the introduction of an abstraction hierarchy of communication, which is typical for the manufacturing of highly integrated technical systems composed from many different parts and

devices (Endy 2005). Standardization of parts and interfaces relieves the engineers from understanding all details of a complex system instead they are responsible only for a certain layer of functional complexity. They design specialized modules and devices that are used by other engineers to be integrated into higher systems. While it may not be necessary to understand the function of those modules and devices in all details, it is of prime importance that the technical specifications and functional parameters of these parts are described quantitatively. Due to this abstraction the standardized parts and modules can be regarded as black boxes as long as they work according to their specifications (Endy 2005). Only with this approach, an engineering technology of highly integrated living systems would be feasible in large scale.

Another important strategy of the engineering branch of synthetic biology is the use of minimal cells as platform (or chassis) for the construction of more complex systems. Minimal cells just contain all genes and proteins necessary to provide the basic living functions. The usual way to define a minimal cell is to strip natural cells from all non-essential functions. This is normally reached by random mutagenesis in combination with identification of the core essential functions by comparative genomics (Juhas et al. 2012; Acevedo-Rochas et al. 2013; Stano and Luisi 2013). Such minimal cells can then be equipped with additional modules that confer specific functionalities.

2.2.2 Orthogonal Biology

The engineering branch of synthetic biology primarily aims at redesigning natural cells to make them more useful for human purposes. Thus, 'natural' molecules are used to create 'artificial cells' that perform 'unnatural functions' (Benner and Sismour 2005). A complementary approach is characteristic for the 'chemical' branch of synthetic biology. Here the aim would be to use 'unnatural' chemical compounds to reproduce biological behaviour without making an exact molecular replica of a natural living system (Benner et al. 2011). Such molecular mimicking of a biological system requires an intimate understanding of the chemical properties of complex molecules and therefore mostly chemists are involved in promoting artificial life based on an alternative molecular design (Luisi 2007). Interestingly, this approach addresses another important aspect of engineering integrated technical systems, the notion of decoupling or orthogonality. Technical systems and devices are called orthogonal if they are completely separated from each other and thus do not show any unwanted interactions or crosstalk. This makes it possible to add parts to a system without creating or propagating side effects. Thus, even large systems containing large numbers of parts and devices can be designed and actually operated successfully. In synthetic biology, orthogonalization refers primarily to the inability of artificial cells to exchange genetic material and/or metabolites with natural organisms. Orthogonality is at odds with the canonical research focus in biology, where most effort is put on the

detection and unraveling of more and more intricate networks of interactions. Orthogonalization aims at reversing this view and implies that system simplification might actually be at the heart of implementing a reliable and robust bioengineering. The prime route to this simplification goes through orthogonalization of biosystems to limit unpredictable (e.g. side) reactions and interactions. Current research in synthetic biology makes a lot of efforts to demonstrate the power of orthogonalization as a biosystems engineering strategy (An and Chin 2009; Schmidt and de Lorenzo 2012).

Orthogonality of biological systems can be reached by different means and at different levels. The most extreme version would be the total construction of a living cell only from unnatural components. To stress the alien nature of these artefacts and their large distance to existing biological entities these cells are also termed 'xenobiotic'. One has to point out, however, that orthogonalization is scalable and already low-level incorporation of unnatural components into existing organisms could result in considerable genetic and biological isolation (Marlière 2009; Mandell et al. 2015). In principle, it would be sufficient to replace only one of the natural base pairs by xeno-DNA bases (XNA) to prevent genetic exchange with other organisms in the natural environment (Malyshev et al. 2014).

The universality of the genetic code is the basis for gene technology since it allows functional expression of heterologous genes in cells of different hosts. Also in the natural environment genes can be laterally transferred between species. Horizontal gene transfer is a major factor of adaptation and speciation and is assumed to have occurred at large scale during natural evolution. Thus, any artificial reassignment of the genetic code is an efficient means to prevent unwanted exchange of genetic information with the organismic world. Recoded cells are genetically isolated and even resistant to the attack of viruses since viral reproduction depends on genetic compatibility. In analogy to computer networks, the genetic isolation of orthogonal cells has been termed a 'genetic firewall' (Marlière 2009). Although not the only advantage of orthogonal cells, this property is largely exploited to justify xenobiological approaches as an ultimate safety tool for synthetic biology (Schmidt 2010; Schmidt and de Lorenzo 2012).

In 2010 the first chemical synthesis of a complete bacterial genome has been achieved (Gibson et al. 2010). Therefore, reassignment of the genetic code is in reach. Just recently, in a recoded *E. coli* strain all UAG stop codons have been replaced by synonymous UAA codons demonstrating the principal feasibility of this approach (Isaacs et al. 2011; Lajoie et al. 2013). Even sense codon reassignments should be possible at least for amino acids whose aminoacyl-tRNA synthetases do not depend on recognition of the tRNA anticodon loop. In principle, genome-wide replacement of a single defined codon together with the respective exchange of the corresponding tRNA anticodon sequence would be sufficient to reassign the genetic code. Although recoded cells would still be able to exchange genetic material with the environment, natural cells taking up this DNA cannot read the genetic information of codon-reassigned organisms.

Furthermore, recoding would also allow to expand the coding capacity of the genetic code towards additional, non-natural amino acids. Therefore, efforts to

expand the genetic code are expected to become a core discipline in synthetic biology as it offers an efficient platform for the transfer of numerous chemistries from the synthetic laboratory into the biochemistry of living cells (Hoesl and Budisa 2012). Any attempt to generate sequence diversity by classical protein engineering is always limited to the set of 20 canonical amino acids. This set of building blocks does not span all dimensions of chemical variability that could be potentially advantageous to diversify the catalytic performance of enzymes or to gain desired features for whole cells. For that reason, synthetic non-canonical amino acids represent an ideal tool to supply proteins and cells with novel and unusual functions and emergent features. Thereby, successful experiments include (1) uptake/import of non-canonical amino acids (2) their intracellular accumulation at levels high enough for efficient substrate turnover (activation and tRNA acylation) by aminoacyl tRNA synthetases, (3) metabolic and chemical stability of imported non-canonical amino acid; (4) tRNA charging (acylation) that must be achieved at a reasonable rate, and (5) the translation of the non-canonical amino acid into a nascent polypeptide chain. A second approach relies on the orthogonalization of protein translation as a tool for protein engineering whereby various orthogonal-pairs were developed (An and Chin 2009; Wang et al. 2014). It enabled the incorporation of around 40 different amino acid analogs, for different application purposes such as crosslinking, site specific coupling reactions etc. (Liu and Schultz 2010).

The generation of artificial genetic systems would certainly require additional and chemically different coding units. For that reason, much work has been focused on biomimetic chemistry of the DNA bases, and recently few research teams demonstrated novel DNA bases and base pair structures capable for stable unnatural base pair formation, with the ability to serve as substrate for polymerase enzymes (Pinheiro et al. 2012; Hunter 2013). This is indeed an important step in the direction to explore the possibilities of employing a new alphabet in order to change/modulate/reprogram already existing replication, transcription and translation machineries of living cells (Malyshev et al. 2014). Here we shortly outline the pioneering research in this field as newest progress is covered in numerous recent reports publications.

As DNA forms stable double helices via weak interactions between only two base pairs (A = T and G ≡ C) and exhibits considerable resistance to hydrolytic cleavage, new genetic alphabets composed of unnatural base pairs can be synthesized by shuffling the hydrogen bonding sites in the nucleobases. It was also shown that chemically synthesized mRNA containing the modified nucleotides is able to direct position-specific incorporation of 3-iodotyrosine into a polypeptide via a suppressor tRNA containing the unnatural anticodon (Chin et al. 2003; Hammerling et al. 2014). In the meantime, many research groups succeeded to greatly expand the number of synthetic base pairs capable of transcription into mRNA. For example, in vitro transcription/translation systems where DNA with unusual base pairs can be transcribed into RNA molecules and subsequently participates in protein synthesis on the ribosome were also reported (Hirao and Kimoto 2012).

2.2.3 Protocell Biology—Recapitulation of Evolution

Darwins explanation for the origin of species by means of natural selection immediately provoked the question for the first appearance of life on earth. The common origin of all living species and the absence of any present-day spontaneous generation of life indicate that this event may have happened only once. Singular events, however, are difficult to treat by any scientific theory due to lack of reproducibility. Nevertheless, experiments have been designed to get clues as to how life might have been spontaneously arisen in the primordial soup. Already in the early 1950ies Miller and Urey demonstrated that common organic molecules such as amino acids and nucleobases are formed spontaneously in a chemical environment that resembled the early reducing atmosphere of the earth if energy was provided mainly in the form of electric discharges (Miller 1953). Based on theoretical considerations, Manfred Eigen proposed a model where a replicating system results from cyclic coupling of enzymatic reactions (hypercycle) (Eigen and Schuster 1977). Therefore many of the approaches in protocell research concentrate on the chemical realization of such life-like reaction networks. It is generally assumed that compartmentalization in lipid vesicles is the most likely scenario that enabled these simple replicators to become subjects of Darwinian evolution (Mansy and Szostak 2009; Loakes and Holliger 2009). There is ample evidence that at a certain stage during evolution RNA formed the material basis of information storage but was also able to catalyse chemical reactions (ribozymes) (Joyce 2002). However, how stable replication and faithful distribution of these complex molecules was accomplished in such early versions of protocells is still largely unknown.

In the present era of synthetic biology, the rational design of protocells may provide an alternative way for the generation of minimal cells that could serve as chassis for the rational design of modularized cells ('bottom-up approach'). However, the lack of any knowledge on how early life forms looked like during prebiotic evolution on earth together with the very limited capacities of simple replicating systems makes it likely that it will be still a long way to a stable protocell endued with the basic properties of a living system. But on this route, important insights into possible scenarios for prebiotic evolution will be gained. Since the formation of a self-sustaining autocatalytic chemical network is a necessary step to establish simple life-like conditions, organic chemists interested in biological evolution have developed numerous self-replicating or autocatalytic systems (Mansy and Szostak 2009; Attwater and Holliger 2014). By careful inspection of metabolic synthetic processes it is almost always possible to detect some basic principles. For example, despite their diversity the amino acid synthesis pathways in extant cells share two basic features (a) the nitrogen of the α-amino group originates from $NH4^+$ and (b) the sources of skeletal carbons are intermediates of the tricarboxylic acid cycle and the other major metabolic pathways that are coupled to the assimilation of CO_2 (in autotrophs) (Pereto 2012). In general, this chemical determinism should be kept in mind when considering the possibility to create

living cells with biopolymers whose structure and function tolerate a systemic chemical change in the backbone such as the substitution of one atom by another: phosphorus by arsenic, carbon by silicon or oxygen by sulphur etc. That implies that all extant cells are characterised by an invariant basic chemical organisation i.e. all the properties of living beings rest on the fundamental mechanism of molecular invariance.

In addition to terminological issues, inquiries into the ways of creating life reveal that there is no consensus among scientists, philosophers, and theologians about the term "life". Thus, the aim of "creating life" is a vague scientific goal as nobody exactly knows what life itself is (Luisi 1998). The different subfields of Synthetic Biology not only differ in their methodological approaches but also in their underlying conceptions of life. The products of synthetic biology itself are at the borderline between living and non-living matter (Deplazes and Huppenbauer 2009). Depending on the different approaches of synthetic biology, certain aspects of "being alive" are emphasized. For chemists trying to reconstruct simple life-like cellular structures, self-maintenance of metabolic reactions already suffices for a minimal definition of life. Luisi has introduced the term "autopoiesis" into protocell research (Luisi 2003). A metabolic process enclosed in a semipermeable spherically closed membrane is called a minimal autopoietic system, if it is able to replace decaying components of its membrane. Depending on the kinetics of generation (V_{gen}) and decay (V_{dec}), this system will be in homeostasis ($V_{gen} = V_{dec}$), grow ($V_{gen} > V_{dec}$) or die ($V_{gen} < V_{dec}$) (Luisi 2003). Autopoietic systems depend only on open dissipative processes that allow the yielding of energy from the uptake and use of nutrients. They even do not require hereditary material, but it is easy to incorporate nucleic acids and enzymes into this theoretical framework (Luisi 2003).

From such simple forms of synthetic life to bacteria "rebooted" with a chemically synthesized genome (Gibson et al. 2010) is a large jump. While the former are close to non-living matter, the latter are indistinguishable from natural bacteria of the same species. This makes it necessary to devise a scheme, in which the different life forms that are produced by the diverse approaches of synthetic biology can be described and compared. This will also help to conceive risk assessment strategies and to give specific recommendations for cellular constructs generated in the diverse fields of synthetic biology. Here, we propose a layer model that should help to get a better picture of synthetic biology and to describe its different approaches.

2.3 A Layer Model to Describe and Classify Natural and Synthetic Organisms in Different Fields of Synthetic Biology

All approaches of synthetic biology outlined above are dealing with living or life-like systems thus expanding the number of potential designs to a great extent. Synthetic organisms will be created that have not been evolved and thus can be regarded as being beyond natural (Elowitz and Lim 2010). Here we develop a

model, which will allow classifying the different artificial living systems according to their position within or outside of the realm of the natural life forms. All existing life forms on earth have arisen by natural evolution. Due to Darwinian evolution by random mutation and selection, natural "life" as it exists on earth is also highly contingent since many crucial inventions that enabled organic life on earth have been "invented" only once (for example the genetic code). It is commonly accepted that all living beings can be traced back to a "last universal common ancestor" (LUCA) and that all extant species are connected by a common genealogy (Kyrpides et al. 1999; Lazcano and Forterre 1999; Woese 1998).

However, from a global point of view, existing life forms represent only a very tiny fraction of all "potential" living beings including those that have not yet been evolved. All existing species depend on DNA as its hereditary material and use (with only very few exceptions) the same (universal) genetic code. Thus, every species is unambiguously defined by its genome sequence. Since DNA sequences consist of four different bases (A, C, G, T) and are of various lengths—typically they range from a few million base pairs to several billion—the number of potential genome sequences is almost infinite. If each (actual or potential) genome sequence is represented by a single point on a layer, all points of this layer represent the sequence space of all organisms that share a common genetic code. Within such a layer, the number of potential living organisms is infinite, since it equals the number of all possible genome sequences. Every point on the layer corresponds to a single species, either to one that exists in nature or to one that have not (or not yet) evolved. One has to point out, that within this layer of natural species exchange of genetic information is possible since all species share a common genetic system, the universal genetic code. Since the number of extant species is small if compared to the number of all possible genome sequences the area covered by natural is tiny and resembles more small islands in a vast empty space (see Fig. 2.1).

According to the modern evolution theory, species evolve by mutation, selection and genetic drift (Kimura 1983). Therefore all new species are closely related to their progenitors and contain only a few number of sequence deviations. Thus, the dynamics of evolutionary change results in a slow but continuous spreading of species that explore the neighbouring uncharted territory of the sequence space. Synthetic biology now provides the new and unique possibility to create any of potentially viable organisms within a layer while both natural evolution and gene technology depend more or less on the limited amount of present-day organisms and genomes.

Since the total number of potential organisms corresponds to the number of potential genomes, it thus equals the number of possible DNA sequences. Simple calculations demonstrate that in the previous billions of years only a very tiny fraction of all potential genomes has been realized by evolution. This is due to the fact that the combinatorial number of sequences is beyond our imagination. Genomes encode thousands of proteins but even for a rather short peptide of only 100 amino acids 20^{100} different sequences are possible. Therefore "the ratio between the possible (say 20^{100}) and the actual chains [realized in nature by evolution] (say 10^{15}) corresponds approximately to the ratio between … all the grains of sand in the vast Sahara and a single grain" (Luisi 2006). This does not imply

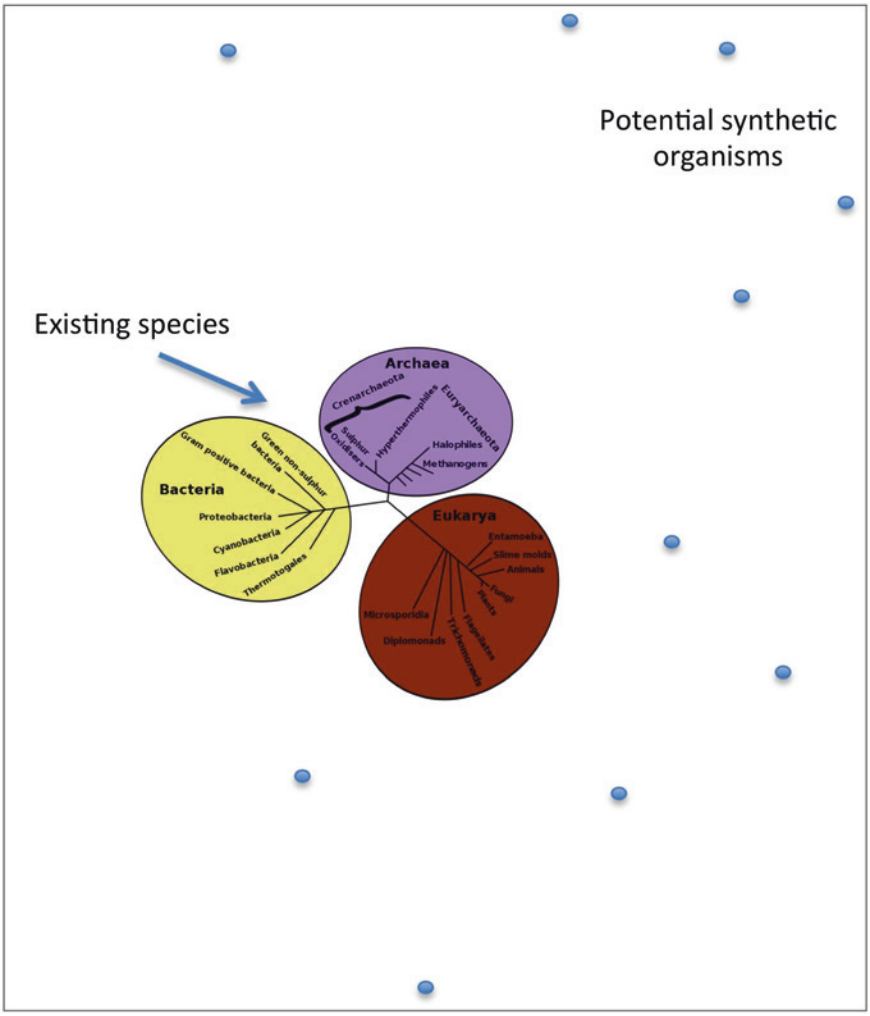

Fig. 2.1 Although life on earth exists for more than 3 billion years, the number of genomes that have been realized by evolution corresponds only to a tiny fraction of the number of possible genomes. Synthetic biology is able to create synthetic organisms that are not derived from pre-existing organisms

that every point on the unlimited sequence space immediately corresponds to a putative living being. In contrary, one has to assume that the vast majority of all potential organisms are not able to "live" in the common sense. Since each point of the sequence space corresponds to one (random) sequence, the chance that it represents a successful living being, would equal that of a monkey typing randomly on a typewriter to reproduce the works of Shakespeare to quote a famous saying.

If the infinite sequence space of potential genomes is depicted as a two-dimensional layer, than only a tiny space is filled by the existing natural genomes that are all connected by evolutionary trajectories (Fig. 2.1). In contrast to natural evolution

that explores the remaining space only in small mutational steps all starting from the existing species, synthetic biology can make immediate use of the complete sequence space, because it does neither depend on mutations nor on any previous gene sequences. The ability to read (sequence) and write (synthesize) DNA in large scale allows to design any sequence and even to assemble whole genomes. This has liberated synthetic biology from the direct connectivity to existing species (Fig. 2.1).

The layer model visualizes the large sequence space that now can be explored by designing novel genomes. In addition, the layer model is also able to accommodate attempts to create orthogonal life forms that are separated from the natural world by a "genetic firewall" (Marlière 2009). Those synthetic organisms that differ from natural ones in their biological and chemical nature can be placed on separate layers. Layers are defined such that within a certain layer all organisms use the same genetic material and use the same genetic code. This allows unlimited exchange of genetic material and information within a layer. One has to point out, that all biological systems created by the engineering branch of Synthetic Biology are based on DNA and the standard genetic code and therefore able to exchange genetic material not only between each other but also with the existing natural organisms. Between the layers no such exchange is possible because they differ in their chemical composition and/or in their genetic code. Therefore organisms that are placed on different layers are genetically isolated from each other and thus have to be regarded as orthogonal. The use of non-standard bases or reassigned

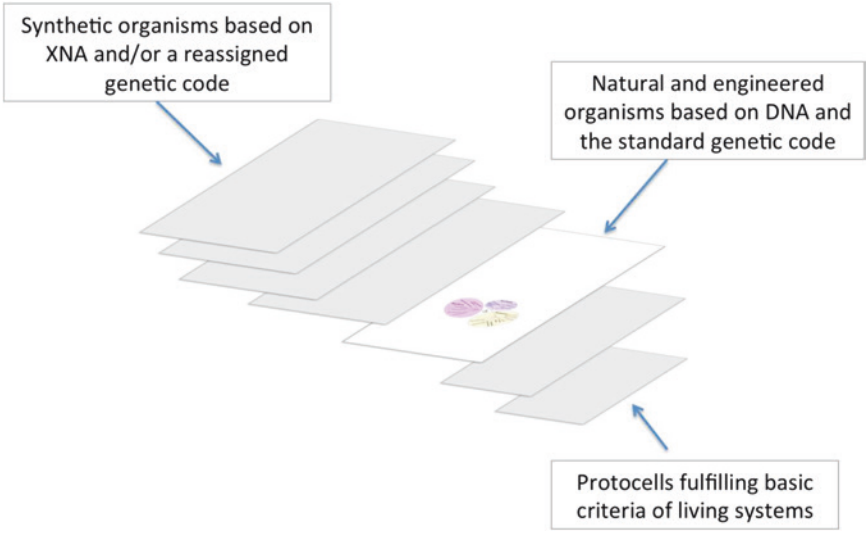

Fig. 2.2 The existing living organisms, produced by natural evolution, cover only a very tiny area of the nearly unlimited space of all potential organisms. The infinite sequence space of potential organisms sharing a common genetic code is indicated by a single layer. Beyond the natural form of life, characterized by DNA as genetic material and the standard genetic code, many other sequence spaces can be imagined, that differ in the genetic code, or in the chemical nature of the hereditary material. While exchange of genetic information is feasible within a plane, no such transfer is possible between different layers

genetic codes in orthogonal synthetic biology is visualized in our model as separate layers of possible genomes.

The layer model is not hierarchical and being placed in another layer does not indicate that some organisms are higher developed than others. However, there might be some layers with a more restricted sequence space, e.g. due to a reduced genetic code encoding a smaller number of amino acids. Such layers most probably correspond to early stages of natural evolution but would also apply to artificial protocells with a reduced genomic content (Loakes and Holliger 2009) (Fig. 2.2).

According to this model, a single layer of the sequence space contains only species that share a common molecular basis of metabolism and reproduction. In particular, all natural organisms belong to one single layer, which might be termed the "natural" layer. It is defined by the use of the "universal" genetic code, which obviously has evolved only once.[1] The universality of the natural genetic code has an important consequence: since it allows the meaningful exchange of genetic information not only between closely related species, but also between those that are only distantly related and may share a common ancestor dating back millions of years. Such events, termed horizontal gene transfer, may occur in nature only infrequently. Nevertheless, they have shaped many species and are an important factor in evolution. Furthermore, several times during evolution novel species have been generated by endosymbiosis events in which complete cells were taken up by another species. The most spectacular case is the fusion of a bacterium with an early archeal cell to form the first "eukaryotic" cell. In these cases it is assumed that a transient uptake of free-living cells lead to an intimate coexistence and finally resulted in intracellular organelles (Sagan 1967). Importantly, this process was accompanied by a transfer of genetic information from the originally free-living but now enslaved bacteria to the nucleus of the eukaryotic cell. This transfer would not have been possible if these two cells, that most probably differed in many aspects, did not share at least the same genetic code.

2.3.1 Layer of Genetically Modified and Artificially Designed Cells

Therefore, all organisms placed within a layer can exchange genetic information, be it via natural mechanisms or genetic engineering. Deletion of endogenous genes, or introduction of additional DNA derived from a heterologous donor organism, would change the genome sequence and thus shift the position of this organism. In general these alterations are small if compared to the size of the total genome, and the genetically modified organisms are still very similar to their natural progenitors. But with increasing number of transferred genes and size of transformed DNA the relationship with the original organism dwindles. If a

[1]Interestingly there exist a few exceptions e.g. in Candida yeasts (Ohama et al. 1993; Santos and Tuite 1995), but these species use a code with some small deviations, i.e. the code is not totally unrelated.

large number of genes, originating from many different species, is used to create a "chimeric" new organism containing an artificially designed genome, this genome sequence has to be placed within the layer of natural species but somewhere in the open space as yet uncharted by evolution. Although the biological and biochemical basis of such synthetic creatures does not differ from their natural counterparts, one may regard such living cells as "Life beyond natural Life" (Elowitz and Lim 2010). Within our scheme it becomes obvious, that there is no principal difference between organisms created by natural evolution, natural mechanisms of horizontal gene transfer, gene technology or by engineering synthetic biology: all are based on DNA as genetic material and the universal genetic code. But the groundbreaking "creation of a bacterial cell controlled by a chemically synthesized genome" has demonstrated: Current synthetic biology does not any longer depend on natural sources of DNA to generate a living organism. It was only the sequence information that was used to direct chemical synthesis and assembly of a complete bacterial chromosome (Gibson et al. 2010). However, this implies that any sequence designed in the computer can be realized by chemical synthesis and may serve as a blueprint for a novel bacterial species. There are no principal technical barriers, at least on the level of genome synthesis, preventing us from designing a synthetic organism whose genome would correspond to any point on the layer, independently whether this designed organism would be viable or not.

Thus, it is not only that all existing biological species can be used as a starting point for genetic modification but also that the vast space of not yet evolved organisms can be explored by synthetic biology. Many critics of the Venter experiments have complained that a mere exact synthetic copy of a natural bacterium just confirms what has long been known, that it is the DNA sequence, which determines the identity and the phenotype of a species. However, the aim of that study was less to demonstrate the creation of novel forms of life, but more to show that it is feasible to synthesize any given genome sequence and to bring it to life with the help of a DNA-free cell. Admittedly, there are and will be many technical obstacles to use this technique for implementing synthetic genomes that are not closely related to existing ones. One of the biggest challenges will be which cells can be used to accept a chemically synthesized genome. It has to be compatible with the incoming DNA to allow for the replacement of endogenous ribosomes by the novel ones. Craig Venter calls this step the "rebooting" of a cell with a new genome. However, "rebooting" may require a high level of genomic compatibility, setting tight constraints on the fantasy of genome designers.

The layer model demonstrates that there are no explicit distinctions between organisms that have been shaped by natural processes such as mutation, recombination and horizontal gene transfer, and those produced by genetic modification or even by introducing a chemically synthesized genome. Even if cells were generated by extreme genetic engineering using many different sources of natural donor DNA or by synthetic biology on the basis of a complete new genome design, in all cases these organism share the same principles of metabolism and inheritance. Therefore they can interact with each other not only in the environment but also by transfer and exchange of genetic information. This has been interpreted by many

to state that synthetic biology essentially is nothing but gene technology. Taking an extreme position, Rob Carlson even claims that natural processes occurring during evolution can be regarded as "technology", as stated in his book "Biology is Technology" (Carlson 2009). He describes "life" as we see it today as organisms that exploit each other to survive and make energy in the Earth's newly formed atmosphere. Eukaryotic organisms utilize mitochondria and chloroplasts acquired through symbiosis for their own purpose. In this sense, bacteriophages appear to be perfect synthetic biologists. Injection of a short piece of viral DNA is sufficient to reprogram a bacterial cell to produce several hundred bacteriophage copies within less than an hour.

Nevertheless, there are good reasons to stick to the traditional scheme of distinguishing organisms derived by natural modes of gene transfer from those modified by classic gene technology or constructed as artificially designed synthetic cells. Especially in the discussion of potential risks for health or environment, we depend on experience and knowledge of both the probability and the damage that might be caused by novel species. We know that natural organisms have existed for as long as life has existed on earth. This does not necessarily imply that these organisms are by any means harmless; mankind was and is always confronted with emerging diseases, including pandemic ones. But we know that all existing organisms, including ourselves, have been shaped by this long record of historical challenges, and we are confident that our immune system will be able to cope with these challenges as it has developed during evolution just for this purpose (if one may use such teleological terminology as a surrogate for selection and fitness). For organisms genetically modified by classic gene technology, it is the close similarity and relationship to existing biological species that can be used to assess potential risks and to decide at which safety level experiments have to be performed. These criteria are not available for synthetic cells that are either composed of DNA fragments originating from many different natural sources, or that have been rationally designed with the help of computers (see Chap. 3 on risk).

Engineering biology aims at a simplification of biological systems into modular and abstract networks to facilitate re-engineering of living systems and even make the technical "synthesis of life" feasible. Philosophically speaking, the belief in an "artificial creation of life" is based on the rigid determinism and reductionism of 19th century materialistic western philosophy. This is best illustrated by a lecture entitled "Life", held in Hamburg, Germany, in 1911 where the American physiologist Jacques Loeb proclaimed his reductionist view that sees living organisms as chemical machines (see Chap. 4). There is indeed a long history of ingenious automatic devices already in the late classical period and during medieval times, not only for popular entertainment but also to serve religious purposes and which later influenced the mechanical natural philosophy of the Enlightenment. For example, Descartes maintained that animals (and the human body) are "automata", mechanical devices differing from artificial devices only in their degree of complexity. With a similar belief in mind, Loeb proclaimed the goal of a controlled and useful design of "synthetic life" by forming new combinations of the elements of living nature by engineering. The engineering version of Synthetic Biology is also

reflected in the prescient imagination of the French biologist Stephane Leduc, who proclaimed a future quantitative biology, which—in analogy to theoretical physics—should give a precise description of organisms whose behaviour is not only completely quantifiable but also predictable and controllable.

From the engineering perspective of modern synthetic biology, a cell is regarded as a complex system of devices and functions that can be reconstructed by rational design. A major goal is the reduction of the complexity of natural cells, which appears to be an unnecessary complication due to historic contingencies. The commonly accepted generalized strategy for this approach is the design and construction of a standardized minimal cell. Such cells can then be used as a chassis to set up artificially designed cells that fulfil useful functions that are novel and have not been seen in nature. The minimal cell itself is equipped only with the basic life-sustaining functions but has been stripped of everything which appears in the eyes of the engineers as contingent or unnecessary. Although there exist no bona fide minimal cells yet, a number of different approaches have been discussed (Acevedo-Rocha et al. 2013). Popular approaches to exploring the "minimal requirements for cellular life" include comparative genomics and massive genome mutagenesis (Forster and Church 2006). Synthetic biologists usually approach this problem by considering "minimal genetic requirements" or by employing information theory to living systems. But, one has also to think of the chemical invariance of living beings: a finite number of basic chemical building blocks (metabolites, amino acids, and nucleotides) participate in endless cycles of transformation between plants, animals and microorganisms in the biosphere. At least one can imagine that one day such a minimal cell could gain the status of a model system like *E. coli*. If one gains enough experience working with such an established minimal cell, it may even be less risky than working with natural cells. This concept could pave the way to a standardized synthetic biology.

2.3.2 Layer of Orthogonal Cells (Xenobiology)

One of the basic assumptions of orthogonality and xenobiology is that the molecular nature of the extant living organism is only one solution to the problem of creating life. If evolution were rerun on our planet, most probably another sequence of molecular events would have occurred. Therefore the specific chemistry of living beings is for a large part due to historical contingencies. In the very moment where the first successful life forms emerged on earth, it was no longer possible to switch to other molecules of life. This suggests, however, that at least potentially there should exist many more molecular structures able to constitute living matter. This view was supported by some early and recent experiments where natural amino acids and co-factors were replaced by related substances without severe loss of function (Cowie and Cohen 1957; Lemeignan et al. 1993; Ma et al. 2014). Therefore the space of yet unexplored alternative life forms is enormous.

This raises the question whether these life forms can indeed be regarded as "alternative" or "beyond natural" (Elowitz and Lim 2010). Here one has to consider that xenobiotic life forms are more or less explicitly modelled on existing living beings. For example genetic information is stored by XNA, which resembles natural DNA not only in its base pairing principle but also in its double helical structure. But this chemical mimicking of natural life, in combination with the inherent genetic firewall of xenobiotic organisms, provides at least one significant advantage to engineering synthetic biology. With regard to their biological safety orthogonal organisms are easier to handle since by design they are unable to exchange any genetic information with natural organisms. They are for example resistant to phages, which could become an advantage in production. It is argued that the farther they are away from natural organisms the safer they are (Marlière 2009; Budisa 2014). This is in sharp contrast to the engineering branch of synthetic biology that argues that artificial life forms constructed on the basis of minimal cells and standardized modules are safer because their behaviour will be much better predictable. Therefore, xenobiotic cells can keep some of the vitality and unpredictability of their natural counterparts.

In contrast to minimal cells that are at least derived from normal cells, it is not so easy to decide whether a cell is still alive if many or all important biological functions are exerted by similar but not identical chemical compounds. If one sticks to a definition of life, which also refers to its material basis, for example that life is defined as being based on DNA as genetic material, then xenobiotic life forms using alternative XNA for information storage cannot be regarded as alive. However, many biological definitions do not make any material constraints but refer only to functional attributions (cf. Chap. 4 by Toepfer). Therefore, replacing certain (or all) parts by those of different chemical composition would not affect the ability of the system to be alive. Even from a practical aspect this is evident, since all realistic approaches to the construction of xenobiotic cells conceptually start with the chemical transformation of an existing organism. Into this undoubtedly living entity, novel components are inserted which then take over certain cellular functions (see Schmidt and de Lorenzo 2012). However, if DNA is replaced by XNA, such an organism would no longer fit into the layer of natural species. Since exchange of genetic information is no longer possible due to the different chemical nature of its material, organisms would belong to a parallel layer, which is neither intersecting nor overlapping with the natural layer.

Although initial steps have been achieved (Malyshev et al. 2014) organisms containing XNA instead of DNA are still far from being realized. Two types of orthogonal organisms, however, might be much easier to achieve: recoded cells with a reassigned genetic code and mirror-like cells based on stereoisomers of natural compounds. In principle, reassignment of only a few codons is sufficient to exclude any meaningful exchange of genetic information. There is good evidence that large-scale reassignment of the genetic code will not be easy to achieve. This is due to the structural constraints in enzymatic realization of different codes, for example in the biochemical structure of proteins involved in charging tRNAs with amino acids. Interestingly, some naturally occurring species (e.g. *Candida*

albicans) have evolved reassignment of a single codon demonstrating that genetic recoding of cells is feasible (Ohama et al. 1993; Santos and Tuite 1995)

An interesting alternative to the difficult tasks of xenobiology or genetic recoding is the construction of mirror-like cells (Church and Regis 2012). Such cells are predicted to behave identically to natural ones since they are based on mirror-like versions of natural compounds. Most organic molecules used in biological systems display chirality, and in all cases nature uses only one of the two possible stereoisomers. Since it is known from physics and chemistry that all properties and reactions are independent of chirality, one can predict that a mirror-like cell will display exactly the same properties and phenotypes as its natural counterpart. However, such cells would fully depend on left-handed compounds whereas a normal cell uses right-handed ones and vice versa. For biotechnology this might be even an advantage. Since all existing natural species are subject to parasite attacks, being mirror-like makes a bacterial cell invulnerable to the attack of bacteriophages. George Church even envisions the creation of mirror-like humans who would then constitute a "parallel" humankind (Church and Regis 2012). With regard to our layer model there exists exactly one orthogonal layer of mirror-like organisms; for every single existing species there exists exactly one that is of opposite chirality. In contrast, with regard to codon reassignments there are almost infinite orthogonal layers if one considers all possible reassignments of the genetic codes. One can even imagine that companies construct their "individual" (and maybe patentable) cells, which are characterized by their highly specific genetic code. These cells are "labelled" by their genetic code and can always be traced back to their inventors in case of alleged misuse of intellectual property.

Although in principle every species might be equipped with an alternative genetic code, there might be serious constraints: regulatory elements such as enhancers depend on DNA sequence. If located in coding regions the sequence of such elements will be altered upon recoding. Therefore it is not guaranteed that replacement of many or even a few codons is compatible with functionality; fitness could be severely decreased. But genetic recoding might be interesting in the construction of minimal cells, which are reduced in their biological complexity anyway. One can imagine different sets of minimal cells constructed on the basis of alternative genetic codes. These constraints are much greater upon the introduction of XNA. Here it is already extremely difficult to establish such an organism. Current scenarios imply a transition from natural to xeno-organisms by gradually replacing natural DNA with its chemical counterpart XNA (Schmidt 2010). This resembles the metaphor of the Delphic boat (Danchin 2003, 2009), where it is not clear whether it is the same after all the planks have been replaced during its long journey. In xenobiology it would be the property of being alive which is maintained (or not), even if all the chemical components are replaced by different versions.

2.3.3 Layer of Simple Life Forms and Protocells

Protocells are cell-like structures that are spatially delimited by a membrane boundary and contain biological material that can be replicated. Ideally they consist of a self-assembling chemical system capable of reproduction. Current research in this field is mainly concerned with the fundamental questions of the origin of life (Rasmussen et al. 2009). The crucial goal is to understand and experimentally master the transition from complex abiotic chemistry to simple biology that is to enable the emergence of complex chemical assemblies capable of fulfilling the criteria for life. In other words, to perform the transition from "non-life" to "life" one needs to define at least basic terminology: "non-life" usually means the maintenance of chemical rules (chemical equilibrium and reaction rates) whereas "life" is described as a system that maintains biological rules (selective pressures from the environment and replication rates).

Another motivation for protocell research is the "artificial life" (AL) field which traditionally includes *in silico* design of systems constructions that exhibit lifelike behaviour ("Soft" AL) and even robotics ("Hard" AL). In this context, "fluid" (or "wet") AL involves the creation of lifelike protocells, based on aqueous carbon chemistry in water with repeatable sets of autocatalytic chemical cycles that are properly coordinated. These autocatalytic cycles include replication of informational material coupled with internal metabolic reactions in a self-maintained manner within the membrane boundary. In an ideal case, the conditions for the coordination of the growth and shape changes of the cell's membrane (that is cell growth and division) are defined. At the level of simple physicochemical laws, the processes of vesicle growth and reproduction occur as a consequence of breaking the spatial symmetry of a synthetic protocell. Finally, protocell research can be employed for the design of drug delivery systems in medicine, for example the encapsulation of drug cocktails in liposomes and various nanoparticle.

If one questions whether protocells are alive or not, the answer very much depends on the criteria that are used to distinguish between living and non-living systems (see Toepfer, Chap. 4). Traditionally, living beings have to fulfil a considerable number of criteria derived from the description of existing biological organisms. In protocell research it is often a single unifying aspect of living systems, which is used to distinguish living systems from non-living ones. Autopoiesis has been proposed to serve as such a unifying concept (Luisi 2006). Autopoiesis is the ability to maintain a system from within by replacing components that are degraded by chemical processes. Such an autopoietic process results in stable maintenance if the number of replaced components equals the number of degraded ones. If the system produces more components than are degraded, the system will grow. This definition does not depend on reproduction or genetic information but resembles more the chemoton definition of life (Gánti 1975) which is modelled along dynamic chemical equilibria. Protocells have a dualistic nature; on the one hand they can be described as a complex chemical system but on the other hand they can be seen as simple forms of life (Rasmussen et al. 2009).

In the layer model, the size of a layer is determined by the number of possible genome sequences. Typically, synthetic protocells carry no heritable material at all (purely metabolic networks) or only very short polymers capable of self-replication (Mansy and Szostak 2009). Therefore, protocells are usually unable to exchange any kind of "genetic" information with each other. This indicates that in our layer model every protocell represents its own small island, separated from other protocells that operate by different metabolic mechanisms. Layers, in a strict sense, are thus only present if some kind of hereditary system will be established allowing the exchange of information between different protocells. Admittedly, with such simple life forms the layer model reaches its limits. But this may actually reflect the unique position that protocells occupy if compared both to natural and engineered synthetic cells.

2.4 Conclusions

One of the major goals of synthetic biology is the design of novel living systems. These can be based on defined minimal cells that are derived from naturally existing cells but with reduced complexity. In essence, this strategy follows traditional engineering principles: reduction of complexity by modularization in combination with quantitative estimation of module parameters is expected to allow total control of such highly integrated "systems". Whether the behaviour of such synthetic cells indeed becomes more predictable remains to be seen since also purely technical systems turned out to be less controllable as originally planned especially if they surpass a certain level of complexity.

The generation of orthogonal living systems follows an alternative rationale: Here it is the genetic incompatibility of these organisms with natural species that confers a certain level of safety. But this is paid by the significantly higher level of unfamiliarity that characterizes such xenobiological organisms. Therefore, in the moment, it is difficult to judge whether this strategy will ever provide a practicable road to synthetic cells that can be used also outside the laboratories.

Thirdly, protocells might be an interesting alternative for certain simple applications that do not require fully living cells. Here it is the defined chemical composition and the inability of these systems to compete with complex organisms that provides some opportunities both with regard to safety considerations and applications.

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