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MaxSynBio: Avenues Towards Creating Cells from the Bottom Up

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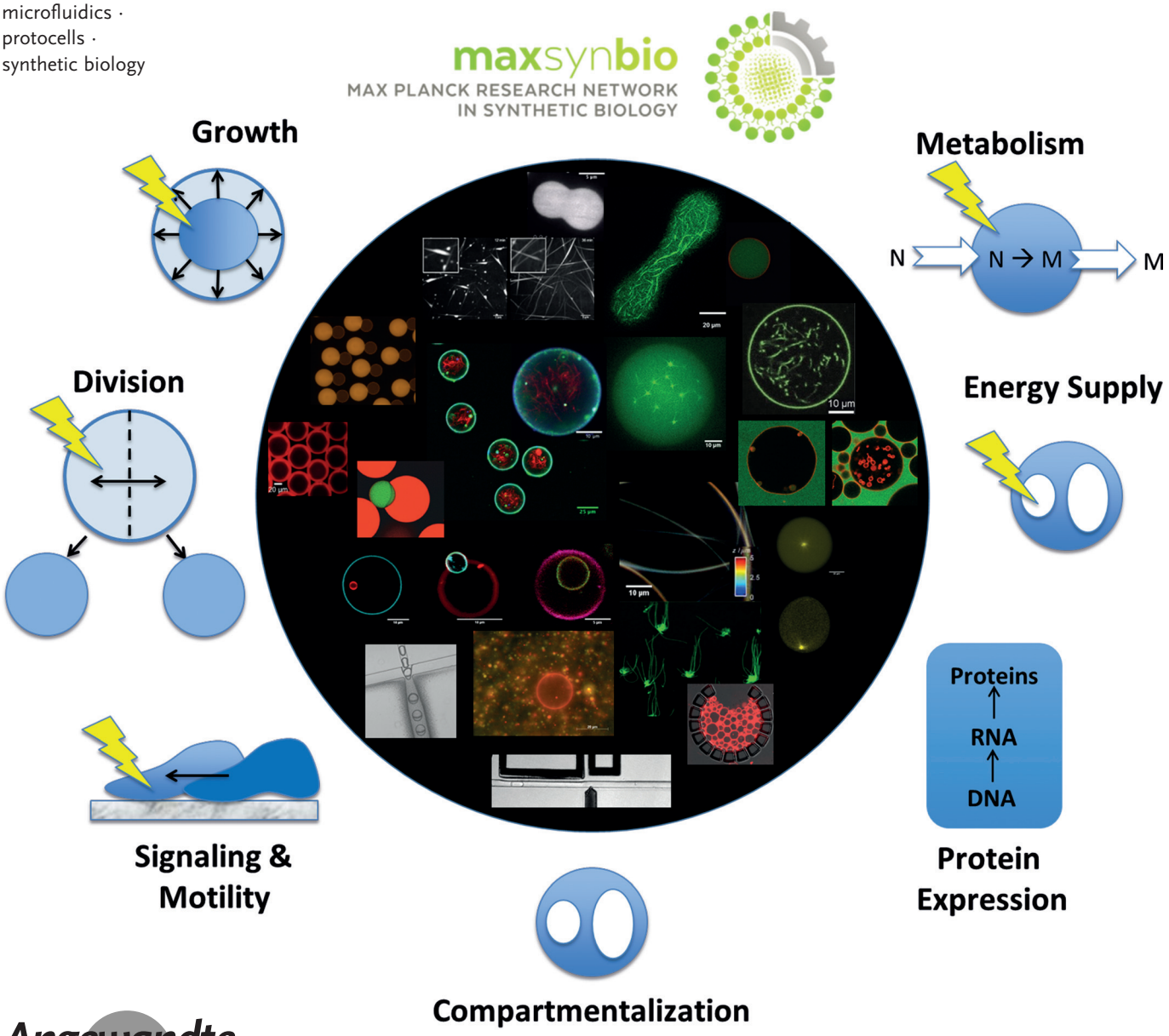

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A large German research consortium mainly within the Max Planck Society ("MaxSynBio") was formed to investigate living systems from a fundamental perspective. The research program of MaxSynBio relies solely on the bottom-up approach to synthetic biology. MaxSynBio focuses on the detailed analysis and understanding of essential processes of life through modular reconstitution in minimal synthetic systems. The ultimate goal is to construct a basic living unit entirely from non-living components. The fundamental insights gained from the activities in MaxSynBio could eventually be utilized for establishing a new generation of biotechnological processes, which would be based on synthetic cell constructs that replace the natural cells currently used in conventional biotechnology.

1. Minimal Cells, Artificial Cells, and Protocells in Synthetic Biology

The emerging field of synthetic biology is considered to be one of the great promises for future biotechnology. This new approach towards biology is partly inspired by the large success of synthetic chemistry over the past century, but also the wealth of mechanistic insights gathered through decades of research in molecular biology and genetic engineering. Currently, biotechnology is limited by the fact that it relies on production organisms that are enormously complex entities, featuring large numbers of components, but also an inherent redundancy and ambiguity in their functional cellular elements and biomolecular networks. Thus, synthetic biology aims to generate simpler life-like entities, that is, man-made systems, which can be predicted, manipulated, and controlled with exquisite precision.

The complexity of natural systems can be understood as the product of a very long "arms race" between living species in their competition for resources. However, it is far from evident whether life as such, including its fundamental features of metabolism and self-replication, could not be implemented and maintained in much simpler predictable systems. Such minimized systems would represent more efficient machineries for the conversion of energy and the production of drugs and smart biomaterials compared to conventional host organisms like microbes. This is the underlying hypothesis of many enterprises summarized under the concept of the "minimal cell".

Consequently, the quest for minimal cells, which would potentially allow maximal efficiency in biotechnological processes, has been at the forefront of synthetic biology for many years. Teams employing the full power of large-scale DNA synthesis, most prominently represented by the Venter group,[1] have come a long way in addressing the minimal set of genes through top-down gene knockout, and by constructing the full genome of a microorganism able to fully take over the live functions of a cell.

While being a valid approach to reach a minimized host chassis, these studies have so far not attempted to define the minimal set of functional elements required to build a living system from scratch. Instead, such a de novo approach has been stimulated by the origin-of-life field, in an attempt to identify the key components of a historically plausible "protocell". Much work on the formation, growth, and division of membrane vesicles,[2] the replication of nucleic acids inside protocells,[3] and primitive biocatalysis[4] was pioneered by origin-of-life researchers, who necessarily had to follow a bottom-up approach.

These fundamental questions at the core of life sciences, namely, what is life and how could it reconstituted in a minimal system, are currently only marginally addressed in the current research in synthetic biology. Although protocells are usually included in all definitions of synthetic biology, active research in this area has been largely underrepresented. Protocell research

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has also suffered from comparably slow progress over the past ten years, in comparison to approaches that involve the development of advanced genetic circuits through genetic engineering in organisms at the systems level.

This difference is certainly due to the fact that biological systems can be much more easily manipulated than fundamentally understood from first principles. It is also the consequence of only moderate interest in synthetic biology from fundamental disciplines such as chemistry and physics up to now. Although there has been tremendous progress in collaborative projects at the interface between chemistry and biology, and physics and biology, only few groups and research consortia worldwide have attempted the bottom-up reconstitution of essential features of living systems, among them our recently founded MaxSynBio initiative.

Within MaxSynBio, we will approach synthetic biology from a fundamental perspective of basic research. This distinguishes our enterprise from other research consortia that aim for a mainly application-driven synthetic biotechnology. Our primary goal is a true bottom-up synthesis of minimal living systems through modular synthesis from well-characterized functional molecular entities, parts, and modules.

In this minireview, we will discuss the manifold of different tasks and aspects covered by our research initiative, which is presently outlined as a six-year project, but will certainly have to extend to a much larger time scale in order to reach its goals. We will also touch briefly upon the state of art of the various research goals, many of which are currently pursued by other groups and consortia worldwide.

2. How and What to Engineer from the Bottom Up?

Living organisms are complex, self-organizing systems featuring the following important properties:

- They are compartmentalized.
- They continuously exchange mass and energy with their environment.
- They self-organize and regulate their spatiotemporal features.
- They can move autonomously and grow, and are capable of development and evolution.
- They are capable of reproduction.
- They sense and communicate with their environment.

In order to reduce the complexity of the objects under investigation, we do not primarily aim at the reconstitution of a whole functional synthetic cell. Instead, our research is subdivided into selected life processes, which we believe are most important for the proliferation of living cells, more specifically:

Energy supply: All active processes in living systems need a continuous supply of energy and materials, either harvested from the extracellular environment or transferred from other parts of the systems. In many cases, energy supply and storage is closely connected to cell metabolism, that is, to the enzymatically controlled conversion of energy into chemical substances required for certain processes and subsystems, or the conversion of nutrients into readily available energy components needed for performing cellular functions.

Metabolism: Metabolic processes are the hallmark of life. Besides their fundamental importance, they are central to industrial production processes. Metabolic reaction cascades and networks in biological cells are of impressive complexity. In MaxSynBio, we aim to reconstitute a fully functional metabolic cascade while reducing its complexity to a minimum. As a proof of principle, but also to demonstrate a practical application, we focus on the CETCH cycle, a synthetic pathway that captures and converts CO_2 into organic compounds.

Growth: The term growth is used here in the context of cell development, that is, it refers to the increase in volume of a single cell. Cellular growth can happen either by gradually acquiring material from the extracellular medium or through fusion. Growth often precedes cell division. We consider both processes as key phenomena of proliferating cells.

Replication and Division: A mother cell divides to produce two daughter cells. Before division can occur, the genomic information stored in chromosomes must be replicated, and the duplicated genome must be separated between cells. Generally, self-replication of an information carrier is considered to be the key causative reaction required for the emergence of life. In a synthetic cell, this information carrier might be DNA that is replicated with the help of polymerases. Alternatively, a self-replicating RNA-based system could also be used for simpler cellular designs. One type of division

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mechanism is binary fission, where the genetic material is segregated equally into two daughter cells. In order to divide, a cell has to be polarized. Cell polarity refers to spatial differences in the shape, structure, and function of cells. Almost all types of cells form polarity patterns that enable them to spatially segregate specialized functions.

Signaling and Motility: Along with control of cellular growth and differentiation, morphogenesis is one of the fundamental aspects of biology. It causes a cell to polarize or an organism to develop its shape. Morphogenetic responses can be induced by environmental chemicals or by mechanical cues as well as cell–cell interactions. These stimuli initiate a spontaneous and active response in cells, for example, adhesion on surfaces, directed movement within their environment, or cytoskeleton organization, which in turn impacts division.

In the context of MaxSynBio, we summarize the entirety of the above life processes under the term "minimal cellular system". The combination of these processes is a prerequisite for a functional living entity. The minimal cellular system is based on all the key aspects outlined above, with the very important initial condition of being compartmentalized. Nearly all life processes take place in compartments (or sub-compartments) consisting of membrane structures functionalized with embedded proteins. It is actually this compartmentalization that ensures that living systems are able to operate far from thermodynamic equilibrium. As a consequence, synthetic life processes also must be reconstituted in cell-like microcompartments.

In principle, there is no restriction concerning the molecules and materials to be used. However, with regard to the nature of functional elements that could implement the complex set of features required, it would make sense to rely on biomolecules, primarily proteins. Thus, the efficient generation and reconstitution of functional proteins in cell-free environments is of paramount importance and probably the greatest challenge for enterprises like this. However, any future engineering approach will also have to look into potential non-natural replacements of these building blocks. In addition, smart new laboratory routines will have to be employed to assemble fluid-based systems on the scale of cells. Thus, the importance of new nano- and microfluidic handling routines in bottom-up synthetic biology cannot be overestimated. We will briefly touch upon these aspects and how we will employ them in our initiative (Figure 1).

It is obvious that only a truly interdisciplinary team of researchers can attempt to solve all these tasks in a concerted fashion. Importantly, this kind of synthetic approach to life-like systems not only poses technical challenges, but also needs communicated to the general public, as it may elicit fears or strong ethical concerns, both justified and unjustified, at various levels of reflection. Included in our consortium are thus partners from the humanities, who accompany our scientific work with awareness and respective ethical considerations.

In the following, we will outline in brief what particular aims and problems generally need to be tackled, and how we propose to address them.

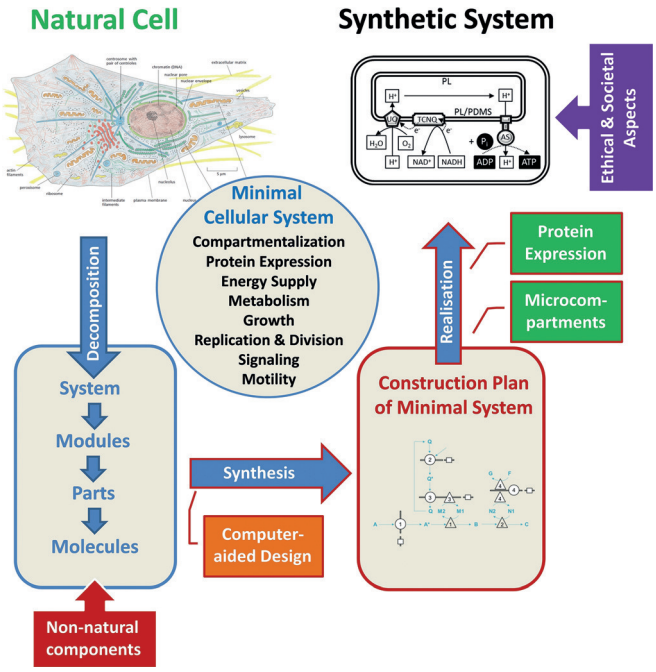

Figure 1. In MaxSynBio, the essential features and processes of a so-called "minimal cellular system" (compartmentalization, protein expression, energy conversion, metabolism, growth, replication and division, signaling, and motility) are designed for a better understanding of the behavior of natural cells. System synthesis is based on natural and non-natural components, and in the long term, it should be assisted by computer-aided design tools to generate minimal system blueprints. The practical realization of the blueprints is supported by technology platforms (protein expression, microfluidics). The bottom-up synthetic biology workflow as a whole is critically monitored and evaluated regarding ethical and societal aspects.

2.1. Energy Supply

A unique feature of living cells is the ability to extract energy from their environment and to use this energy to carry out activities such as growth, movement, or reproduction. In general, energy from nutrients (in cellular respiration) or light (in photosynthesis) is transformed through respiratory or photosynthetic electron-transfer chains into a proton gradient across the cell membrane, which is finally utilized for adenosine triphosphate (ATP) synthesis. Similarly, to sustain life-mimicking processes in synthetic cells, a continuous supply of energy is required. Therefore, we aim to design and construct energy regeneration modules that are specified to continuously supply energy in the form of ATP to an artificial cell.

In nature, ATP regeneration is coupled to the recycling of nicotinamide cofactors [NAD(H) or NADP(H)]. Although in some archaea, these processes might be decoupled where the necessary proton gradient for ATP synthesis is generated by the light-driven proton pump bacteriorhodopsin (BR). To date, few attempts to mimic energy regeneration under in vitro conditions have been reported.[5] Most of them concentrate on the conversion of light energy to ATP.[5a–c] To this end, the combination of ATP synthase (ATPase) and BR has attracted a lot of attention.[5b] Recently, a combination

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of photosystem II, a protein complex that is able to split water photocatalytically, and ATPase for light-driven ATP regeneration was demonstrated.[5c] The conversion of chemical energy into ATP has been less studied.[5d] Until now, no synthetic system capable of chemical-energy conversion from an imported substrate (e.g., glucose), coupled to ATP regeneration via nicotinamide cofactors recycling has been demonstrated. Nicotinamide cofactors are the most abundant redox cofactors in living systems. They are involved in many enzymatic transformations and their recycling is of high practical relevance.

The literature shows that the energy supply issue in bottom-up synthetic systems is largely unsolved. Clearly, the design of synthetic energy-converting systems from biological and chemical components is a task of significant complexity. In order to cope with it, smart simplifications are necessary. We aim to develop such strategies for the assembly of functional parts and their integration into functional modules. Our functional part consists of a membrane protein or a chemical catalyst embedded in a suitable container (a membrane or hybrid vesicle, or a polymersome). As a first step in this direction, we used a high-throughput microfluidic method to generate stable, size-defined liposomes termed droplet-stabilized giant unilamellar vesicles (dsGUVs) and functionalized them with ATPase.[6] Application of a pH-gradient causes production of ATP. Furthermore, a complex chemical-energy-driven ATP-regenerating functional module, an artificial mitochondrion, has been constructed.[7]

2.2. Metabolism

Metabolism is the dynamic chemistry of cells that provides the energy and building blocks required for the three- and four-dimensional self-organization of life. In the cell, metabolism is organized as a complex interplay between catalysts (enzymes) and substrates (metabolites). A long-term goal of MaxSynBio is to construct complex metabolic networks within defined compartments that can be coupled to energy modules. From an application point of view, the assembly and operation of multi-enzyme cascades in compartments is an attractive goal, which will pave the way to the tailored construction of minimal "cellular factories" for the customized production of value-added compounds. Such bottom-up efforts could serve as an alternative strategy to conventional top-down efforts pursued in biotechnology, such as metabolic engineering of microorganisms.

Our efforts to create metabolic compartments are fueled by recent developments. On the one hand, it has become possible to incorporate and operate enzymes inside polymersomes and vesicles,[6,8] thus demonstrating that it is in principle possible to build and control chemical reaction networks within defined space. On the other hand, the reconstruction and operation of complex reaction networks for the production of value-added compounds in vitro has also become feasible. Notable examples are the use of complex enzyme cascades in vitro to produce monoterpenes, isobutanol and polyhydroxybutyrate from glucose as a feedstock.[9]

While there has been recent progress in the reconstruction and control of complex enzyme networks in vitro, most approaches have thus far focused on exploiting naturally existing reaction cascades. However, the full potential of synthetic biology can only be realized when it becomes possible to assemble customized, non-natural reaction networks in a rational fashion. In a proof of principle, we recently designed and realized a synthetic pathway for the capture and conversion of carbon dioxide. The so-called CETCH (crotonyl-coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA) cycle is an in vitro metabolic network of 17 reactions that was established with enzymes originating from nine different organisms, including three engineered enzymes.[10] While it was possible to draft and assemble a simple version of the CETCH cycle through the concept of metabolic retrosynthesis,[11] a robust operation of the system was only possible after several rounds of optimization, which included enzyme (re-)design and the principle of metabolic proofreading.[11,12] In Version 5.4, the artificial CO_2 fixation cycle is slightly faster and requires 20% less energy per CO_2 fixed than the naturally evolved Calvin cycle that operates in photosynthesis.

Next efforts will focus on further optimizing the CETCH cycle with model-based approaches, known from chemical engineering, as well as coupling the artificial reaction network to energy (and co-factor) regeneration modules[7] to allow its continuous operation. Altogether, these approaches aim at establishing a synthetic alternative to photosynthetic CO_2 fixation to access the greenhouse gas CO_2 as a future carbon feedstock for a sustainable, low-carbon bio-economy.

2.3. Growth

Growth of proto-cellular compartments can be established either by fusion, or by gradually acquiring material from outside. In addition to just increasing the cell size, growth delivers new energy, information, and nutrients for cell development. In the context of MaxSynBio, we are developing various strategies for growth of droplets, liposomes, and polymersomes as fully synthetic analogues (Figure 2).

Vesicles represent a relatively close approximation to the compartments of living cells. The pioneering studies on vesicle growth[13] have relied mainly on fatty acid micelles and vesicles, which have been investigated extensively as protocell models. The spontaneous uptake of fatty acids into preformed vesicles has been often considered as a primitive growth mechanism. However, while fatty acids are efficiently incorporated, phospholipids, the constituents of modern living cells, are highly insoluble, and this mechanism for growth is not applicable. Additionally, the incorporation of membrane proteins into fatty acid bilayers, as well as the synthesis of proteins within fatty acid vesicles, as further steps in protocell design have not been achieved so far.

The continuous search for realistic protocell models based on natural phospholipids has led to the approach of phospholipids being supplied from the outside. Vesicle growth has been demonstrated by adding a cationic precursor hydrolyzed

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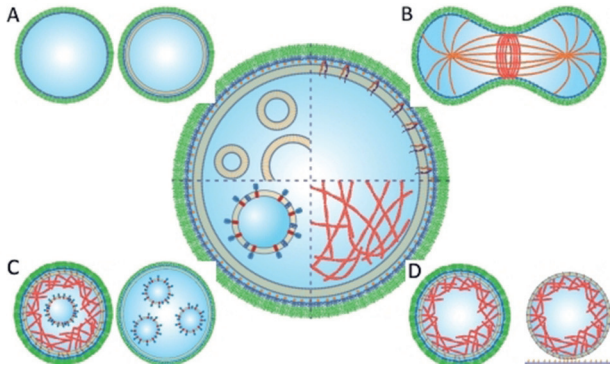

Figure 2. Schematic representations of modular engineering approach for bottom-up assembly of cell-like compartments (A) which can grow, divide (B), generate energy and forces (C) and perform cellular function such as adhesion and migration (D).

into a membrane lipid by a catalyst embedded in the membrane.[14] However, the quite exotic chemistry of the membrane may hinder interactions with other biological species (e.g., membrane proteins). In the case of authentic phospholipids, vesicle size increase has already been achieved by vesicle fusion, initiated by various triggers.[15] Thus, membrane fusion could be employed as a simple growth mechanism, not only for increasing the membrane area but also for supplying other necessary chemical species.

Our primary aim is to establish a dynamic and controlled increase of the microcompartments size. The various possible model containers based on fatty acids, phospholipids, or polymers, require conceptually different approaches, but all largely rely on concentration gradients as driving forces, and electrostatic interactions and light as external stimuli, as well as combinations of these. Controlled growth of droplets can be accomplished by coalescence of two droplets by means of manipulation through microfluidics or optical trapping. In the case of vesicles, we rely on vesicle fusion driven by osmotic, electrostatic, and other stimuli and modulated by membrane tension. The growth of polymersomes will be established with stimuli-responsive nanoparticles containing amphiphilic material, which is released upon the application of a stimulus and integrated into the polymersome membrane, thereby leading to growth.

2.4. Replication and Division

Division and replication are the most obvious and distinctive features of living cells. These two primary aspects are functionally separate but need to be concerted for the successful generation of offspring: the dramatic mechanical transformation that leads to full splitting of a compartment, and the replication of the genome, copies of which need to be faithfully distributed into the two new compartment fragments. In modern cells, large protein machineries are devoted to the spatial organization and orchestration of these processes. Thus, full reconstitution of a replicating and dividing membrane compartment containing DNA remains a grand challenge for the creation of an artificial protocell. In addition, self-replication of a protein-based living cell will require replication and synthesis of more than a hundred active genes needed for translation and ribosome biogenesis[16]—a monumental task, the completion of which is still distant. Nevertheless, replication and division of semi-autonomous cellular designs could be achieved by using in vitro translation coupled replication of minimal genomes based on DNA. RNA is an alternative attractive information carrier that can support self-replication and even evolution in cell-like systems.[17] It remains to be explored how such self-replicated RNA molecules could be faithfully separated to the daughter cells.

Exciting work along the lines of coupling compartment growth and division has been performed with RNA enclosed by fatty acid vesicles.[2b] However, due to their relatively low stability and "leakiness", fatty acid vesicles have limited potential for implementing more sophisticated functional (protein) modules, and vesicles made of phospholipids offer a more widespread functional variability.

Over the past few years, it has been acknowledged that in their interior, cells form biochemical membraneless compartments (droplets) through liquid–liquid demixing of biomacromolecules.[18] Studied examples are the P-granule protein PGL-3 and the stress granule protein FUS.[19] When expressed in a test tube, these proteins phase separate to form droplet-like compartments. These droplets can grow through steady uptake of material from their environment, and there are indications that they may also divide under certain circumstances. Hence they provide another promising concept for the construction of an artificial minimal cellular system.[20]

With regard to the mechanical transformation of phospholipid compartments, much work has been devoted in the past years to the recruitment of membrane sculpting and membrane-transforming protein coats and machineries to giant vesicles,[21] whereas the incorporation of proteins involved in cell division has been rather challenging. Thus, the task of full large-scale vesicle division through protein activity has hardly been approached. Of particular interest is the incorporations of well-known eukaryotic and prokaryotic division machineries based on actomyosin and FtsZ,[22] but archaeal divisomes have also lately come into focus.[23]

Towards possible DNA replication in membrane compartments, it has been shown that budding and fusion of vesicles can occur in encapsulated polymer solutions by entropic depletion volume and wetting effects.[15b] Such self-reproduction could be combined with the amplification of encapsulated DNA.[14] Partitioning of the large chromosomal DNA in vesicles may be at least partly achieved through purely entropic repulsion.[24] Alternatively, spindle-like structures for actively segregating DNA can be reconstituted from purified components of the bacterial plasmid-segregation machinery.[25]

The ongoing work in our consortium covers a number of these mentioned aspects of minimal cell division and replication. We focus on physical, in particular temperature-induced, transformations of droplets, and highlight the aspect of droplet transformations by force-transducing protein machineries that have been segregated into the droplets.[26]

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We combine theoretical and experimental measures to investigate how vesicles can be transformed by physical cues, particularly by light, and how this transformation can progress into true fission based on local and global membrane properties. There will be a focus on the characterization of how exactly cargo is distributed into two daughter compartments during an enforced division process. Finally, we aim to reconstitute chromosome replication and segregation in vitro and couple it to the mechanical process of compartment splitting.

2.4. Signaling and Motility

Although attaining a fundamental characterization of cellular signaling in minimal systems is a compelling goal, there has been little progress, mainly due to the amazing complexity of these processes. The geometry of biological membranes is tightly intertwined with the signal-processing capability of a cell.[27] The plasma membrane represents a surface that actuates signaling through the dimensionality reduction resulting from the recruitment of cytosolic effectors, for example, to membrane-bound GTPases.[28] However, the local geometric properties of a membrane also determine the ability to recruit these cytosolic effectors. For example, signaling from GTPases is affected by the deformation of the plasma membrane by the cytoskeleton. In turn, cytoskeletal growth that deforms the plasma membrane is guided by cytoplasmic signaling gradients that emanate from the recruited enzymatic effectors.[29] Self-organized information processing at cellular membranes arises from recursive dependencies in the triad of membrane shape, cytoskeletal dynamics and signal transduction, thereby enabling context-dependent motile and morphogenic responses.[27,30]

Recently, we developed GUVs with reconstituted α2bβ3 integrins. Upon activation of integrins by Mn^{2+} ions, the integrins responded specifically to the externally presented fibrinogen matrix by spreading on top of it.[6] This is a first demonstration how engineered compartments can receive signals from the outside and translate it into an active response of the compartment. As a next step, the coupling of integrins to a force-generating molecular network such as F-actin/myosin or microtubules/kinesin might cause compartment mobility initiated by an external signal.

We aim to synthetically reconstitute these stigmergic systems fundamental to life on artificial membranes. In particular, we attempt to synthesize a microcompartment that is able to receive external signals, transmit these signals across its boundary, and translate them into morphological changes and motility of the compartment. Besides intracompartmental molecular networks, compartment mobility might be also generated by externally attached cilia. The general concept is to reconstitute a synthetic system that generates signaling activity in combination with spontaneous or signaling-directed mobility.

3. Enabling Technologies

3.1. Microfluidics to Generate and Manipulate Compartments

As outlined above, droplets are conceptually the simplest minimal system usable for liquid compartmentalization. Droplets are easily generated in two-phase systems when an external source provides sufficient work to overcome the energetic cost of the creation of a droplet interface: simple agitation of an aqueous phase in oil will lead to the formation of a dispersion of one phase in the other. However, bulk hydrodynamic forcing leads to a polydisperse distribution of the compartments. In addition, basic thermodynamic arguments reveal that the dispersion is only metastable and will spontaneously decay towards two bulk phases separated by a surface of minimal area. The use of droplets as minimal compartments relevant in a synthetic biology approach therefore requires a means to stabilize the droplets in a metastable state and to control the droplet sizes accurately over large populations. These two problems can be solved by employing droplet-based microfluidics.[31]

All microfluidic devices used in our research are fabricated from poly(dimethylsiloxane) (PDMS) using photo- and soft-lithography methods.[32] PDMS is a common material in microfluidic technology due to its low price, good biocompatibility and permeability to gasses, high transparency, and low fluorescent background. Droplets are generated in a flow-focusing geometry junction, in which an aqueous phase is cut off by a surfactant-containing oil phase. Following the formation, water-in-oil droplets are stabilized by the accretion of block-copolymer surfactants at the water–oil interface, which leads to to reduction of the oil/water interfacial tension.[33] The droplet diameter is mainly controlled by the channel dimensions, but can also be regulated to some extent by varying the flow rates of the aqueous phase and oil phase.

To allow precise delivery of various biological components into preformed droplets, the microfluidic devices can be integrated with small and compact electrodes to apply electric fields in the microchannels. These electric fields induce destabilization (poration) of the surfactant (mono)layer and facilitate controlled injection (pico-injection) of aqueous phase into the droplets. The design of our droplet-based pico-injection unit is adapted from Abate et al.[34] A microfluidic flow-control system is used to introduce droplets into the pico-injection unit, in which isolated droplets pass an electric alternating current (AC) field. This process destabilizes the droplet interface and allows the introduction of biological reagents through a pressurized injection channel (Figure 3). The injection volume can be controlled precisely between 1 to 100 pL, dependent on the applied pressure in the injection channel.

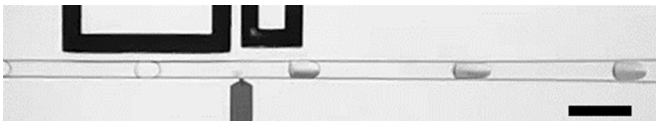

Figure 3. Example of pico-injections of around 15 pL of dark liquid into droplets passing the side channel. Scale bar: 50 μm.[6,34]

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In addition to offering novel methods for the creation of synthetic compartments, microfluidic systems can also provide a means to handle lipid vesicles for subsequent analyses. Devices with pairs of PDMS posts make use of the hydrodynamic forces within microfluidic channels to immobilize single or pairs of GUVs.[35] Individual pressure-controlled chambers designed on-chip provide an additional level of confinement for the fast and reliable control of chemicals flowing around the GUV, which is useful for parallelized long-term studies.[36]

3.2. Cell-Free Protein Production

The production of purified functional proteins is both a key stage and a bottleneck for the research of many groups in the life science community, including for the bottom-up assembly of minimal biological systems. In spite of great advances in the standardization and parallelization of protein purification, the highly specific purification conditions required for individual proteins precludes the development of general methodologies. Suitable conditions for the purification of a novel protein often cannot be identified by extrapolation from homologous systems, and as such, many of the integral and peripheral membrane proteins key to our working tasks remain notoriously challenging to express and purify.

An alternative bottom-up strategy to integrate proteins into a system is through cell-free protein synthesis (CFPS). Here, bacterial and eukaryotic cell extracts or recombinant systems can be programmed with RNA or DNA to produce proteins directly in an in vitro environment. In addition to simple protein production, CFPS can be used to establish synthetic gene circuits,[37] generate integral and peripheral membrane proteins,[38] and even produce large viral assemblies capable of infection.[39] Remarkably, co- and post-translational protein modifications have been achieved in eukaryotic extracts by using additional microsomal membranes.[40] The absence of these modifications is a key shortcoming in standard bacterial expression systems.

In recent years, CFPS systems have become increasingly amenable to encapsulation by the various microcompartments used within the MaxSynBio network, such as liposomes/vesicles,[41] emulsion droplets,[42] polymersomes,[43] and coacervates.[44] Due to these advances, many groups have successfully used CFPS in the development of cellular mimics.[45] Many valuable manuals and reviews discussing the practical challenges of these systems are available.[46] Due to recent developments, the design of microfluidics systems[47] has promoted the development of droplet-encapsulated CFPS.

Within our consortium, there is long-standing interest and expertise in the development of droplet-encapsulated CFPS,[42b] which is now being exploited to develop novel minimal biological systems based upon cell-free expression. The coupling of transcription and translation makes it an appealing choice for the realization of a partially or fully self-encoded synthetic minimal cellular system.[48] However, the creation of such a complex self-replicating system based on CFPS remains a long-term goal, since it requires the parallel integration of many different modules such as recursive genome replication, ribosome biogenesis, lipid synthesis, division, and energy production. Although considerable progress, for example, with respect to de novo ribosome synthesis during CFPS, has been made,[49] significant improvements to the yield and lifetime of CFPS systems and to the physicochemical compatibilities of the different modules are still needed. These issues may be addressable through optimization of the CFPS components using mathematical modeling and computational tools.[50]

4. Design of Biosystems from Functional Modules: Towards an Engineering Workflow

In the future, great progress along the bottom-up design route towards self-organizing biosystems is expected, and the toolboxes of synthetic biology will be filled with an increasing number of functional building blocks. In this way, libraries of molecules, parts, and modules will be established, which is one of the major prerequisites for the systematic synthesis of artificial life-like systems.[51] At the far end, the computer-aided design of life-like systems at the three main system scales involved (molecules, parts, modules) is a big vision from the bioengineer's point of view. The targeted design of functional systems requires: 1) a blueprint, that is, a clear definition of the functionality of the system to be constructed; 2) quality assurance, that is, experimental validation of the functionalities of the molecules, parts, and modules from which the targeted system is assembled; 3) standardization, that is, well-defined interfaces connecting the functional components at each level of the system's hierarchy in a stable manner; and 4) mass-production platforms, that is, technologies enabling the manipulation and assembly of all system entities in a reproducible manner. These four elements will be essential for the bottom-up production of biosystems from functional units in a bio-engineering workflow, in analogy to workflows established in other engineering disciplines such as chemical, electrical or mechanical engineering.[52]

First successful examples along a computer-aided design route towards synthetic cells have already been shown. Schneider and Mangold[53] demonstrated the modular assembling process of a computationally modeled protocell consisting of a membrane proliferating module, a membrane contraction module, and a positioning module. Theoretical hypotheses were tested in order to merge the module models to a protocell model with synchronously working parts. Otero-Muras and Banga[51] have proposed an automated design framework for 1) forward design by finding the Pareto optimal set of synthetic designs for implementation, and 2) reverse design by analyzing and inferring motifs and/or design principles of gene regulatory networks from the Pareto set of optimal circuits. The authors illustrated the capabilities of their framework by considering different case studies, including an oscillator system. The two selected publications show that the establishment of engineering design concepts is very helpful for a systematic approach towards the modular assembly of biosystems from functional modules. In this area,

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we expect new exciting developments when designing cellular systems featuring complex behaviors, including division, cognition, and motility.

As discussed by Tayar et al.,[54] recent advances in cell-free protein expression systems allow the reconstruction of self-organization phenomena in reaction-diffusion systems, in particular 1) turnover mechanisms for continuous and prolonged gene expression reactions, 2) programmable gene expression reactions using biological regulatory elements, and 3) spatial distribution and communication between compartmentalized reactions. As concluded by the authors, defining design rules for self-assembly in synthetic biosystems is essential for paving the way to the realization of autonomous self-replicating systems.

Bottom-up synthetic biology is not restricted to reproducing and mimicking the features and behaviors of living systems existing in nature. It has already started to explore hybrid systems combining natural and non-natural molecules, parts or modules. For example, Otrin et al.[7] have assembled a chemical-energy-driven ATP-generating artificial module through the bottom-up reconstitution of ATP synthase and terminal oxidase in novel nano-containers, built from graft copolymer membranes and from hybrid graft copolymer/lipid membranes. These containers might be usable as a versatile tool for membrane protein reconstitution in more complex compartmentalized systems, for example, protocells equipped with modules for energy supply and metabolic networks. In this way, synthetic biology could also contribute to the construction of systems featuring self-organizing behaviors that are not observable in nature.

5. Ontologies of Life and Ethics

Within recent years, there have been different agendas and approaches in order to identify possible societal challenges for and within synthetic biology.[55] The following four aspects can be described as most important in dealing with the ethical challenges.

First, safety and security problems have to be addressed. A frequently discussed issue is the problem of a possible misuse of results and products. Since information, reagents, and new technological developments have the potential to be used for both beneficial and harmful purposes, this first challenge is designated as "Dual Use Research of Concern" (DURC). Many scientific organizations have elaborated and implemented codes of conduct as a kind of self-regulating set of standards in order to influence the work of the respective researchers. Nevertheless, there are four aspects, especially with regard to the top-down approach, that need peculiar and ongoing awareness, particularly concerning possible ecological effects: 1) differences between the physiology of "natural" and "synthetic" organisms, 2) hitherto unknown possible alteration of synthetic organisms in different habitats, 3) possible evolution and adaptation of the "produced" synthetic organisms, and 4) the possibility of microbes taking up free DNA from the environment or exchanging their genetic material with other organisms. However, up to now, the existing regulation frames, especially with regard to the protocell approach, can be said to sufficiently cover the current research activities.[56]

Second, especially with regard to the protocell approach, ethical issues from a possible blurring of cultural concepts and distinctions such as "living versus non-living matters" or "natural versus artificial" have become subject to societal, conceptual, and ethical studies. Notions and metaphors such as "creating life" or "playing God" can be understood as society's attempts at finding expressions for the present significance and impact of technological developments. One source of potential unease linked to synthetic biology is the fact that the logical value of a statement like "X does (not) belong to the class of living systems" may well turn out to depend not only on X, but also to some extent on the respective observer and the ontological and epistemological concepts that they take for granted.

Our analysis of different metaphors used by science and society could identify two different processes that are caused by the emergence of new biotechnologies. On the one hand, the capacity of biotechnologies may lead to profound transformations in the respective social, economic, or physical environments and therefore may have significant implications for the different ways of life. On the other hand, the generation of novel objects not found in nature may disturb and alter established schemes of meaning and value and thereby gain potential for causing societal unease.

Third, the ethical and societal debate about dealing with emerging biotechnologies in general and synthetic biology in particular moves towards the question of who must and should be involved in making decisions pertaining the stated questions. Thus, it is not only whether the scientific promises will be fulfilled that is at stake, but likewise how and by whom they will and should be propelled. For that reason, public participation in science is not only another "nice to have" item on the agenda of assessing emerging biotechnologies but will be decisive for the future trajectory of synthetic biology.

Fourth, by trying to create artificial life out of synthetic chemicals in order to better understand the process of how life evolves coincidently, the question arises of how such endeavors might change basic epistemological and ontological concepts such as life, biodiversity, or evolution. Against this background, one of the most urgent questions is to develop a feasible framework to theoretically stress the achievements within bottom-up synthetic biology towards the conceptual challenges.[57]

6. Final Remarks

According to cell theory, every cell is derived from another living cell, and nothing is known about how a cell could come into existence de novo from an intricate interplay of non-living constituents. However, such a process must have occurred at least once, and armed with today's knowledge and technology, could potentially be within reach following a bottom-up engineering approach with well-defined modular components on the micro- and nanoscales. Naturally, our joint six-year research project within MaxSynBio can only mark the beginning of a much larger and longer research enterprise

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that transcends national initiatives and demands further intensive Europe- and world-wide cooperation. The quest for creating living cells from the bottom up has just begun.

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Conflict of interest

The authors declare no conflict of interest.

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