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COMMUNICATION

MaxSynBio – Avenues towards creating cells from the bottom-up

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Abstract: A large Max Planck-based German research consortium ('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, via their 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 can eventually be utilized for establishing a new generation of biotechnological processes, which would be based on synthetic cell constructs that replace natural cells currently used in conventional biotechnology.

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 during 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 thrives to

generate simpler life-like entities, i.e. 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 entertained 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, potentially allowing 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 by 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, so far these studies have not attempted to define the minimal set of functional elements required to build a living system from scratch. Instead, such a *de novo* approach was stimulated by the Origin-of-Life field, in the attempt to identify the key components of a historically plausible "protocell". Much work on the formation, growth and division of membrane vesicles,[2] 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 life is, and how it could be reconstituted in a minimal system, are currently only marginally addressed in the current research on Synthetic Biology. Although

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protocells are usually included in all definitions of Synthetic Biology, active research in this area has been largely underrepresented. Protocell research also suffered from a comparably slow progress over the past ten years, in comparison with 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 easier 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 towards minimal living systems via the 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 carried out as a 6 years 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.

How and what to engineer 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, 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 the syntheses of 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 the cell's metabolism, i.e. 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, i.e. 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 by fusion. Growth often preceeds 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 as 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 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 which enable them to spatially segregate specialized functions.

Signaling and Motility: Along with the 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 of cells, e.g. adhesion on surfaces, directed movement within their environment or cytoskeleton organisation, 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 the prerequisite for a functional living entity. The mimimal 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. Its is actually this **compartmentalization** that ensures that living systems are able to operate far away from thermodynamic

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equilibrium. As a consequence, synthetic life processes also must be reconstituted in cell-like micro-compartments.

In principle, there is no restriction concerning the molecules and materials which are to be used. However, with regard to the nature of functional elements that could implement the complex set of features asked for, it suggests itself 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 building blocks. In addition, smart new lab 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 are going to employ them in our initiative (Figure 1).

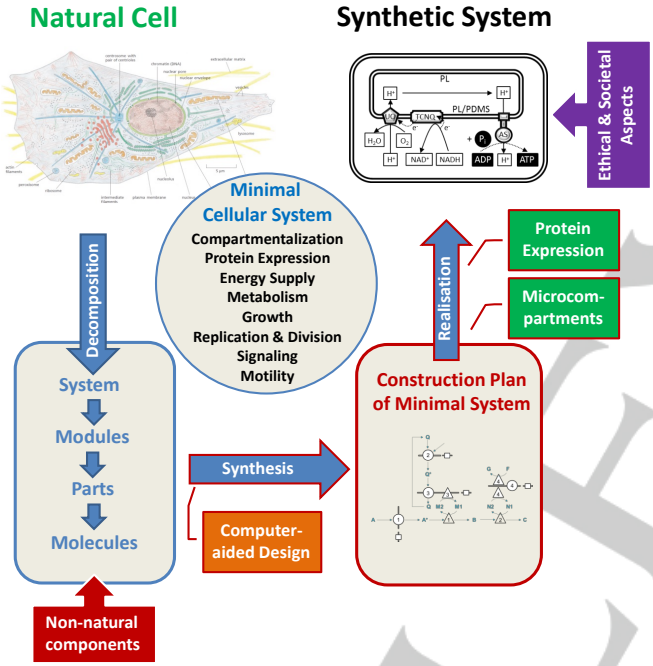

Figure 1: In MaxSynBio, essential features and processes of a so-called "minimal cellular system" (compartmentalization, protein expression, energy conversion, metabolism, growth, replication and division, signaling and motility) are synthesized for a better understanding of the behavior of natural cells. System synthesis is based on natural and non-natural components, and on the long term, it should be assisted by computer-aided design tools generating minimal system blueprints. The wet-lab 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.

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 does not only pose technical challenges, but needs to be well communicated to the general public, as it may face fears or strong ethical concerns, justified and unjustified, at various levels of reflection. Included in our consortium are thus also 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.

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 of nutrients (in cellular respiration) or light (in photosynthesis) is transformed via 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, which are specified to continuously supply energy in the form of ATP to an artificial cell.

In nature, ATP regeneration is coupled to nicotinamide cofactors (NAD(H) or NADP(H)) recycling. Though 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). Up to date, few attempts to mimic energy regeneration under *in-vitro* conditions have been reported.[5] Most of them concentrate on light energy conversion to ATP.[5a-c] At this end, the combination of ATP synthase (ATPase) and BR attracted a lot of attention.[5b] Recently, a combination of photosystem II, a protein complex that is able to split water photocatalytically, and ATPase for light-driven ATP regeneration was demonstrated.[5c] Chemical energy conversion into ATP was less studied.[5d] Up to 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 examples thus demonstrate 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 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, defined sized liposomes termed droplet-stabilized giant unilamellar vesicles (dsGUVs) and to functionalize them with ATPase.[6] Application of a pH-gradient causes production of ATP. Further, a complex chemical energy-driven ATP regenerating functional module, an artificial mitochondrion has been constructed.[7]

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 bio-technology, 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 of polymersomes and vesicles,[6, 8] demonstrating that it is in principle possible to build and control chemical reaction networks within defined space. On the other hand, the re-construction 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, respectively, from glucose as feedstock.[9]

While there has been recent progress in the reconstruction and control of complex enzyme networks *in vitro*, most approaches thus far focused on exploiting naturally existing reaction cascades. Yet, 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 its 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.

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's 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 living cells' compartments. The pioneering studies on vesicle growth[13] relied mainly on fatty acid micelles and vesicles, 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 in 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 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 been already 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, electrostatic interactions and light as external stimuli, as well as combinations of those. Controlled growth of droplets can be accomplished by coalescence of two droplets by means of manipulation via 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 leading to growth.

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 successful generation of offsprings: the dramatic mechanical transformation that leads to full splitting of a compartment, and the replication of the genome, whose copies are supposed to be faithfully distributed into the two new compartment fragments. In modern cells, large protein

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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 of active genes needed for translation and ribosome biogenesis[16] – a monumental task, whose completion 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 can 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.

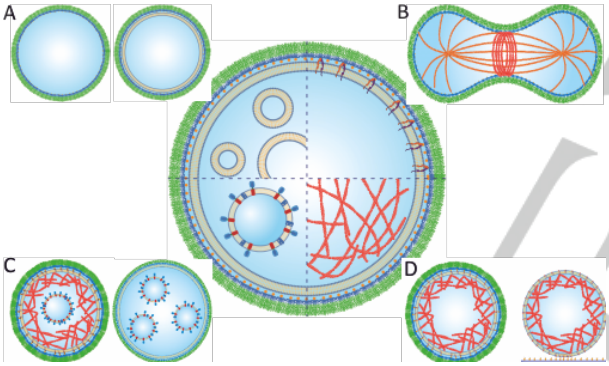

Figure 2: Schematic representations of modular engineering approach for bottom-up assembly of **(A)** cell-like compartments which can grow, **(B)** divide, **(C)** generate energy and forces and **(D)** perform cellular function such as adhesion and migration.

Over the past few years, it has been acknowledged that in their interior, cells form biochemical membrane-less compartments (droplets) by 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 by 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 proliferome.[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 onto 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 are the incorporations of well-known eukaryotic and prokaryotic division machineries based on actomyosin and FtsZ,[22] but also archaeal divisomes have lately come into focus.[23]

Towards possible DNA replication in membrane compartments, it has been shown that budding and fusion of vesicles can result from 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 actively segregating DNA can be reconstituted from purified components of bacterial plasmid-segregation machinery.[25]

The ongoing work in our consortium covers various 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] 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 will reconstitute chromosome replication and segregation in vitro and couple it to the mechanical process of compartment splitting.

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 by the dimensionality reduction resulting from the recruitment of cytosolic effectors to e.g. membrane-bound GTPases.[28] However, the local geometric properties of a membrane also determine the ability to recruit these cytosolic effectors. E.g., 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 thereby arises from recursive dependencies in the triad of membrane shape, cytoskeletal dynamics and signal transduction, enabling context dependent motile and morphogenic responses.[27, 30]

Lately, we developed GUVs with reconstituted $\alpha 2b\beta 3$ Integrins. Upon activation of Integrins by Mn^{++}-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

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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 microtubule/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 which 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 which generates signaling activity in combination with spontaneous or signaling-directed mobility.

Enabling Technologies – 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: a simple agitation of an aqueous phase in oil will lead to the formation of a dispersion of one phase into 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 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 accretion of block-copolymer surfactants at the water-oil interface leading 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 the variation of 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 introduction of biological reagents via 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 ca. 15 pL of dark liquid into droplets passing the side-channel; scale bar is 50 μm.[6, 34]

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, usable for parallelized long-term studies.[36]

Enabling Technologies – Cell-free Protein Production

The production of purified functional proteins is both, a key stage and bottleneck for the research of many groups in the life science community, including 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 preclude 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 use of 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 labs 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 realisation of a partially or fully self-encoded synthetic proliferome.[48] However, the creation of such a complex self-replicating system based on CFPS remains a long-term goal, as 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 *e.g.* with respect to *de novo* ribosome synthesis during CFPS has been made,[49] still significant improvements to the yield and lifetime of CFPS systems and to the physicochemical compatibilities of the different modules are needed. These issues may be addressable through the optimization of the CFPS components using mathematical modeling and computational tools.[50]

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 continuously with an increasing number of functional building blocks. Thereby, 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: a) a *blueprint*, i.e., a clear definition of the system's functionality to be constructed; b) *quality assurance*, i.e. the experimental validation of the functionalities of the molecules, parts and modules from which the targeted system is assembled; c) *standardization*, i.e. well-defined interfaces connecting the functional components at each level of the system's hierarchy in a stable manner; and d) *mass production platforms*, i.e. technologies enabling the manipulation and assembly of all system entities in a reproducible manner. These four elements (a-d) 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 been shown already. Schneider and Mangold[53] demonstrated the modular assembling process of an *in-silico* 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 (i) forward design by finding the Pareto optimal set of synthetic designs for implementation, and (ii) 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, we expect new exciting developments when designing cellular systems featuring complex behaviors, including division, cognition, and motility.

As discussed by Tayar *et al.*[54] the recent advances in cell-free protein expression systems allow the reconstruction of self-organization phenomena in reaction-diffusion systems, in particular (i) turnover mechanisms for continuous and prolonged gene expression reactions, (ii) programmable gene expression reactions using biological regulatory elements, and (iii) 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 does not stick to reproducing and mimicking the features and behaviors of living systems existing in nature. It 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 via 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, e.g. 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 not observable in nature.

Ontologies of Life and Ethics

Within the past years, there have been different agendas and approaches in order to identify possible societal challenges of 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 both for beneficial as well as for 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 – which need peculiar and ongoing awareness, particularly concerning possible ecological effects: First, the differences of the physiology of 'natural' and 'synthetic' organisms, second, the hitherto unknown possible alteration of synthetic organisms in different habitats, third, the possible evolution and adaptation of the 'produced' synthetic organisms, and fourth, the possibility of microbes to take up free DNA from the environment or to exchange their genetic material with other organisms. However, up to now, the existing regulation frames – especially with regard to the protocell approach – can be rated 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 disinctions such as 'living vs. non-living matters' or 'natural vs. 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 of finding expressions for the present significance of and impact on the technological development. One cause of the potential unsettlement 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 his/her ontological and epistemiological taken for granted concepts.

Our analysis of different metaphors used by science and society could identify two different processes, which 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 societal unease.

Third, the ethical and societal debate about dealing with emerging biotechnologies in general and Synthethic Biology in particular moves towards the question about who must and should be involved in making decisions pertaining the stated questions. Thus, it is not only at stake if the scientific promises will be fulfilled, 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 how such endeavours might change basic epistemological and ontological concepts such as life, biodiversity or evolution. Against this background, it is one of the most urgent questions to develop a feasible framework to theoretically stress the achievements within bottom-up Synthetic Biology towards their conceptual challenges.[57]

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 the intricate interplay of non-living constituents. However, such a process must have occurred at least once, and armed with todays knowledge and technology, could potentially be within reach following a bottom-up engineering approach with well-defined modular components on the micro-and nanoscale. Naturally, our joint 6 years research project within MaxSynBio can only mark the beginning of a much larger and longer research enterprise transcending national initiatives and demanding further intensive Europe- and world-wide cooperation. The quest for creating living cells from the bottom-up has just begun.

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