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Origin of life from a maker's perspective – focus on protocellular compartments in bottom-up synthetic biology

Authors: Ivan Ivanov^{1,2}, Stoyan K. Smoukov³, Ehsan Nourafkan³, Katharina Landfester⁴, Petra Schwille⁵

¹Max Planck Institute for Dynamics of Complex Technical Systems, Sandtorstraße 1, 39106, Magdeburg, Germany.

²Universitat Politècnica de Catalunya, Rambla Sant Nebridi 22, TR14 08222 Terrassa, Barcelona, Spain.

³Active and Intelligent Materials Lab, SEMS, Queen Mary University of London, London E1 4EN, UK.

⁴Max Planck Institute for Polymer Research, Ackermannweg 10, 55128, Mainz, Germany.

⁵Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152, Martinsried, Germany.

Correspondence: ivan.ivanov@upc.edu, s.smoukov@qmul.ac.uk, landfester@mpip-mainz.mpg.de, schwille@biochem.mpg.de

Abstract:

The origin of life is shrouded in mystery, with few surviving clues, obscured by evolutionary competition. Previous reviews have touched on the complementary approaches of top-down and bottom-up synthetic biology to augment our understanding of living systems. Here we point out the synergies between these fields, especially between bottom-up synthetic biology and origin of life research. We explore recent progress made in artificial cell compartmentation in line with the crowded cell, its metabolism, as well as cycles of growth and division, and how those efforts are starting to be combined. Though the complexity of current life is among its most striking characteristics, none of life's essential features require it, and they are unlikely to have emerged thus complex from the beginning. Rather than recovering the one true origin lost in time, current research converges towards reproducing the emergence of minimal life, by teasing out how complexity and evolution may arise from a set of essential components.

The question how life originated is difficult both because direct evidence is long gone and because modern definitions of life reveal it is a grayscale continuum phenomenon, not a binary yes/no test. One humbling yet motivating point of agreement is that life is a complex phenomenon that is not sufficiently understood and not yet possible to build in the lab – despite the Miller-Urey experiment^{1,2} and the technological advancements in combinatorial chemistry,^{3,4} fast-forwarding billions of years of stochastic conditions and interactions on a planetary scale. In an effort to strip down the skeleton of life, top-down synthetic biology approaches have modified living cells by, e.g. paring genomes down to a set of 473 genes capable of supporting replication, though the functions of a third of those is still unknown.⁵ Bottom-up synthetic biology, in contrast, starts with few components, performing increasingly complex functions, with the aim of creating life.⁶ Furthermore, it has the ability to take under its umbrella research from a number of scientific fields such as colloidal science, biochemistry, biophysics, genetic engineering, active matter, etc. and can be considered as a conceptual offspring of abiogenesis research. Thus, it has inherited many of the tools and aims of the latter, and the terms proto-, synthetic, artificial, and minimal cell are often used interchangeably. However, bottom-up synthetic biology is not burdened by the command of the evolutionary timeline – its molecular toolbox ranges from simplistic presumable predecessors to highly evolved biological machinery. It takes advantage of the newest developments in molecular biology and relies rather on controlled production, than on environmental cycles and chance. In addition, it is not limited to natural building blocks and architectures but augmented by the power of synthetic chemistry and subjected to modular reorganization at will, with the overarching aim of reproducing essential biological features with a minimal set of parts.

Similar two-sided approaches in the exploration of life's emergence and evolution are attempted all the way from defining the chemistry of proto-life, to the higher functions above mostly considered the domain of biology, with a palpable tension. On one hand there is a desire to “recognize life when we see it” often implying not only DNA but most of its other modern properties, as if in the Greek myth seeing Aphrodite (Venus) emerging fully formed from the ocean foam⁷. On the other, it is a self-evident postulate that the intricate constituents and complexity of modern biology did not emerge at once in that prebiotic time. Therefore, it has been necessary to separate and conceptualize the component functions by which we might recognize life more abstractly, including not only the above movement, metabolism, communication, reproduction, and evolution. Paring down the origin of more advanced cell properties, such as compartmentation, current metabolic cycles, or the present DNA-RNA-protein machinery will

bring additional fundamental insights but may also trigger new applications. For example, minimal chemical systems have been proposed to randomly generate predecessors and plausible molecular pathways to the molecules of life ⁴. But such systems also have the highly sought application of designing the synthesis for any molecule. Equally simple “biological” ensembles are put together from few components to mimic ever larger collections of features of life. In both chemical and biological systems (we note the increasingly arbitrary discrimination) scientists aim to reproduce central life processes, while hunting for emerging phenomena, self-assembly, reaction networks with autocatalytic properties, etc. Furthermore, life is energy-demanding and from a thermodynamic point of view requires constant external drive to maintain its out-of-equilibrium state. However, even minimal chemical systems may exhibit complex energetic transformations, leading to apparently living phenomena. For example, two-component swimmers in water recently demonstrated a novel elasto-hydrodynamic propulsion mechanism and showcased that even such simple systems can harness energy from temperature fluctuations and store it to recharge ⁸. Thus more and more complex processes are moving out of the exclusive domain of biology.

In this chapter, we start with a comparison of the two, in our opinion complementary, synthetic biology approaches, followed by discussion of selected advances in the bottom-up approach. We show how bottom-up synthetic biology combines physical, chemical and biological tools to yield ever more complex artificial structures in a rationally directed and accelerating evolution process, driven by human ingenuity, which may turn out to be a more instructive, definitive, and functional approach than historical speculation. Thereby, our aim is not a comprehensive literature overview but to present a few major developments, revolving around compartmentation, which exemplify the reconstitution and understanding of key living features via minimal synthetic systems made of both natural and man-made constituents. We also attempt to address the limitations and the missing parts in the present blueprint for assembly of an artificial cell as the pieces of a yet undefined puzzle.

The question of origin of life looms large in the last decades and there is optimism among researchers that the problem of synthesizing life in the lab can be solved in the next few decades. Several scientific fields are trying to approach the question from different angles, and synthetic biology has taken a relatively new role in this endeavor. The latter research field has long been dominated by a more top-down approach, modifying existing life and thus developing biotechnological solutions of great practical importance. On the other side, bottom-up synthetic biology – a recently coined and still not firmly established term – shares many of the aims and problems of the origin-of-life research even though in its current iterations

it may seem removed from its conceptual ancestor. However, we believe that the ability to build complexity from few components is key to answering “what is life?”, and thus to creating life via abiogenesis. All of synthetic biology struggles with the overwhelming complexity of biology, which in turn often seems to doom both approaches’ efforts to dead ends. Consequently, for better understanding and control they both resort to the fundamental scientific method of reductionism, too. We illustrate the central features of both concepts in Fig. 1, and compare them in the chapter. The top-down approach always starts with living biological cells and reduces their complexity step by step, while still monitoring principle viability as the decisive feature. It dominates the current perception of the term “synthetic biology” in the biosciences, and also in the society, partly because it has made practical advances in sophisticated genetic engineering methods⁹. The stripping down of genes in that approach to expose the functional skeleton of life, however, has been daunting and not ready yet to serve as a simple chassis on which to install new features. For example, the celebrated genome reduction of *Mycoplasma mycoides* has yielded a “minimal cell” with 473 genes, acknowledging that hundreds (one third) of these are with still unknown function⁵. Thereby, the top-down requirements for success have been somehow more generous since they are defined not by understanding but by apparent biotechnological figures of merit, e.g., the production of a given protein, and not by mechanistic blueprints from molecular and structural biology that serve as incomplete instructions for building a cell. On the contrary, bottom-up synthetic biology usually addresses initially non-living systems with up to 20 chemicals in a bilayer membrane or other physical confinement, where most of the component interactions are known. Remarkably, such dramatically simplified systems have recently been brought to successfully mimic several curiously complex features of living systems. These include motility⁸, metabolism¹⁰, communication¹¹, reproduction¹², and evolution¹³, as covered in more detail below. There are also combinations of both top-down and bottom-up approaches, which have intermediate complexity – for instance, ready-to-use protein expression machinery in form of a cell extract, encapsulated in minimal compartment.

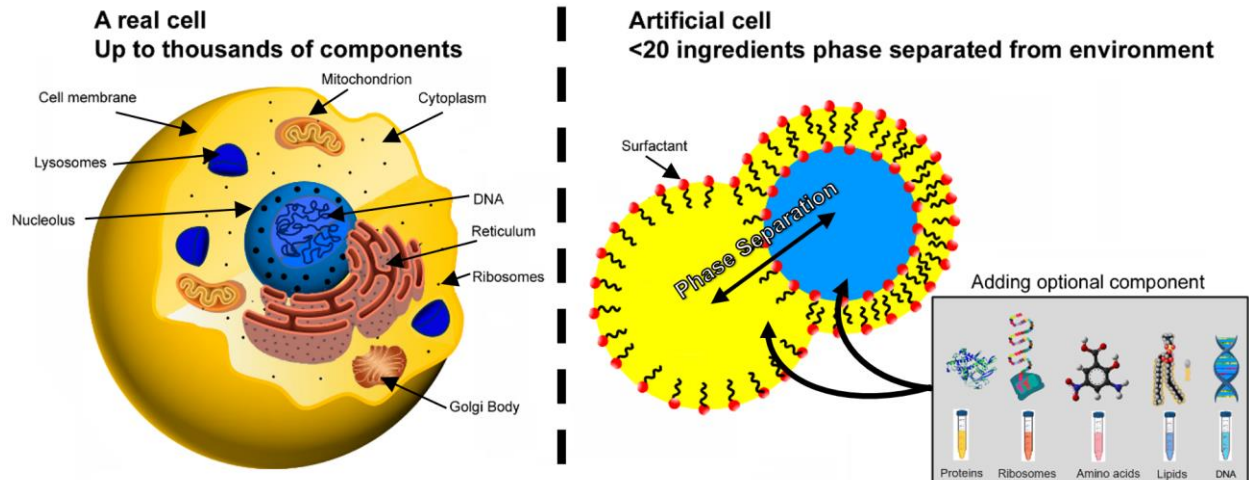


Figure 1. Schematic comparison of minimal biological vs. artificial cells. The most minimal biological cells still have > 1000 components, whereas artificial models have only up to 20, allowing great control and understanding of processes. Of course, artificial cells have yet to become alive – with all the properties expected of life.

For the purpose of engineering features of life, the bottom-up approach within synthetic biology has an unlimited arsenal of building blocks at its disposal. Thus, next to primitive molecules from the speculative toolbox of abiogenesis, highly evolved or engineered protein machinery and even fully synthetic alternatives, products of modern chemistry, are being employed. The bottom-up method aims to pick up prokaryotic and eukaryotic functional units like LEGO pieces, borrows proteins across all kingdoms, tunes and blends, disassembles and reassembles them in minimal or novel ways. In parallel, synthetic chemistry and material science deliver additional means, deliberately or accidentally biomimetic, which may fuel bold speculations about other possible life forms^{14,15}. Here, the creative aspect during the quest for the physicochemical principles of life is particularly emphasized, not only as a search for what happened long ago, but how we could use building blocks to start life *de novo*. In other words, studies of molecular assemblies with life-mimicking collective properties should not only help defining life and understanding how it appeared, but will also allow building a living cell, or possibly a tissue, organ or even organism from scratch. Furthermore, ever more complex biological phenomena are becoming addressable in the bottom-up approach, enabled by the evolved molecular toolbox.

Unlike the abiogenesis research, which firmly serves a fundamental role, bottom-up synthetic biology is able to incorporate parallel efforts in colloidal and material science, which also have great application potential. In the long run, once we learn what life is, how to put it together and jump-start it, we may succeed in engineering the perfect platform organism – assembled and tuned to maximize yields and minimize the metabolic penalty for self-preservation, unchained from the muddle of evolutionary

heritage. Both top-down and bottom-up approaches are driven in parallel towards this goal. Metaphorically speaking, the top-down biologist pulls cards from a house tower until it falls, while the bottom-up artisan stacks cards until the tower is raised. To tackle this problem, both lines of research in synthetic biology employ a modular tactic, *divide et impera*, which is conceptually reminiscent of conventional mechanical and chemical engineering, hence the parallels to building a car or a chemical plant. In fact, we argue that the modularization of the traditionally fluid, interpenetrating and messy concept of life is one of the hallmarks of the umbrella term synthetic biology. The other defining feature is directly evident from the semantics of “synthetic” and exemplifies the desire to assemble and create at will, analogous to the established field of synthetic chemistry.

Reductionism is a valid and proven method, intrinsic to science, and not exclusive to any research field. Bottom-up synthetic biology tries to reduce biological complexity by creating putative biological entities with certain key functions of life. It can be considered as an evolved form of traditional *in vitro* reconstitution experiments in biochemistry, enabled by continuous technological developments and accumulation of knowledge. We note that abiogenesis is not the only motivation for bottom-up synthetic biology and that such experiments build up the understanding of cells and biology in general. We note in the same way, that top-down synthetic biology has been characterized as nothing but a more powerful genetic engineering, continuously evolving since the first attempts of selection and cross breeding. However, although there may be some truth in synthetic biology being partial rebranding of other fields, we believe that there is increasing momentum and awareness for an impending paradigm shift. Its connection with the famous Feynman quote “*What I cannot create, I do not understand*” will have multiple implications for life sciences and biotechnology. Thus, by creating and understanding new systems, synthetic biology will help reveal the underlying molecular and physical principles of life and will guide the elucidation and discrimination of conflicting abiogenesis hypotheses.

In Table 1, we present a brief summary comparison of the tradeoff made between the top-down and bottom-up approaches in terms of functionality and understanding. Though not alive, the featured bottom-up constructs have drastically fewer, well-characterized components, to which one can reduce the mechanism for the functionality in question and possibly understand the component actions.

Table 1 – Function vs number of components needed

| System type/ Functionality | Top-Down | | Bottom-up | | Representative Photo of bottom-up examples |
|-------------------------------|------------------------------|-----|------------------------------|-----|---|
| | # of chemical components* | Ref | # of chemical components* | Ref | |
| | | | | | |

| | | | | | |
|---------------------|--------|----|------------------------------------|-----------|--|
| Reproduction | > 1000 | 16 | <10 | 12 | |
| Enclosed metabolism | > 1000 | 16 | 10–50 | 17 | |
| Division/Splitting | > 1000 | 16 | < 20 (vesicle), 2 (oil droplet) | 18, 19 | |
| Communications | ?? | ?? | <5–6 | 20 | |
| Swimming | ?? | ?? | 2 | 8 | |
| Shape-changing | > 3000 | 21 | 2 | 22 | |
| Evolution | > 1000 | 16 | 5 | 23 | |

* Minimum # of distinct chemical species needed in addition to water

Below we present a concise kaleidoscopic overview of some recent efforts towards the creation of a cell, termed interchangeably proto-, minimal or synthetic, largely depending on the preference of the researchers. We focus on recent progress milestones in compartmentalization, reflecting our own and attempt to relate to other key abiogenesis questions like metabolism and cycles of growth and division. We firmly refrain, however, from claims for comprehensiveness, let alone favoring metabolism-first or RNA-first hypotheses.

Unifying the plausible protocells in line with the crowded cell

Some form of compartmentation is indispensable for life, even if it should only serve to overcome the dilution of molecules. In fact, compartments do much more – they segregate reactions, enable gradients and bestow individuality, among many other functions. From the simplest protocells, compartments have evolved to today's highly compartmentalized and crowded cells. There are many parallel efforts in trying to decipher their physicochemical and biological origins, and whether membranes came first, or the liquid-liquid phase separation of coacervates. Bottom-up synthetic biology (as a conceptual offspring of the origin-of-life research) has generally not been concerned about the evolutionary timeline and prebiotic plausibility of their building blocks²⁴. But by constructing more and more functional models of life, it will show their relative importance for *de novo* origins of life.

Thus, vesicles are being formed from phospholipids, their presumable predecessors fatty acids, and combinations thereof, but also from synthetic molecules such as amphiphilic polymers²⁵, showcasing the omnipotent molecular principles of self-assembly. These polymersomes, as well as their hybrid combinations with phospholipids, substantiate the chemically synthetic aspect of bottom-up biology, striving to achieve improved properties by deliberate design of building blocks^{26,27}. However, they also mimic fundamental phenomena like membrane phase separation^{28,29}, associated with the raft hypothesis as a means for two-dimensional compartmentation of membrane proteins, which has in turn numerous implications for the organization of life. Different vesicles are now routinely used to accommodate advanced biological processes like mimicking replication via PCR³⁰ or reverse-transcription PCR³¹ and protein synthesis³².

Alternatively, it may be reasonable to assume that phase separation of an active “proto-cytosol”, potentially stabilized by viscosity, has preceded the membrane as a universal boundary, or at least that both variants of compartmentation emerged simultaneously in a synergistic manner. This hypothesis is reinforced by the recent findings and emerging consensus on the ubiquitous role of liquid-liquid phase separation in biology³³. In parallel to sequestering nanoparticles and biomolecules, and the enhancement of enzyme kinetics³⁴, coacervates may be coated with fatty acids as a primitive form of a membrane³⁵. Such an architecture bridges the apparent dichotomy (largely determined by the preference of the researchers and not so much by conflicting hypotheses) with respect to possible prebiotic compartments, and has been realized with phospholipids and short polyamines³⁶ but also with fully synthetic polycations³⁷.

An interesting but still underrepresented aspect is the deliberate control of compartment morphology, as both membraneless and membrane-bound compartments tend to relax to spherical shapes. Thus, the transient non-spherical shape of biomolecular condensates³⁸ has not been arrested yet, unlike the diatom-reminiscent morphology attainable in hydrocarbon emulsions²². Notably, though the exact mechanisms are still under debate and investigation,³⁹ the causes of shaped archaea may be due to physical phase transitions rather than biological programming^{24,40,41}. Control of surface-induced rotator phase transitions has been used for the generation of desired shapes in mixed oil systems⁴², bottom-up polymerization of shapes⁴³, and also triglyceride oils with chain lengths found in the membranes of living cells⁴⁴. Fundamental studies have found that such transformations occur near or above the CMC of the surfactants, ensuring a well-packed layer on the droplet surface.⁴⁵ Highly dynamic phase transformations have been harnessed even to replicate biomimetic flagellate-like movement⁸. In parallel, there is a growing number of studies on cytoskeleton reconstitution, which should lead to dynamic morphological asymmetry. However, the shape control is largely still not intrinsic but achieved by microfluidic manipulation of droplets⁴⁶ and vesicles⁴⁷ instead, to study the assembly of actin and tubulin homologues. Nevertheless, the first successful examples have been already reported – the known bacterial determinant MreB has induced rod-like shapes of PEGylated vesicles⁴⁸. Even though in that case the interactions between cytoskeleton and membrane have been tuned by synthetic means, by consulting the established area of membrane sculpting by proteins and other biomolecules, plausible primitive morphogenesis phase diagrams may be generated in the future. Similarly, non-biological reaction-diffusion processes have been used to transfer patterns from soft to hard materials,⁴⁹ or grow nanoscale patterns and three-dimensional structures.⁵⁰ The process of artificial morphogenesis has been used to grow prescribed geometric shapes bottom-up – oil droplets transforming due to internal phase transitions – has been shown to apply to a variety of oils⁵¹, some used in biology, which may have been the basis of protobiological shapes. The smallest genome of a modern non-spherical bacterium so far is of the helical *Drosophila* symbiont *Spiroplasma poulsonii* sHy, with 1584 genes²¹, though mapping between genome sizes and shapes of organisms is an open problem that could shed some light on which shapes are more difficult than others.

Self-sustained cycles of growth and division

In addition to structures that help sustain it, life is composed of highly dynamic and far out-of-equilibrium processes, which both origin-of-life studies and bottom-up synthetic biology seek to reconstitute. These range from a transient single biochemical reaction⁵² to complex interactions and feedback loops enabling

oscillatory behavior⁵³. Separately, the processes of growth, copying of genetic information, or division have recently been achieved in many contexts. However, the combined process of reproduction of growing entities together with heritable information has been elusive. It is notable that of 7 genes determined required for a normal cell division of the smallest cell so far, only two (*ftsZ*, *sepF*) have known function¹⁶. Thereby, continuous self-reproduction deserves a particular attention, as it is recognized as one of the cornerstones of life and constitutes the basis for autopoiesis, heredity and evolution and thus it is a fruitful area of current inquiry. We provide a short overview of milestones in this direction, as well as other recent perspectives of approaches, suggesting possible solutions of the problem^{54,55}.

With respect to self-reproduction of protocellular compartments, consecutive cycles of growth and division have been demonstrated predominantly in membrane-bounded compartments. This is also the more challenging case when compared to coacervate growth, where existing frameworks from emulsions (e.g., Ostwald ripening) can be readily applied. The growth of fatty acid vesicles by simple uptake of the same is experimentally attainable thanks to the fast dynamics of these simpler amphiphiles⁵⁶. In some cases, it is succeeded by division⁵⁷, while for instance, the temporal coupling with RNA replication⁵⁸ would be a matter of synchronization. The realization of primitive strategies for growth of phospholipid vesicles, however, is not as straightforward⁵⁹. In this regard, bottom-up synthetic biology may force the revisiting of mixed approaches, involving both single- and double-chain amphiphiles⁶⁰. Even though the motivation behind such studies might have been different in the past, mixed phospholipid-fatty acid systems bear significant biological resemblance, related to the uptake of exogenous fatty acids and their use for membrane synthesis. In this regard, phospholipid vesicles were shown to grow upon addition of oleic acid, which was then converted to phospholipids by an in vitro assembled eight-enzyme cascade⁶¹. Similar cascades, encapsulated in liposomes, have been also encoded, translated and even self-regulated via genetic programming of cell-free systems⁶². Cytosol-confined transcription-translation machinery has been also used to grow peptide compartments⁶³, where the somehow exotic membrane building blocks bear a remote analogy to viral capsid proteins. Polypeptide compartments have been also shown to fuse and used to accommodate DNA ligation⁶⁴. Recursive reproduction of liposomes has been coupled with RNA replication via freeze-thaw cycling over ten iterations⁶⁵. While this approach is not cell-centric but populational, in the sense that the progeny cannot be traced because the reproduction relies on random exchange of membrane and nucleic acids, it holds abiogenetic relevance with respect to the messy conditions at the onset of life. In parallel, membrane growth and division has been realized via synthetic precursors, at the borderline of biology. In one example, the membrane self-reproduction was coupled with DNA amplification⁶⁶, where nucleic acids participated in the formation of the catalytic complex, while

the combination with fusion also allowed for recursive cycles, referred to as ingestion, replication, maturity and division ⁶⁷. In another report, triazole derivatives were used as phospholipid-embedded autocatalysts that drove potentially inexhaustible membrane formation ⁶⁸. Synthetic chemistry has been also employed for the formation of polymer membranes, like template polymerization of polyaniline ⁶⁹ or ring-opening metathesis polymerization coupled to fusion ⁷⁰, and both approaches remarkably resulted in traceable growth. These examples are obviously unrelated to the origin of life, yet worth mentioning in this context, as they appear to breathe some life into the otherwise inert polymer compartments. From a current standpoint, the fact that most of the enzymes responsible for phospholipid biosynthesis are membrane proteins may appear as a chicken-and-egg problem, which motivates efforts towards *de novo* membrane formation, reenacting the emergence of the first compartments. This has been realized by putative abiogenetic means, such as enzyme-free synthesis of phospholipids ⁷¹ but also via engineering of the soluble enzyme FadD10, which is normally responsible for fatty acid activation and coupling with CoA ⁷².

Growth is also attainable via vesicle fusion, which can proceed in the absence of complex synaptic machinery, driven for instance by simple physicochemical cues like oppositely charged phospholipids ⁷³ or membrane tension by osmotic triggers ⁷⁴. Fusion has been mainly investigated in relation to secretory pathways and protein trafficking, and while it could be tentatively put in the context of eukaryotic lipid trafficking, there are growing indications that this is not a major mechanism for plasma membrane expansion ⁷⁵. Nevertheless, fusion of protocellular compartments can be still hypothesized as a primitive mechanism for information exchange and integration of functions at the early onset of abiogenesis. Such a phenomenon has been demonstrated via the fusion of complementary gene circuits ⁷⁶. These examples, even if they resulted from deliberate manipulation via modern and sophisticated biochemical means, build on evidence for the possibility of the simultaneous and independent emergence of membrane-forming agents on the one side and life-central biopolymers (i.e. nucleic acids and polypeptides) ⁷⁷ on the other, and reproduce later stages of speculative abiogenetic scenarios. In a purely phenotypical line of thought, spontaneously formed biomolecular condensates could sequester relevant molecules like protopeptides ⁷⁸ and become decorated with primitive membranes. Such a containment would provide evolutionary advantage via protecting and arresting the fairly dynamic condensates, while transition from fatty acids to phospholipids might have been catalyzed by segregated protoenzymes. If different catalysts are available at the same time, they could chemically “evolve” with the membrane by simple partitioning, following its altered amphiphilic properties, which does not require the central biological dogma of gene expression. Membrane internalization would also turn the protocells fitter with respect to reproduction

⁷⁹, while potentially catalyzing the polymerization of aminoacids ⁸⁰ or information carriers ⁸¹, alongside other indirect (protective) advantages for the latter ⁸². We note that although many of the currently investigated coacervates rely on nucleic acids, other “life-inert” phase-separating molecules ⁸³ may be also sought as potential seeds for life “crystallization”.

In an oversimplified analogy to modern cells ⁸⁴, once the protocell has metabolized and acquired sufficient material, it is ripe to split into two. Thus, next to reconstitution of membrane formation, one of the other current challenges in bottom-up synthetic biology is to reconstitute the machinery for binary fission, even though the precise mechanisms for regulation of the cell size have not been fully elucidated ⁸⁵. This bold aim has come into reach by the recently acquired experimental ability to reproducibly place and study cytoskeletal machinery inside vesicles ⁸⁶. There has been a significant progress in minimal divisomes, e.g., division of liposomes by bacterial Z rings ⁸⁷. However, the assembly and orchestration of several processes ⁸⁸, including complex reaction networks for positioning and geometry sensing (sizers) ⁸⁹ or machinery for DNA segregation ⁹⁰, will require ingenious craftsmanship and will cover the concluding stages of cell shaping. In fact, prebiotic division should have resulted from much simpler physicochemical and intrinsic triggers, and was very likely far from being symmetric. Moreover, modern organisms still exhibit budding, hyphal growth or daughter cell formation.

Rudimental division of liposomes is often accompanied by an imbalance between volume and area and relies on some minimal “cytosol”. Such examples have been demonstrated with encapsulated PEG ⁹¹ and dextran/PEG, whereby the latter led to asymmetric budding, aided by the phase-separated aqueous system ⁹². Biochemically active cytosols also may lead to division – enzymatic hydrolysis of urea enabled the splitting of mixed phospholipid/fatty acid vesicles thanks to the resulting osmotic gradient and susceptibility of oleic acid to pH change ¹⁸. In another case, osmotic deflation was induced by external enzymatic reaction, while the main driving force for parting the liposomes was phase separation of the membrane ⁹³. While deflation of hollow liposomes has proved as a practical trigger for division and it serves as an indispensable tool when studying membrane physics, it is difficult to ascribe the first division events solely to a floppy, relaxed membrane, provided the simultaneously required nutrient uptake and metabolism in the crowded cytosol. In fact, HeLa cells were found to increase their surface and hydrostatic pressure during mitotic rounding, which might expel water, but the concomitant osmoregulation masked any measurable changes in volume ⁹⁴.

Other bottom-up division strategies focus on the manipulation by proteins in the framework of membrane remodeling. One such artificial approach employed the adsorption of His-tagged GFP to NTA-

functionalized phospholipids at low density and enabled recording morphology diagrams based on the degree of deflation (i.e. the surface-to-volume ratio) and the spontaneous curvature, whereby the curvature also affected the constriction force, necessary for neck fission ⁹⁵. This is an example how the theoretical milestones for division become attainable in a minimal system, even though GFP has been externally supplied. The physicochemical fundamentals of primitive division can be further solidified by testing other membrane effectors, even if they normally exercise quite different roles in biology. One such example, facilitated by the decreasing cost and wide availability of DNA synthesis, is DNA origami as it currently enables attaining various shapes in a predictable and straightforward way ⁹⁶. Thus, DNA origami that mimicked the prominent BAR protein domains resulted in vesicle tubulation for instance ⁹⁷. In this regard, computational leaps in protein folding open new horizons for protein design ⁹⁸, which will hopefully mature to “protein origami” in the near future and will thus revolutionize structural biology. DeepMind’s AlphaFold 2 has already achieved prediction of protein structures on par with single crystal experimental methods ⁹⁹ and now University of Washington researchers have made an open-source framework RoseTTA with similar accuracy available to all ¹⁰⁰. In 2021 the AI structure prediction has deservedly been chosen as Science Magazine discovery of the year, and has enabled not only protein structure prediction but also docking of substrates and protein-protein complex interactions ¹⁰¹. Among numerous other applications, this synthetic approach will allow identifying minimal and repeating functional sequences in order to draw a family tree back to the first peptides with catalytic or structural functions. The implications of protein folding on the understanding of life and its origin obviously reach far beyond membrane sculpting or cytoskeletal dynamics. Yet, this serves to show the parallel with a readily available technology like DNA origami and exemplify the huge potential in respect to self-reproduction too. Lastly, division relies on mechanical forces, which have been even speculated to precede biochemical energy ¹⁰², and therefore only the simultaneous investigation of metabolically and mechanically active membrane and proto-cytosol will lead to more conclusive theories. From a thermodynamic point of view, living processes have been thought necessary to split droplets and vesicles in a controlled way (rather than via mechanical shear). Yet, recently oil droplets subjected to temperature fluctuations have harnessed energy of rotator phase transitions and split spontaneously into higher energy (smaller diameter) droplets ¹⁹. Molecular dynamics methods have only been designed this year to try and capture the molecular details of such solid-solid rotator phase transitions ¹⁰³. This and other chemical ratchets are among the many abiotic strategies that still wait to be discovered in the area of reproduction and may be potentially transferrable to systems comprising today’s or ancestral building blocks of life.

At the same time, other paradigmatic patterns are being assembled from scratch too. On the level of proteins, the reconstitution of the cell-division-related bacterial MinCDE system has yielded insights into how chemical energy, such as ATP, can be harnessed to promote spatiotemporal protein self-organization, resulting in the emergence of micron-scale pattern formation^{104,105}, directional protein transport¹⁰⁶ and even the mechanical transformation of membranes¹⁰⁷. On the level of transcription-translation, a minimal large-genome replicator, variation of the PURE system, was also able to regenerate its proteins for translation and transcription¹⁰⁸, while a similar construct was chemostatically operated in a microfluidic device to sustain the activity¹⁰⁹. Moreover, the ability of the former system for regeneration of essential proteins was tested in serial transfer experiments¹¹⁰, which conditions mimicked cellular reproduction in a few generations. Furthermore, the autocatalytic replication of a Φ 25 bacteriophage genome was performed successfully, encapsulated in liposomes¹¹¹. Integrating these studies in a thought experiment, along with the potentially attainable recursive self-reproduction of compartments that was discussed above, substantiates the hope for experimental verification of minimal life, which may in turn streamline the search for genesis.

Transport and energy generation at the interface

Compartmentation necessitates transport in and out to enable the continuous processing of matter, analogous to the microfluidic chemostat just mentioned above. In coacervates, this would be simply driven by the interplay of chemical equilibria and partitioning effects, but in membrane-bounded compartments, there is a barrier to be overcome. It can be anticipated that this transport was a stochastic process at the early onset of life, when various environmental or intrinsic triggers randomly caused membrane defects. Defects can also be artificially and reliably induced by electric fields in electroporation, a useful current technique for studying membrane biophysics¹¹². It is also a practical tool for vesicle loading, e.g., to produce protein and genetic-material packed nano-vesicles (exosomes) which are useful in gene-therapy and an essential vehicle in cellular communication¹¹³. Interestingly, in recent experiments, while the loading efficiency of DNA in the nano-sized exosomes depended on the DNA size in the latter case, there was no difference in translocation (loading) efficiency in the range of 25–20,000 bp for micron giant unilamellar vesicles¹¹⁴, implying a size-dependent difference in mechanism. A natural pore opening is not a guarantee for prebiotic genetic translocation, as it may occur on very long timescales and potentially lead to rupture. However, the presence of some mechanical reinforcement on the inside could maintain compartment integrity, as demonstrated by the resilience of liposomes loaded with artificial DNA-cytoskeleton against osmotic shocks¹¹⁵. Provided that some degree of integrity is ensured,

membrane defects could be reproduced by means that are more abiogenetically relevant – for instance by UV exposure that would degrade phospholipids. In this regard, transient permeability was speculated as a mechanism for DNA exchange between liposomes subjected to freeze-thaw cycles, as opposed to secretory fission and fusion ¹¹⁶.

Energy and complexity There is a dichotomy between the smooth running metabolic engine of biology and understanding how one could design it. Its complexity is one of the mysteries of life, no less because it did not emerge fully formed either, but evolved over time to what we see today. Though many life forms use similar reactions in their metabolism, not one life form can do them all. We live in an ecosystem that life itself has built over billions of years. Until the second world war, virtually all (90–95%) of the nitrogen life used was being produced by no more than 13 phyla of bacteria, capable of making nitrogenases and most of them relying rare metals, either Mo or V ¹¹⁷. The Haber-Bosch process has enabled intensive agriculture and in recent years has almost matched the volume of Nature’s fixing of nitrogen ¹¹⁸. Yet the industrial catalysts can only perform the reaction above 400 °C and at high pressures of 50–200 bar, whereas nitrogenases can perform the reaction at room temperature and ambient pressure around 1 bar ¹¹⁹. The long-standing mystery of nitrogenases has been unraveled in the last few years ¹¹⁷ and parallel efforts have now allowed us to design small molecule catalysts that also perform nitrogen fixation at room temperature ¹²⁰. But we are still far away from understanding the complex symbioses that the nitrogenase-synthesizing bacteria have formed with the roots of legumes and a select few other plants. The main point we would like to exemplify here is about complexity. Besides the extreme complexity of life, there could be major, simple (even if less efficient) milestones that we can create bottom-up. Thus the bottom-up creation of a minimal nitrogen-fixing system would not only create an industrially desirable catalyst but if integrated in a simple metabolic cycle, it would also help create a simplified version of current biology, perhaps not unlike the simple proto-metabolic cycles present at the origin of life.

Complexities across the metabolism have made truly astounding transformations possible. Glycolysis and its associated electron transport chain can split the energy of a glucose molecule into 30–32 molecules of ATP. This raises the intriguing question, is there a fundamentally smallest unit of energy that life can harvest? Is that environmentally determined, and how many kT of energy is it? In modern living systems this capability is spectacular, it has been tuned down to about 3 kT in some circumstances, modern cells living on just formate as a source of energy. These cells are able to access chunks of energy smaller than the barrier of translocating a single ion across their membranes, by coupling opposing ion pumps in complex structures called antiporter proteins ^{121,122}. Then, it is possible for cells to subsist on energy spikes

that are barely large enough to stick out of the thermal background motion. But did these capabilities emerge together with the first life forms? Unlikely. The high functionality in biology is accompanied by complexity that we are barely starting to understand, and one that certainly did not come overnight. The electron transport chain alone incorporates over 60 associated proteins that would have evolved from many less efficient processes. Even small parts of it, e.g. the ATP Synthase enzyme have extremely complex structures to have emerged at once, and would have needed simpler functional precursors. In the absence of finely tuned coupled processes, a key technique used by evolution was likely again said compartmentation.

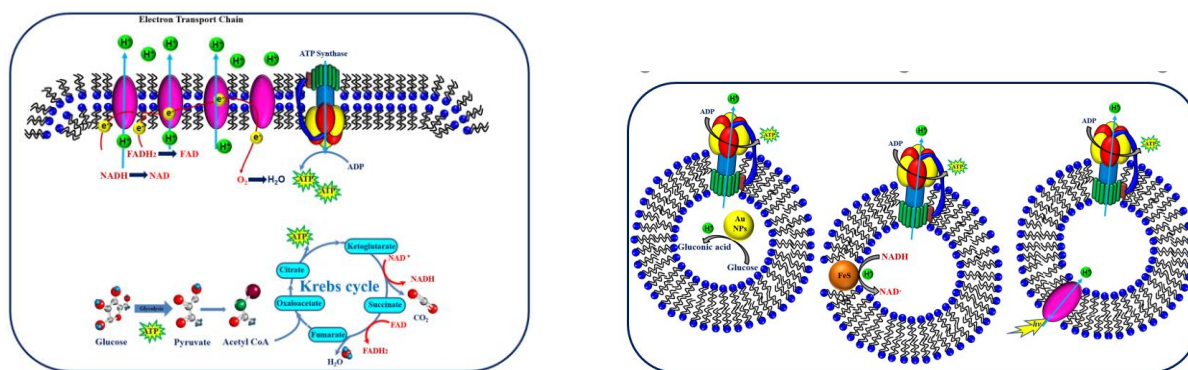


Figure 2. Comparing metabolic complexity in living cells and attempts to engineer artificial cells. The many dozens of coupled reactions and proteins in current living cells could not emerge at the same time. The much simpler to understand and control systems attempted in synthetic biology are also closer to a potential origin of life, which later evolved to the kind of complexity we see today.

Energy compartmentation The evolution of selective protein pores is apparently difficult to be traced back to the early timeline of life, which in fact holds true for all proteins, but today's engineering capabilities¹²³ may allow for identification of minimal motifs, aided by combinatorial screening of peptide libraries¹²⁴. Combinatorial testing can be also applied with respect to the membrane, in order to determine key mechanical properties that influence protein folding, such as bending rigidity¹²⁵. Synthetic chemistry can turn out to be useful for this endeavor, because it potentially allows obtaining more degrees of design freedom – an exemplary approach linked the surface pressure of different polymer/phospholipid mixtures to the distribution of potassium channels¹²⁶. Even though the motivation behind such studies is application-driven, and these polymers have no biological relation whatsoever, the concomitant generation of data will lead to a better understanding of the required membrane properties, which can be then extrapolated to the early membranes.

The reconstitution of unspecific pores like hemolysin has proved useful in multiple instances of assembled biomimetic constructs. Cell-free expression of the toxin inside liposomes enabled prolonged synthesis of GFP due to the enhanced uptake of nutrients ¹²⁷, while in another case it facilitated the export of glucose as a chemical signal for communication with proteinosomes ¹¹. Reconstituted hemolysin has been also used to activate intracellular signaling thanks to Ca permeation and mechanosensitive channels ¹²⁸. The preference for this relatively simple protein pore is largely due to its facile insertion in membranes, which has even allowed for evolution of hemolysin mutants via liposome display from encapsulated translation machinery ¹²⁹. Yet it unequivocally demonstrates the importance of permeability mechanisms, after the eventual sealing of the early membranes, alongside the fact that many processes can be entertained without high specificity. Upon conceptual merging of the above examples, one could easily imagine liposome-encapsulated cell-free expression of simple peptides for screening of their pore-forming properties, whereby compartment hits that ensure more efficient nutrient uptake would have evolutionary advantage.

In the field of energy metabolism, bottom-up constructs also generate complementary knowledge. From an origin-of-life standpoint, the stabilization of FeS clusters with peptides resulted in primitive catalysts that could reproduce the functions of complex I with respect to NADH oxidation, electron transfer to quinones, and change of pH arising from the redox reaction, which provided a plausible roadmap for the chemical origin of energetic machinery ¹⁰. These prebiotic catalysts essentially mimicked the functions of non-pumping (type 2) NADH dehydrogenases, which are the single entry point for electrons in several organisms, while proton translocation in vesicles can be accomplished by redox shuttles alone ¹³⁰. Interestingly, shuttles with fully synthetic spiropyran chemistry have even enabled the light-driven generation of transmembrane proton gradients ¹³¹ to mock rhodopsins, which suggests that proton pumps might emerge from relatively simple chemistry. In this regard, light was also used for NADH oxidation by a polymeric photocatalyst through the generation of superoxide ¹³² (accompanying also the natural oxidation by complex I as a side reaction ¹³³), while subsequent coupling with a silica-encapsulated enzyme system for cellular defense counteracted the oxidative stress ¹³⁴. This synthetic example is difficult to be conceptually linked to possible cellular energetics, as the electrons from NADH would be wasted in vain. However, it motivates an abiotic revisiting of the reverse reaction, in line with the reported photocatalytic activity of TiO₂ ¹³⁵ and Au ¹³⁶ nanoparticles towards NAD⁺ reduction, and synthesis of nucleoside bases ¹³⁷, within the search for primitive and decoupled pathways for carbon and “hydrogen fixation”.

On the other hand, the experimental modularity and tunability of a fully functional, yet minimal construct for respiration and oxidative phosphorylation (referred to as artificial mitochondrion, comprising complex I, oxidase and ATP synthase embedded in phospholipid vesicles¹³⁸) might be employed towards better mechanistic understanding. For instance, it has been suggested that protons generated in the final step of respiration traverse along the membrane, as evidenced by the effect of lateral distance between *bo*₃ oxidase and ATP synthase on the ATP synthesis rates¹³⁹. This partially conflicted the textbook cartoon of cytoplasm acidification with a direct “kinetic” coupling instead, and underlined the importance of protein clustering¹⁴⁰. The association of protons with phospholipids is known and has been already experimentally reflected in the altered buffering capacity of liposome suspensions. These findings have implications for the overall architecture needed for oxidative phosphorylation and may turn obsolete the putative need of periplasmic (or mitochondrial intermembrane) space as proton storage in the context of protocells. In other words, just the interface by itself might have been sufficient at the beginning. Moreover, the proton gradient may be stoichiometrically generated from multiple other reactions, as shown for instance by the integration of gold nanoparticle-catalyzed glucose oxidation with ATPase¹⁴¹. Similar minimal assemblies like the Arc cascade, that included an arginine antiporter, have been built to reproduce substrate-level phosphorylation too¹⁴². However, the gap between purely mineral¹⁴³ or chemical scenarios¹⁴⁴ and modern ATP synthases and kinases has not been bridged yet by constructs of intermediate complexity, although studies in the field of enzyme mimics can prove helpful in this regard¹⁴⁵. The protein apparatus for light-¹⁴⁶ and chemically¹⁴⁷ driven ATP synthesis has been also reconstituted in polymer vesicles. Apart from a purely creative rationale, such demonstrations once again help to determine the necessary membrane properties (thickness, fluidity, softness) to accommodate protein machinery¹⁴⁸, which in turn should limit the chemical landscape of potential membrane-forming molecules. Photosynthetic ATP production via bacteriorhodopsin and ATPase has been also addressed in combination with cell-free expression in giant liposomes, which provided a mechanism for partial replenishment of the bioenergetic apparatus¹⁷.

Origin-of-life studies (extensive discussion in¹⁴⁹) and bottom-up synthetic biology tackle the energy metabolism from two different directions, while the necessity of compartments, regardless of their particular nature, and the segregation of redox chemistry to the interface is recognized in both fields. Yet the experimental proficiency and modular assembly, highlighted in the bottom-up approach, may be employed to shed further light on abiogenesis. This will require expansion of the state-of-the-art biochemical toolbox towards plausible early energetic scenarios by consulting and reproducing cryptic metabolisms and not clinging to the currently prevalent ones. In this line of thought, the complexity of

the rotor-stator-type ATPase conflicts its evolutionary conservation, while oxygen, which might not have been widely available, is not a must even for eukaryotic mitochondria ¹⁵⁰, let alone for the numerous microbes relying on iron respiration ¹⁵¹. On a practical note, oxygen depletion is in fact a common hurdle in multiple in vitro experiments. Moreover, the nearly ubiquitous phosphate bond, epitomized in the universality of ATP for energy storage, can be confronted with the possibility of phosphate-free metabolism ¹⁵². Thus, a synthetic rationale to redirect the assembly of synthetic cells to underexplored simpler and ancient energy pathways may prove especially useful for the abiogenesis conundrum, while potentially providing unexpected utilities.

Synergistic effects towards the origin of life

Two conceptual scenarios crystallize from the above bottom-up studies: either an entirely biological (modern-day) toolbox, yet minimized and reorganized, or on the other hand combinations of biologically evolved and potential primitive parts from a presumed abiotic genesis. Both approaches contribute to the understanding of life by the identification of minimal assemblies that reenact biological functions. Furthermore, some works engage entirely synthetic building blocks. These man-made materials may have had no abiogenetic aspirations, however they also aid the elucidation physicochemical principles underlying overarching “biological” processes and patterns. With increasing sophistication synthetic chemistry nowadays is creating nucleic (DNA, RNA) sequences, peptides of increasing length and sophistication, phospholipid-like amphiphiles, as well as many other molecules associated previously only with the domain of life. Judicious interpenetration has resulted in artificial muscle polymers with additional functions,¹⁵³ including even self-sensing materials.¹⁵⁴ New developments, such as sequence-defined polymers ¹⁵⁵ promise peptide-like functionality from non-peptide chemistry, and perhaps if life is created *de novo* in the lab, it may augment rather than just recreate existing life. The ambitious aim of abiogenic creation of life by straightforward experimental verification, in contrast to the more speculative nature of historical origin-of-life research, promises to bring such origins in the realm of modern synthetic biology ⁶. In line with its engineering aptitude, bottom-up synthetic biology often resorts to technological platforms for compartmentation, such as droplet-based microfluidics for the production of vesicles and coacervates or cell-free systems for protein expression. These are useful tools for studying later snapshots of abiogenesis, after life is seeded via compartmentation, and allow for addressing multiple evolutionary effects. However, the experimental reenactment of the true transition from chemistry to biology will require integrated efforts. As many of the fundamental principles of that transition are still poorly

understood, a partial withdrawal from deliberate manipulation to achieve life, towards combinatorial and autonomous approaches, may allow greater chances of discovery and success.

We conclude with a conceptual comparison between a popular literature reference – Frankenstein’s monster¹⁵⁶ – and the anthropomorphic *golem* as a recursively appearing theme in mythology and religion. The latter creature is made of clay and somewhat amorphous, clearly echoing the abiogenic approach to life. While its animation is a subject of wide interpretation, in many of the recorded legends golems have been made to do useful hard work, also mirroring motivations in biotechnology and bottom-up robotic design.^{157,158} On the contrary, Mary Shelley’s fictional character was assembled from human parts, bolted together by artificial materials, and brought to life by electricity. Thus, we can metaphorically summarize the contribution of bottom-up synthetic biology to the origin of life by using these parallels, whereby we note that the prevalently negative connotation of Frankenstein’s creature is a close-minded rejection based on superficial unfamiliarity and bias, but it is, as a matter of fact, a human being, capable of emotions. Thus, the ongoing fabrication of Frankenstein’s creatures, whose limbs are occasionally replaced by clay mockups or biomechatronic prosthetics, may help us discover how Adam was created from mud.

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