Building Synthetic Cells – from the Technology Infrastructure to Cellular Entities

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Abstract

The *de novo* construction of a living organism is a compelling vision. Despite the astonishing technologies developed to modify living cells, building a functioning cell “from scratch” has yet to be accomplished. The pursuit of this goal alone has – and will – yield scientific insights affecting fields as diverse as cell biology, biotechnology, medicine and astrobiology. Multiple approaches have aimed to create biochemical systems manifesting common characteristics of life, such as compartmentalization, metabolism, and replication and the derived features, evolution, responsiveness to stimuli, and directed movement. Significant achievements in synthesizing each of these criteria have been made, individually and in limited combinations. Here, we review these efforts, distinguish different approaches, and highlight bottlenecks in the current research. We look ahead at what work remains to be accomplished and propose a “roadmap” with key milestones to achieve the vision of building cells from molecular parts.

Keywords

Synthetic Cells, Artificial Cells, Minimal Genome, Cell-Free System, Characteristics of Life, Origin-of-Life

Introduction: What is a Cell and Why Build One?

What is a Cell?

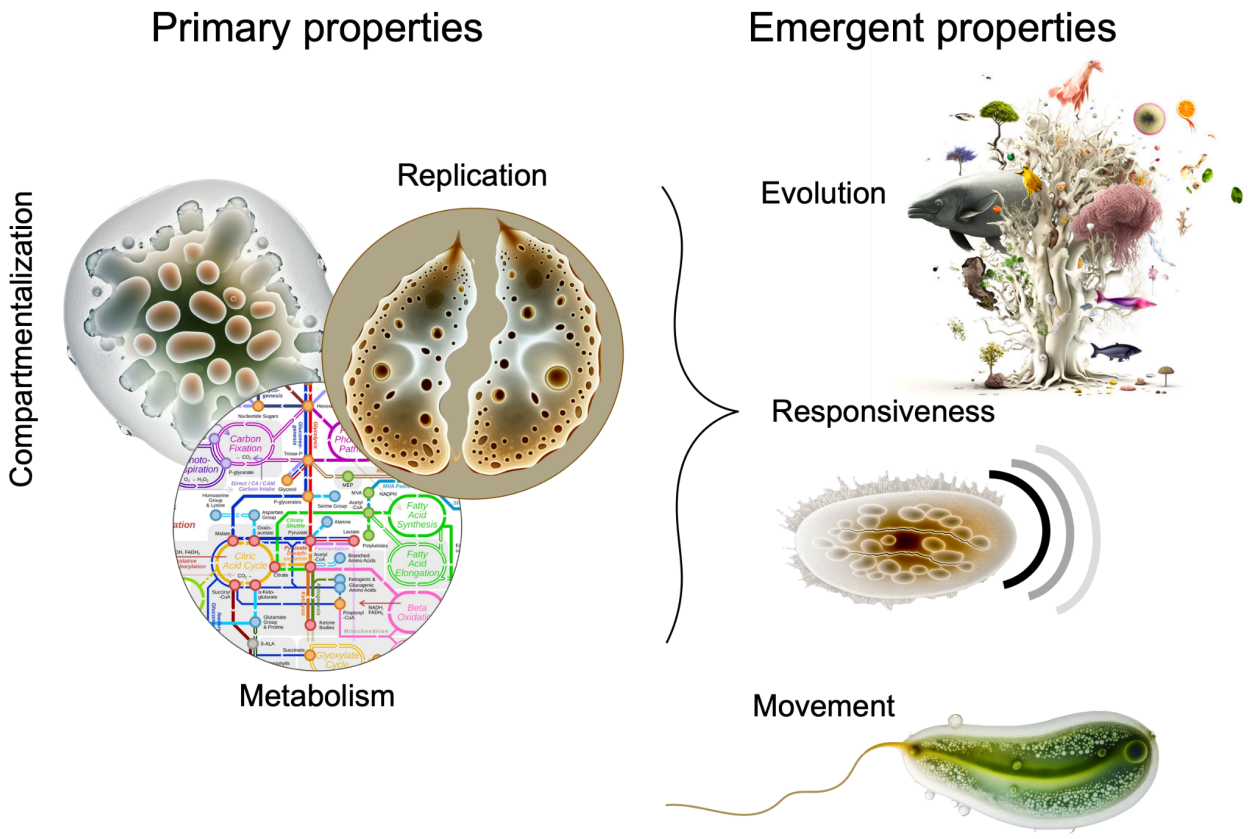
Cells are discrete, compartmentalized units of living systems that are distinct from their surrounding environment and other cells. As individuals, they can interact with each other, the environment and act as distinct units of selection in evolution. While most living cells comprise lipid-bounded compartments, other biological entities, such as complex viruses, rely on protein capsules. Growing evidence suggests that compartmentalization via liquid-liquid phase separation (i.e., coacervate formation) may also be sufficient for compartmentalization.[1]

The flexibility of this definition of a cell begs the question, "What is life?" While this has been debated for decades, if not centuries, even a modern, more complete understanding of biological systems at the molecular level has not yielded a consensus definition.[2] This is for good reason. Earth's organisms (the only ones known so far) demonstrate breathtaking diversity in their ecology, phenotypes and biochemistry. Definitions of life have tended to search for commonality in life, and thus are repeatedly re-written, following discoveries that disprove previously established rules.[3,4] Perhaps a generalized universal definition of life is even impossible: a 'natural kind' in philosophy is a category that reflects the actual world and not just human interests or properties of a group.[5] For example, water is a natural kind, whereas chairs are not. Life is possibly not a natural kind, and thus there may never be a natural definition.[6]

To anchor our discussion, we use Gánti's Chemoton model of life,[7] which uses three criteria: replication, metabolism, and compartmentalization. From these criteria emerge additional features, such as evolution, responsiveness to stimuli, and directed movement, which are strongly indicative but not strictly required for life.[8] Although the latter three emerge from the three main criteria, they are not strictly limited to 'fully living' systems. For example, a replicating moving system need not necessarily show compartmentalization. The primary criteria are interdependent. For example, replication cannot occur without the compartmentalization of "self" genetic material from "non-self" genetic material. The merits of these three essential criteria have been discussed at length elsewhere.[9,10,11,6] Gray areas are immediately apparent. What about "replication" through mechanical processes? Metabolism for how long? Evolution is a derived characteristic of populations, not individuals, and not all cells have the ability for directed movement and responsiveness to stimuli. Ultimately, the key question is: if we were to engineer a living system *de novo*, what are the desired final parameters by which we declare success? This process itself may force another re-examination of what it means to be alive.

For simplicity, we propose that any engineered synthetic system that meets the three criteria and subsequently demonstrates evolution and possibly other emergent behaviors should qualify as "alive", as depicted in Figure 1. The word "system" is key, as life is a population-level phenomenon. For example, not every member must replicate, and certainly no individual undergoes evolution. This definition also works well with other working definitions of life. The

so-called NASA definition of life, “a self-sustaining chemical system capable of Darwinian evolution”[12,9] includes the critical phrase “self-sustaining”, which we include in our discussion. Note that in this context “self-sustaining” does not mean either completely autotrophic as all organisms require the intake of water, as well as elements such as carbon, nitrogen, and other nutrients. Thus, this includes everything from diazotrophic cyanobacteria to parasites. In reality, “self-sustaining” refers to being able to live without outside intervention. Probably a better way to put it is that while alive an organism sustains itself in the face of entropy. We expect that as more discoveries are made, especially if life is discovered beyond Earth, the three criteria may continue to evolve, potentially allowing us to formulate a natural definition of life.



<u>Figure 1</u>: Our working definition of living systems, based on Tibor Gánti’s Chemoton model of life. Compartmentalization, replication, and metabolism together make up minimal criteria for a living system. From these other features emerge, including evolution, responsiveness to stimuli, and directed movement.

Here, we discuss the varying strategies and approaches to building synthetic cells. We then describe the motivations for and impact of building synthetic cells by highlighting the features of life that make biology attractive as a technology. Finally, we identify the progress and most

critical current and future challenges in the field. This leads us to propose a roadmap towards the design and synthesis of a wholly engineered living system that embodies the criteria of life as put forward by us. We envision this endeavor to be accomplished within a decade.

Why Build a Cell?

What is to be gained from building a cell? While the breadth of life on Earth is awe-inspiring in its diversity, the exercise of building a cell *de novo* offers lessons and perspectives that are unique from the scientific interrogation of extant living systems: it could enable entirely new science and may empower the engineering of living systems that are orthogonal and contextually superior to those that have emerged naturally. Examples of scientific gains from this pursuit include insights into how life on Earth may have begun, as well as what may permit life's emergence elsewhere in the universe. Practical applications could include the creation of better platforms for industrial biotechnology and biomedicine.

The tools for building cells and dissecting the function of biological mechanisms into modules will entail bioengineering advances that affect multiple areas of the life sciences,[13] if the development of tools for the experimental modification of living systems is a guide. Such experiments interrogate cellular systems by endowing them with novel functions and expanded capabilities. While similar to metabolic engineering efforts, such gain-of-function experiments are distinct in that they primarily aim to answer questions about the system and seek to replicate characteristics of life from non-living components to shed light on fundamental questions about life's origins, components, and workings.[14,15]

Building a cell would likely have profound scientific ramifications: a fundamental understanding of all cellular components and their organization required to build a living cell would significantly advance our ability to comprehend how the highly complex, dynamic, interacting system called 'life' operates. Equally important, the ability to build cells would expand the range of cell-types beyond the one shared by all life on Earth, thus giving scientists the ability to study not just life as it is, but as it could be. Currently, we have one example of a successful cellular system: that from which all life on Earth descends. Surely, there must be other feasible compositions of functional cellular systems. Others may well have existed on Earth, but we have yet to recognize any traces they left – alternatives may also exist in places beyond Earth, but also those have yet to be discovered.

The first synthetic cells will likely be very basic and thus extremely sensitive to environmental changes. They would be 'minimal' as compared to the functions of common model microbes, such as *Escherichia coli, Bacillus subtilis,* and *Saccharomyces cerevisiae*, which have evolved naturally and are thus robust in respect to a wide range of environmental conditions. It will likely require the infrastructure of generations of professionals with the enabling technologies to build, tune, and enhance or optimize features of many different synthetic cells for particular applications. For instance, a synthetic cell optimized for industrial biotechnology, such as the

production of chemicals, is unlikely to also be optimized for therapeutic uses, such as killing cancer cells. Thus, the greatest values of cells created *de novo* will not be represented in the functions of the first iterations of a synthetic cell, but in the opportunities it opens up.

Advantages of Synthetic Cells over Cell-Free or Liposome Systems

Why are existing technologies with widespread commercial availability, such as cell-free transcription/translation (Tx/Tl) systems, not sufficient? What are the limitations of encapsulated cell-free systems in studying life? To answer this we have distinguished four principal systems as shown diagrammatically in Figure 2, while their advantages and disadvantages are summarized in Table 1.

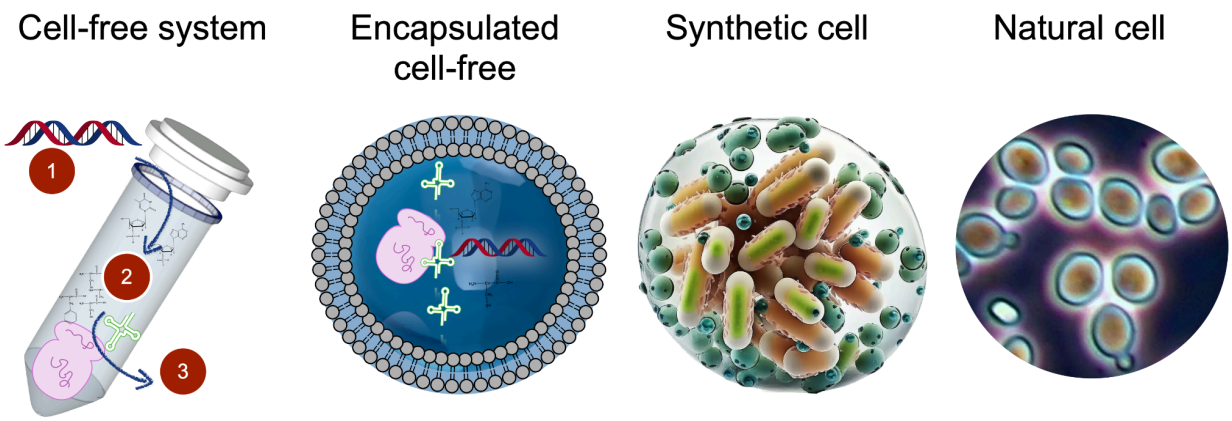


Figure 2: Four different molecular platforms for studying life. Outgoing from externally provided template DNA (1), a cell-free transcription/translation (Tx/Tl) system uses complex substrates and a chemical energy-donor (2) to synthesize RNA, proteins and potentially other biocatalytically (enzymatically) formed products (3). An encapsulated cell-free system is often a liposome enclosing a cell-free Tx/Tl system. A synthetic cell would function more like a natural cell in that it could be a self-sustaining system that is capable of replication.

Table 1: Advantages and disadvantages of using different molecular platforms for studying life.

	Cell-free	**Encapsulated cell-free**	**Synthetic cell**	**Naturally evolved cell**
Defining life / Origin-of-life	Helps understand certain processes	Helps understand a broader range of processes	Ideal to understand the transition from non-living to living	Non-controversial example of life
Alternative cellular systems	Most easily modified for	Non-canonical components	No constraints of prior	Changes are constrained by

	non-canonical components	functioning in a unit	evolution, genetics and development	evolution, genetics and development
Studying genes of unknown function	Testing of genes against a defined background	Testing of genes against a defined background	Testing of genes against a defined background	Too many to study; throughput of traditional means too low and slow; reached limit of previous approaches, better to use synthetic biology tools
Prototyping metabolic pathways	Ideal for simple pathways; no concern about lethality to a cell	Enables compartmentalization for more sophisticated pathways	Most orthogonal while sufficiently capable for more complex pathways	Least orthogonal but most capable for complex pathways
Molecular biosynthesis development	Testing of metabolite transformation	Testing expansion of metabolite transformation	Integrated multi-gene pathway testing in controlled/known environment	Analysis of alternative/competing molecular pathways
Production of toxic compounds	Ideal / unrestricted	Bio-contained delivery of biotech	Ideal if not toxic to synthetic cell; potential to study mechanism of toxicity	Prohibited by definition
Preservation of biodiversity	Understand key metabolic pathways with ecological importance, (e.g., plant-microbe interactions).	Study and engineer key evolutionary and ecological processes (e.g., symbiogenesis).	Study and engineer symbiogenesis, preserve extant and extinct life (e.g., boot-up endangered or extinct genomes), generate new biodiversity.	Preserve organism with intrinsic value and natural producer of valuable compound

Designing environmental stress responses	Protein stability testing	Basic functional tests (e.g., protection of DNA from radiation), subcellular localization and compartmentalization, transport phenomena	Response testing in controlled/known environment	Interaction of stress response with other cellular mechanisms
Therapeutics production / delivery / testing	Distributed production, logic-gated control	Compartmentalization and targeted delivery, logic-gated control	Allow testing against a known genetic background; could provide orthogonal system for drug delivery	May have superior compatibility with life
Studying cellular function	Dependent on system used for Tx/Tl	"Toy" system amenable to modeling; reintroducing confinement/organelles/scaffolding in controlled way	Provides modular, defined system	Intact systems for realistic studies
Evolution studies	Component evolution (e.g., genes)	Component evolution (e.g., transport proteins)	Studies with well-defined cellular systems	Natural evolutionary processes
Workflows for engineered biological "parts" (modules)	Testing parts in a simplified system towards use in living cells	Testing parts in a simplified system towards use in living cells, with more realistic spatial confinement/biophysics than bulk cell-free	Testing of parts against a defined background	Difficult/impossible/unmet challenge to predict *a priori* how engineered part will function *in vivo*

The key difference between a cell-free system and a cell is compartmentalization. This allows for the emergence of individuality, in metabolism, heredity and evolution. Thus, a synthetic cell could be the superior tailored platform for basic science and bioengineering. Like

naturally-evolved life, a synthetic cell may survive in a suspended state for longer periods of time than typical cell-free systems, which is critical when preservation of cellular components is key. Additionally, a synthetic cell could provide a unit of selection for the evolution of desired processes, whereas a cell-free system is not capable of that by a homogenized whole.

Is there an Ideal Synthetic Cell?

What are the ideal properties of a synthetic cell? Biologists and engineers dream of an organism in which every chemical interaction is defined and understood so that the phenotype can be predicted from the genotype. The resulting genetic transparency is somewhat congruent with the idea of a "minimal cell". Such an organism would serve as a genetic "base operating system" onto which any desired function can be "installed". In further analogy to computer science, difficulties in the engineering of natural cells arise from their "closed-source" programming and the difficulty to reverse-engineer them and accurately back-translate their compiled "machine-code". In contrast, synthetic cells would be "open access" making the "source-code" available and thereby allowing the more precise "programming" of functions before compilation.

Transitioning from the theoretical attributes of a synthetic cell to a chemical understanding and implementation may be difficult. Because life is an emergent property of chemistry, it is by definition stochastic and unpredictable.[16] On another level that accounts more for context, engineers tend to have specific, application-dependent needs, relating to growth-rate and biomass-yield, source of energy, carbon, and other nutrients, as well as robustness within an environment (e.g., temperature and pH) and ecology. These are determined by the intended purpose of the organism (foundational studies, metabolic engineering, biomedicine, etc.). In short, there is no single "ideal" synthetic cell, as the design depends on the need and intended application / use case. Thus, not all synthetic cells will necessarily be the most minimal.

Strategies for Building a Cell

The ultimate goal of building a cell is to create a biochemical system recognizable as alive from its non-living component parts *de novo*. Different design strategies for building a cell offer relevant approaches to provide platforms for the development of foundational technologies and advancing understanding of cellular structures and functions towards the *de novo* creation of a cell. These alternative approaches can be organized into "bottom-up", "top-down" and "middle-out". Each approach is characterized by unique advantages, drawbacks, and challenges. A schematic overview of all three strategies is represented in Figure 3.

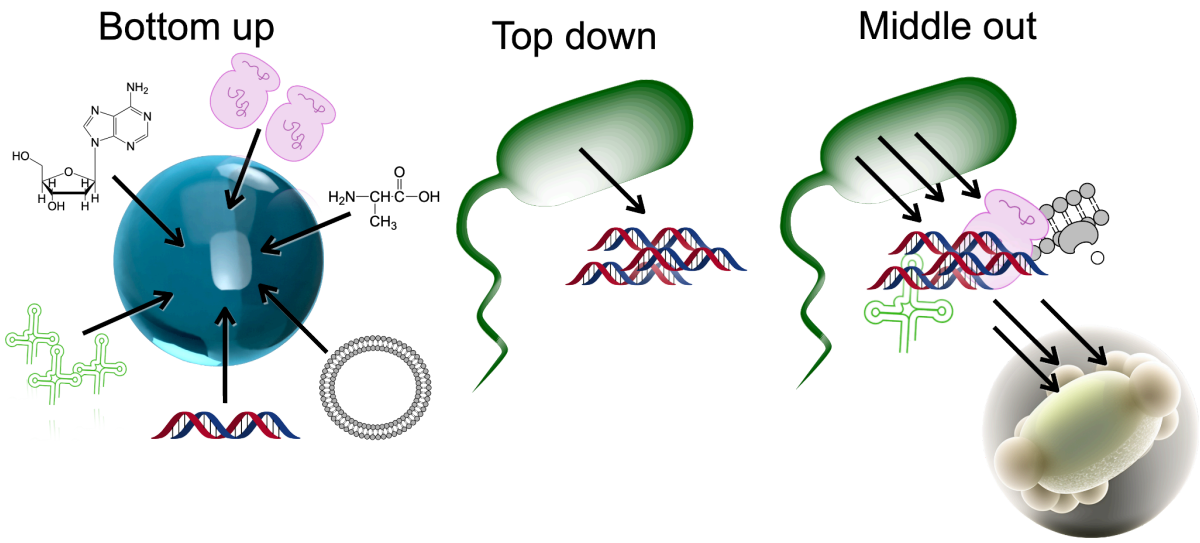


Figure 3: Major approaches to building a synthetic cell: bottom-up (a), top-down (b), and middle-out (c) are distinguished as the basic concepts of currently ongoing research. Top-down approaches generally seek to find a "minimal genome" of an existing organism, while the bottom-up approach aspires to *de-novo* create a cell "from scratch" or based on macromolecules. Middle-out approaches utilize modules of known function (e.g. organelles, extracts) to assemble a new cell or major elements/mechanisms thereof.

To help illustrate these different approaches, consider the analogy of building a car that can drive. The bottom-up approach is like building a drivable car from the most basic components, such as metal sheeting and screws. The top-down approach is akin to stripping a drivable car piece-by-piece, discarding "non-essential" parts, modules, and subsystems (e.g. the radio) while retaining the ability to drive. Sometimes, which modules are "non-essential" is unclear until the car stops driving. The middle-out approach is akin to putting a car together from subsystems and modules (e.g. the engine) whose intricacies the assembling engineer does not need to fully comprehend. However, unlike a car, a living cell displays emergent properties that are extremely difficult to predict and are profound in their implications.

In the following section, we briefly introduce the different approaches and outline the efforts to-date that have been undertaken on each. We also compare the merits of the different approaches and discuss the scientific and technical challenges.

Bottom-up Engineering

The bottom-up approach comprises the step-by-step chemical synthesis of a living system *de novo*, from isolated or synthesized (macro)molecules that can comprise membranes, genetic material, and proteins to carry out the functions of a cell. This gives the researcher complete control of the components, whether to try to create an orthogonal system with non-biological components, such as D-amino acids and L-sugars, or to try to replicate the origin-of-life here or

elsewhere. This approach operates on the hypothesis that the right configuration of molecules will display life-like behaviors, or ultimately create a system we recognize as “life”. It arguably best exemplifies the ultimate goal of the field but probably requires the greatest depth of knowledge and technical skill to be accomplished.

There has been much work towards bottom-up engineering to understand the origin-of-life, creating liposomes that encapsulate various portions of the genetic machinery.[17,18,19] Polymersomes, artificial vesicles that self-assemble from amphiphilic copolymers and enclose an aqueous cavity,[20] and synthosomes, polymersomes that contain channels to allow for selective transport of chemicals, are more recent advances that stray further from mimicking natural organisms. Polymersomes can be engineered as protocells to mimic cell functions and build cell-like structures.[21] However, there is still a substantial gap between the abiotic production of macromolecules, or even macromolecular assemblages such as vesicles loaded with biochemicals, and living systems. Also, as origin-of-life research specifically seeks to recreate life as it likely occurred on a young Earth, the field often does not explore radically different alternatives that might interest an astrobiologist, synthetic biologist, or biomolecular engineer.

Top-down Engineering

Systematic top-down approaches commonly aim to remove extraneous genetic modules from extant genomes to generate a “minimal genome” that contains a subset of its initial functions.[22,23] The objectives of these range from enabling a simplified platform for bioengineering to creating a cell with only the essential functions required to sustain cellular life for foundational studies.[24,25,26] The approach has been applied to various species and genetic systems to varying extents. For example, the genome of *E. coli* was reduced by 15% and 30%, respectively.[27,28] The most minimal genome was created from the bacterium *Mycoplasma mycoides*, whose genome is already one of the smallest known with only 915 genes. From this species, the synthetic genome JCVI-syn1.0 was created by developing a series of enabling synthetic biology technologies.[24,25,29,30,31,32] There, a synthetic bacterial genome containing an antibiotic resistance marker was installed in an existing living bacterial cell to produce a new cell with the genotype of the installed genome. Consequently, a near-minimal organism was created with a synthetic genome that comprised only 531 kbp and 473 genes, a genome smaller than that of any autonomously replicating cell found in nature. While several species exist that have fewer genes than *Mycoplasma genitalium*, none of them are free-living. Notably, the progenitor, JCVI-syn3.0, can only be cultivated in a laboratory environment under strictly controlled conditions.[26]

Middle-out Engineering

The middle-out or “semi-synthetic” approach extracts and repurposes extant modules of living cells to reconstruct a living system.[33] Much of the research that is now designated as a

middle-out approach was previously considered bottom-up. The classic example is the use of complex fractions of cell extracts, such as organelles and other modularly functional fractions of living material that enable functional biochemistry, to reconstitute features of living systems, such as gene expression, replication, and sense/response behavior.[34] Examples include the encapsulation of cell-extracts in liposomes to achieve cellular functions,[35,36] decorating nanoparticles with cell membranes,[37] and re-engineering and transferring complex molecular systems such as the glycosylation machinery, to synthetic systems, such as a microsome.[38] Synthetic biologists have recreated fairly complex systems of replication and transcription-coupled translation *in vitro*.[39,40] Encapsulating such systems into membranes and coupling their function to cytokinesis and metabolism is still a profound challenge.[34]

The middle-out approach is distinct from the top-down approach in that it does not rely on the minimization of naturally evolved cells. It is distinct from the bottom-up approach in that it does not rely on purified or synthesized, well-characterized molecules with defined functions but instead utilizes only partially understood multi-molecular assemblies to perform such functions. Such middle-out systems can be treated as modular black boxes with defined inputs and outputs. Despite being incompletely understood and some lack of reproducibility, these systems can still be leveraged to achieve life-like behavior and are nonetheless useful in piecing together the essential features of living systems, either in conjunction with other complex mixtures or purified molecules. In fact, the reconstitution of a living cell from its components following extraction has yet to be accomplished and would provide a worthy milestone as a technical achievement for the insight it could give into what differentiates living entities from non-living systems.

Applications of Synthetic Cells

Synthetic cells are anticipated to have a range of applications, from fundamental research to applied science. For bioprocess engineers, synthetic cells are of interest as an infinitely scalable manufacturing platform for chemicals or for environmental applications. Synthetic cells could also offer an alternative platform for biomanufacturing that is not readily performed by existing cellular chassis. For example, synthetic cells could allow the unbiased implementation of non-evolved elements like non-standard (proteogenic) amino acids (NSAAs)[41], alternate chiralities, and xeno-nucleic acids (XNAs)[42] that are not, or hardly accessible with extant biology. Cell biologists would be able to use synthetic cells to understand the fundamental workings of living cellular systems. Evolutionary biologists and astrobiologists could use them to answer the “what if?” questions, ranging from the origin and early evolution of life to whether life may exist off-Earth, studying not just the present diversity of life, but potentially what came before and what comes after, that is, what else is possible. The following examples are meant to illustrate the impact synthetic cells could have and thus the importance of the endeavor to build a cell.

Understanding the Origin, Evolution and Diversity of Life

The physicist Richard Feynman famously wrote, "What I cannot create, I do not understand." Building a cell may thus be the only way to truly provide a mechanism for the ultimate validation of our foundational understanding of life's components and functions. Our inability to create a synthetic cell from molecular components – even from components derived from previously alive cells – illuminates gaps in our understanding.

Most likely, all extant life on Earth descended from a universal common ancestor.[43] The exercise of building a cell could allow us to postulate what life might have looked like elsewhere in our solar system and beyond, perhaps permitting us to answer one of our most profound scientific questions: are we alone? Further, synthetic cells could offer a platform for understanding how life might operate on specific extraterrestrial bodies, such as for example the subsurface oceans of Europa and Enceladus. In addition to "how" we are left with many of the "why" questions of evolution. Why does life on Earth only use left-handed amino acids in ribosomally synthesized proteins? Why D-sugars? Did evolution sample other approaches and simply discard them out of pure chance? Or were they outcompeted? Or did alternatives never arise because none were functional? Building synthetic cells would allow us to distinguish among these alternatives for a basic scientific understanding of life. It can further provide insight into the biochemical and structural intricacies of cellular functions to guide application-focused bioengineers. Evidence that the molecules involved in the central dogma on Earth are not the only "way of life" is provided in the form of DNA with unnatural base pairs (UBPs), consistent of e.g., six bases instead of four.[44] This DNA also seems to be energetically superior, as the two non-natural bases are less prone to oxidation and epimerization. The creation of an *E. coli* strain that actually uses unnatural base pairs shows the feasibility of such hypotheses.[45]

Characterization of Cellular Parts and Elucidation of their Functions

Synthetic cells could allow the testing of biological parts outside of their native context. Such "parts" may range in size and complexity from lipid composition to the genetic code to whole organelles. If a module is necessary and sufficient for a particular desired function, a synthetic cell with minimal complexity could provide an excellent chassis to evaluate how that module affects or is affected by basic cellular functions. This is because the synthetic cell chassis is by concept a defined background free from the constraints, complexity, and legacy of evolution with the confounding variables and added complexity found in natural cells – in brief, synthetic cells could be entirely orthogonal. Dissecting the cellular machinery of a natural cell through deletion is not always possible, or preferable to adding components to a synthetic cell. Currently, there are considerable efforts and hypotheses to understand adaptation and regulatory processes involving domestication and biodiversity of natural organisms.[46] Synthetic cells may also be useful as foundations for the synthetic domestication of biodiversity traits – an approach that constitutes a sustainable and viable option for conservation and development of value-added processes and products from biodiversity.[47]

Development of Engineering and Design Tools

Just as cell-free Tx/Tl systems are used for the rapid prototyping of genetic circuits, synthetic cells will prove similarly useful for prototyping at higher levels of organization and cellular interactions, for example, between components or membrane-less compartments. Synthetic cells may offer a broad array of traits found in natural cells that can ultimately be combined in an "*à la carte*" format to engineer uniquely tailored cell systems. Consider how five *Spiroplasma* genes were added to a non-motile organism, yielding a mutant of the minimized bacterium JCVI-syn3B capable of movement.[48] This is not dissimilar to how evolution has operated with bacteria, which have an extraordinary ability to exchange DNA through horizontal gene transfer, or for eukaryotes who have formed temporary symbioses or even incorporated symbionts that ultimately became heritable, such as mitochondria.[49] Progress towards engineering synthetic cells also involves developing orthogonal biochemistries. Examples include: NSAAs, XNAs,[50] and an expanded genetic code with UBPs,[51] as well as *de novo* designed proteins, potentially enabling the formation of proteins with new structures and novel functions.[52]

Metabolic Engineering and Biomanufacturing

The ability to predict, design, and construct reliable and robust biochemical pathways is key to efficient metabolic engineering. Systems Biology, quantitative synthetic biology and integrated multi-omics techniques (i.e. genomics, transcriptomics, proteomics, and metabolomics) grant insights that can significantly accelerate the development of microbial cell factories.[53–55]. This is achieved less by facilitating strain-construction, but rather fosters the understanding of engineered cellular systems, providing quantitative data of interest that can be used to project outcomes. The concept, known as systems metabolic engineering, reduces the Design-Build-Test-Learn (DBTL) cycles required to reach a strain that performs as desired and enables the rational creation of microbial cell factories. Nevertheless, it is still not (yet) possible to exactly predict outcomes of genetic interventions in even the best-studied model organisms. Already, cell-free systems have been shown to accelerate design cycles [56,57]. A fully-defined synthetic cell may go beyond such approaches to provide a predictive platform, allowing bioengineers to further streamline systems metabolic engineering strategies. This may not only improve control over the process parameters (e.g., rate, titer, yield),[58] but also the precision and efficiency of biomanufacturing.[59] Minimization of crosstalk between peripheral and intersecting heterologous metabolic functions may allow formerly inaccessible biosynthetic pathways, for example of complex natural products, to be (re)constructed.[60,61,62] The semi-synthetic near minimal JCVI-syn3A (and derivative strains), is already being used as a chassis to evaluate the effects of adding new metabolic pathways on basic cellular biology. Its extreme metabolic simplicity makes it useful for such purposes; however, synthetic or semi-synthetic cells that are similar to common model organisms could be much more useful than current minimal cells.

A fully defined biomanufacturing system tailored to its purpose may also allow for better process-control through enhanced biosafety, as compared to natural cell systems: requirements for containment may be relaxed due to a lower chance of survival, in case of unintentional release. Intrinsic biological controls could be considered, such as "kill switches" after a certain

number of generations, or in response to a particular environmental change.[63,64] Alternatively, or additionally, a synthetic cell could be designed to be incompatible with natural life, therefore unable to interact with or contaminate it (for example by using D- rather than L-amino acids, L-sugars instead of D-sugars, or UBPs with XNAs and/or NSAAs). *Vice-versa*, synthetic systems that are different enough from nature may not be susceptible to "intruders" such as bacterial- or phage-contamination. Such microbial chassis are already in development and could provide a firewall to prevent any exchange between the recoded organism and natural ecosystems. A recent example is an *E. coli* strain with an alternative codon usage where artificial tRNAs recognize different amino acids.[65]

Food Production and Bioremediation

Microbial Biotechnology will play a crucial role in advancing food-production systems (agriculture, livestock) towards greater efficiency and sustainability.[66] Because existing genetically modified organisms cannot persist indefinitely and are often outcompeted by natural systems in a short period of time, synthetic cells could become a viable tool to e.g., replace chemical fertilizers,[67] or tailor microbiomes of plants,[68] and animals.[69,70] Similarly as for biomanufacturing / metabolic engineering applications, orthogonality and intrinsic safeguards of the synthetic cells could ensure the biosafety of the synthetic system.

Natural cells have been used successfully in environmental remediation efforts, such as treating and recovering concentrated nutrients, sequestering heavy metals, and decomposing plastics.[71,72,73,74] Synthetic cells may provide opportunities to improve and expand these efforts, when engineered to survive environments that preclude naturally-evolved life. For example, synthetic cells could be created to be impervious to compounds that are toxic to life. Natural cells excel at processing natural compounds, but synthetic cells may fill an important niche in bioremediation by being tailored to function with and act on synthetic compounds.

Synthetic cells could also become field-deployable detectors for environmental monitoring to detect contaminants, such as pathogens or heavy metals.[75] These "biosensors" through their portable sizes and scalability could provide a warning system in hazardous situations where there is a risk of exposure to harmful levels of toxic chemicals or pathogens.

Diagnostics and Therapeutics

Our understanding of the molecular biology of cells, though incomplete, underpins much of modern medicine, shaping our comprehension of disease and pathogenicity mechanisms and influencing the development of diagnostics and therapeutics. Several diagnostic procedures rely on identifying cellular components or products, while many therapeutics act by influencing cells, cell products, or altogether replacing cells and tissues. It is clear how diseases and their treatments often lead back to the cell. In the words of the physician-scientist Rudolf Virchow "*no matter how we twist and turn, we shall eventually come back to the cell. Every pathological disturbance, every therapeutic effect, finds its ultimate explanation only when it is possible to designate the specific living cellular elements involved*."[76,77] Similar to the way molecular biology

contributed to modern medicine, one can expect insights from synthetic cell endeavors to further advance medical practice, improving our understanding of disease pathologies, and informing novel diagnostics and therapeutic strategies.

Synthetic cell research may enable the development of novel biological functions that do not currently exist in nature but may be relevant for the treatment of different disease conditions. Today, natural cells form the basis of cellular therapies, spanning multiple therapeutic areas including regenerative medicine, immunotherapy, and cancer. There are 29 FDA-approved cell- and gene therapies with others at various stages of clinical development.[78] However, there have been major limitations with the manufacturing of natural cell-based therapeutics with variability in product specifications as well as other sourcing, supply-chain, and storage challenges.[79] For instance, CAR T-cell therapy, while promising, is restricted by these limitations, leading to extensive duration and high costs.[80,81] Synthetic cells that are able to mimic the functions of these natural cells could significantly improve access to these therapies, allowing better supply, storage, and manufacturing at scale.[82] Towards that objective, droplet-based microfluidics techniques for producing synthetic cells are very promising, as they can combine complex designs (e.g., compartmentalization) with high consistency and large-scale production.[83]

Extracellular vesicles have also become an active area of research towards therapeutic applications. One limitation of these vesicles is the difficulty sourcing them, and their considerable variability, which limits the ability to control their quality. Current synthetic cell research is already tackling these challenges with the bottom-up assembly of synthetic vesicles for wound healing, or regenerative therapies.[84] Further synthetic cell research may lead to the development of synthetic vesicles for other disease conditions for which natural extracellular vesicles have demonstrated effect in preclinical studies.

The potential of synthetic transcription and translation systems to provide a greater shelf-life and stability, by being freeze-dried and later re-hydrated, has already been recognized: such medicines and vaccines could mitigate regional healthcare disparities, overcome geographical and environmental barriers, and improve global healthcare access.[85,86] Better yet would be the ability to create a synthetic cell system that is amenable to long-term desiccation, to address the problem of maintaining stem cells or other cellular-based manufacturing systems in an active or even semi-active form.

Another promising potential application of synthetic cells are specialized medicines and therapies. The rapid advancement of synthetic biotechnologies and research techniques has increased the accessibility, reliability, and prevalence of personalized medicine, resulting in treatments tailored to a patient's unique needs, physiology, and genetics.[87] A personalized medicine regime may be vital for the successful treatment of disease effectively, while maintaining quality of life.

With tissues and organs being multicellular organizations of cells, another potential area of impact for synthetic cell research is organ and tissue transplantation. Terminal irreversible damage of the kidneys, lungs, heart, liver, etc. are treated with organ transplantation. However, there are limitations in the supply of these organs to patients. New synthetic tissue approaches based on synthetic cell research may one day provide alternative organ sources for transplantation.

Bioprinting

Bioprinting is the use of 3D-printing technology with materials that incorporate viable living cells as a component of bioinks to create structures – in essence, biological additive manufacturing. To date, the focus has primarily been on tissue and organ regeneration,[88] but other uses such as printing of plant or algal cells have been explored.[89] Challenges exist that potentially could be remedied by the use of synthetic cells as components of bioinks.[88] For example, they could be engineered to withstand conditions such as temperature or chemical composition of the carrier compound that would be difficult or impossible for naturally evolved cells to survive. There could be situations where cell division is not desired. A synthetic cell could be engineered to be more biocompatible with others or be made immune to infection from viruses and bacteria, and remain undetected by a host immune system.

Space Exploration

Space exploration is limited by mass and volume constraints during launch, as much of the launch-mass is fuel. In the absence of resupply, storage and reliability issues are key. As life is self-replicating and synthetic biology can transfer the ability to convert available resources into a more tractable form (say a single cell that could produce wood or rubber), biotechnology has the potential to be the key to human survival off-planet.[90,83] Cells, especially microbes, are exquisitely good at nanotechnology and comparatively low-maintenance.[91] The attainable savings in up-mass can be huge.[92] Creating synthetic cells that are better suited to off-planet environments than naturally evolved life could be the key to life-support and *in situ* resource utilization for manufacturing of items with regular demand on long-duration space missions, such as food, drugs, and materials.[93,94]

Research Advancements Towards Fulfilling Criteria of Life

To refine a framework for bioengineers to understand current challenges in creating synthetic biological life, we review progress and begin to frame target specifications. For that, we adhere to the foregone criteria of life ‘compartmentalization, metabolism, and replication’ as well as the emergent features ‘evolution, responsiveness, and movement’. While we discuss each of these separately, in many cases research has also demonstrated their combination. For example, various synthetic compartmentalized systems already exhibit some form of metabolism and replication.[95,96,97,98]

Compartmentalization

Compartmentalization of cells into discrete units is required to distinguish life from the physical environment, from each other, and other cells-types (especially in multicellular organisms). From a biological point of view, physical separation allows distinct individuals to experience differential selection. From a physical point of view, compartmentalization allows a separation and concentration of cellular components and compounds in a manner that facilitates metabolism. Compartmentalization also allows for different metabolic activities to occur within the same cell that would otherwise interfere with each other, such as e.g. oxidative phosphorylation and nitrogen fixation,[99] or β-oxidation.[100]

Internal compartmentalization exists in all domains of life. In prokaryotes, there are two major classes of organelles: those formed by a protein shell or lipid monolayer (e.g., lipid bodies, polyhydroxyalkanoate (PHA) granules, carboxysomes, magnetosomes, and gas vacuoles) or lipid bilayer.[101] Eukaryotic compartments in the form of membrane-bound organelles (e.g., nucleus, mitochondrion, chloroplast) are well-known. Additional compartmentalization occurs through biomolecular condensates formed by liquid-liquid phase separation.[102,103] Examples from eukaryotes include membrane-less organelles in the nucleus, such as the nucleolus, Cajal bodies, nuclear speckles, and paraspeckles, and in the cytoplasm, such as P-bodies, stress granules, and germ granules.[104] Some eukaryotic organelles, such as mitochondria and chloroplasts, arose via the assimilation of bacteria into eukaryotic cells through endosymbiosis.[105] Synthetic biology approaches have even enabled the establishment of a bacterial endosymbiosis in yeast that functioned as an endosymbiotic organelle and was stable for more than 40 generations.[106] Formation of these cellular compartmentalizations can be achieved in synthetic systems, such as polymer-based aqueous two-phase systems and hydrogels.[107,108,109,110]

Synthetic compartmentalization has been achieved through different techniques – e.g., encapsulation in amphiphilic lipids, peptides, or polymers, protein capsids, and liquid-liquid phase separation – and is technically relatively facile. Applied methods include, for example, thin-film hydration, biphasic centrifugation, extrusion, and sonication.[111] Generating liposomes containing genetic material is as simple as adding a solution of plasmid to a dried thin film of phospholipid. The challenge with compartmentalization has been the relatively low efficiency of encapsulating molecules at consistent quantities within compartments. Stochasticity often generates wide variation in the composition of individual liposomes encapsulating cell extract.[112] The size of the artificial compartments themselves can vary considerably depending on the method used, as can the relative spatial connectivity. These technical hurdles stymie the production of cell-like compartments with sufficient reproducibility to study their behavior. While microfluidic tools and methods are available, improved methods are needed to create compartments of consistent size, composition, and connectivity to enable more systematic and quantitative studies.

In many cases it will be desirable to build compartments within other compartments and then manipulate those, for example, through fusion or fission. Forming multiple compartments within a larger liposome is possible by microfluidic double emulsion liposome construction using different reagents for the de-wetting process.[113] Some researchers have encased live cells within liposomal cells to allow for compartments with different internal properties.[114] Genetic cascade reactions can be compartmentalized for controlled modular reactions. Incompatible reactions can be allocated to separate liposomes and then fused together to allow for concurrent reactions.[35]

A variety of other techniques, beyond the use of lipid membranes, have been demonstrated for compartmentalization. The most common alternative to membranes are water-oil emulsions created with microfluidics. Those systems enable the compartmentalization functionalities of liposomes, using membrane proteins. As these emulsions tend to be unstable outside of carefully controlled environments, their utility for applications such as drug delivery is currently limited.[115] Peptide-based compartments have also been used to contain biochemical reactions and reaction networks.[116,117,118] Single-stranded DNA has also been used to form complex flexible coacervates and double-stranded DNA can favor coacervate formation in the presence of cationic polymers.[119,120] Additionally, low-complexity RNA molecules can reversibly compartmentalize peptides and oligonucleotides by modifying the temperature.[121] Zeolites, microporous crystalline aluminosilicates, can compartmentalize molecules of choice.[122] Understanding how membrane-less compartments are created within cells through self-organization will also suggest engineering strategies to build a cell.[123]

Metabolism

Metabolism is the chemical means by which life harnesses energy to organize and maintain itself in a non-equilibrium state at the cost of increasing the entropy of the external environment. This set of chemical reactions sustains life through the assimilation of substrate and harvesting of energy for conversion into compounds that allow the biosynthesis of cellular components.

Individual biochemical reactions can be straightforward to replicate. However, recreating a carefully balanced, self-sustaining network of biochemistry that mobilizes energy and matter and commits it to growth and replication is arguably one of the most difficult aspects of building a living cell. First, the construction and dynamic balancing of a complex system of catabolic and anabolic chemistries is by itself a formidable hurdle. For replication, a net-excess of matter (i.e. fixed carbon, nitrogen, and other elements) must be produced. Second, the metabolic system must be encoded genetically if it is to be propagated continuously, which creates the additional challenge of engineering the timing and amount of each component to be formed throughout the life-cycle of an organism. In known extant cells, metabolism must be coupled in some way to replication. Hence, metabolism depends on itself in what could be conceived as an arbitrarily complex differential equation. Despite this, primordial cells could have evolved with only a

tenuous link between metabolism and replication. Metabolism may have resulted in passive cell division, mediated by forces exerted by membrane curvature.

To date, minimal synthetic metabolism has been demonstrated in various forms. Basic redox reactions and energy metabolism are key for cells to generate new chemical products as well as their own energy for sustaining life. In most whole cells, adenosine triphosphate (ATP), the main energy-carrier of biology as we know it, is primarily regenerated by a trans-membrane ion-gradient that drives an ATP synthase. The presence of a membrane is therefore crucial in the function of these enzymes. However, production of synthetic membranes and correspondingly membrane-bound proteins, remains a challenge. Promisingly, cell-free expression can quickly generate membrane-bound proteins.[124] Alternatively, energy in the form of photons can be used to phosphorylate ADP. One method for achieving this is through the encapsulation of rhodopsin, a light-gated ion-pump, and ATP synthase. Encased in vesicles together, they convert light energy into a proton-gradient that generates ATP via phosphorylation.[125] Other, cell-free systems have been developed that use pyruvate,[126] maltose,[127] 3-phosphoglyceric acid,[128] and phosphoenolpyruvate,[129] to provide energy to maintain biochemical reactions. This area has been extensively developed and is reviewed elsewhere.[130]

Replication

Replication requires the existence of genetic material that stores information and can be inherited by any progeny. This information minimally comprises instructions for replication, such as, for example, the genetic code of a virus. Achieving replication of a synthetic cell requires engineering of both a system for replicating the genetic material and a mechanism for subdividing a complete copy of the genetic material into a new compartment. Also needed is sufficient (bio)chemical activity to generate or scavenge resources for the synthesis of both the genetic material and the components of its containing compartment. Integration and coordination of each facet of replication is ultimately required to ensure its lasting over generations.

Replication of internal biochemistry and cellular membranes is essential for cell perpetuation. Several methods of accomplishing this include extrusion through primordial clay and cell-free expression of a minimal divisome.[131,132] Liposomes can be fused and fissured through a freeze-thaw method to generate larger liposomes, which can then be used for encapsulation of larger compounds.[133]

Nucleic acid polymers are currently the only known genetic material. DNA, the predominant form of genetic material on Earth, is highly stable (half-life of millennia) and dense in information.[134] RNA, which is prone to autocatalytic degradation and alkaline hydrolysis, has a shorter half-life *in vivo* (only minutes) than DNA but becomes more stable when bound to ribosomes.[135,136] Self-replicating RNAs have been demonstrated in multiple experiments.[137,138] DNA, however, requires extensive protein machinery to replicate, and deoxyribonucleotides are converted to

ribonucleotides by ribonucleotide reductase. This has been a powerful argument for the existence of an “RNA world” prior to the evolution of DNA as the most widely used genetic material on Earth.

RNAs have been shown to retain catalytic activity when encapsulated in lipid vesicles.[17,139] Because of this, and because no autonomous self-replicating DNA is known to exist, RNA may be the more promising genetic material for a truly bottom-up synthetic cell in the near future. However, DNA-based replication should remain an engineering goal, because of its much greater stability. In addition, proofreading and repair is more common for double stranded DNA than for RNA: *in vivo* replication of DNA replication has an error rate of less than one per 100 million base pairs. Viral RNA replication and transcription of DNA produces an error every thousand bases. Furthermore, DNA molecules can be up to 250 million base pairs large, for example in the human genome, whereas most RNAs are not more than a few thousand base pairs, with the current record length belonging to a nidovirus, which has an RNA genome of 41 thousand bases (single strand).[140] A DNA-based synthetic cell would require a number of additional factors, greatly increasing its complexity. For example, *E. coli* replication requires at minimum helicase, primase, DNA polymerase, and ligase, all of which would need to be encoded and expressed. This, in turn, requires machinery and raw material for transcription and translation to produce each enzyme. In addition, the progeny material must be produced faster than the parent material is degraded.[141] Still, a synthetic cell with a DNA genome that recapitulates features of Earth’s first cells might be much less complicated. Were helicase, primase, DNA polymerase, and ligase enzymes present in the first cell with a DNA genome or was a simpler mechanism the progenitor?

Living cells have elaborate pathways that orchestrate the physical partitioning of copied genomes into new compartments. While careful partitioning of the old and copied genomes has evolved in most organisms, this is not strictly required. Multiple copies of genomes can persist in a single compartment and then be randomly segregated during division. This is the case for the majority of bacterial multicopy plasmids.[142] Without partitioning mechanisms, this would result in a high fraction of progeny having either none or multiple copies of genomes. As long as some fidelity (and diversity) is maintained across generations of synthetic cells, the population can persist. The spontaneous division of lipid vesicles has been demonstrated in multiple systems, however, none have been coupled to the replication of genetic material.

Box 1: Assembly of artificial genomes based on natural or synthetic DNA

Assuming the first synthetic cell will rely on DNA as the genetic material, then this coding sequence must be either chemically synthesized or derived from an extant organism. The middle-out approach of using existing DNA from a living organism is appealing, since it does not require detailed design knowledge of the genetic code itself. *De novo* design of every base pair in even a simple genome is a daunting prospect, requiring holistic understanding of not only the function but also interaction of all genetic elements and gene regulatory networks.[143–146147]

Hence, the coding of the first synthetic cell genome is likely to be naturally derived, while the physical DNA itself will almost certainly be chemically synthesized. This is due to the rapid progress that has been made in recent decades both in the reduction of the cost of synthetic DNA and technical leaps that have enabled stitching relatively small oligonucleotides into massive assemblies of genes, chromosomes, and even whole genomes. The size of the DNA for the first synthetic cell could vary wildly from a few thousand to a few million base pairs. A quarter of a century ago, the first synthetic genome, an 8 kbp DNA copy of a hepatitis C virus sub-genomic replicon, took months to assemble from synthetic oligonucleotides.[148] Less than a decade later, scientists had developed DNA synthesis technologies that allowed completion of the 583 kbp *Mycoplasma genitalium* genome.[30] This became was possible with the invention of homology based assembly strategies relying on chemically synthesized sub-genomic DNA fragments of 1 to 5 kbp.[149] The fragments we recombined into shuttle-vectors and then transformed into *Escherichia coli* to obtain assemblies of 10 to 30 kbp. In a second assembly step, these fragments were transformed into yeast where homologous recombination allowed formation of artificial chromosomes of 20 to 140 kbp. Outgoing from these, fragments can be isolated and used to create a living or non-living synthetic cell.[150]

The booting-up process of synthetic genomes, is not trivial and genome transplantation has only been accomplished for the species *Mycoplasma*.[29,31,32]. The only other organism for which a completely synthetic genome has been loaded is *E. coli*.[65,151] Additionally, *Saccharomyces cerevisiae* strains with multiple synthetic chromosomes have been made where native genomes are replaced with corresponding ~50 kbp synthetic segments that are iteratively transformed into cells where the swap takes place.[65,15115265,151].

While the cost for the construction of large DNA fragments have decreased substantially with the emergence of DNA foundries, transplantation of non-mycoplasma genomes via a similar or equivalent universally applicable technique is still limited to non-living cells with coding sequences that are much less complex than those of natural cells [150].

Emergent Features of Life

Evolution

Evolution is a process that emerges when a population of individuals shows differential survival based on a heritable phenotype; an individual cell does not evolve. For synthetic cell engineers, this means that evolution cannot be observed to occur unless a population of synthetic cells with a heritable phenotype has been created. Evolution also requires that the organism's phenotype is the result of its genotype. The evolution of individuality, referred to as the

"Darwinian threshold",[153,154] was key to the origin of predominantly vertical rather than horizontal transmission of genetic material.

For example, in the case of the JCVI minimal bacterial cell, it was hypothesized that a bacterium whose genome only encoded genes essential for cell-viability might be unable to evolve, mainly because of the absence of DNA-repair mechanisms that would lead to the rapid accumulation of fatal mutations. This idea was disproved recently in adaptive laboratory evolution experiments, demonstrating increased fitness of JCVIsyn3A and JCVI-syn3B after thousands of generations in laboratory culture.[155,156]

Evolution should inevitably emerge from a population of individual cells where there is some relation of phenotype to heredity, whether by natural selection, drift, or another mechanism. If the environment changes or more than one organism competes for a resource, taxa must evolve to even "stand still", according to van Valen's "Red Queen Hypothesis".[157,158] Evolution requires individuals, so it can occur at subcellular levels as well where mitochondria and chloroplasts can count as individuals, all the way down to the genes. For example, the usage of NSAAs may arise that result in new and diverse chemical properties of proteins. This may expand the functional ability of proteins, resulting in a potential evolutionary advantage to the host cell.[159]

Responsiveness

Sensing stimuli and responding by altering system behavior is an emergent trait of life. This enables organisms to adjust their behavior to a changing environment. The unique ability of cells to sense and respond to stimuli has provided inspiration for engineers, for example, in the creation of biomimetic materials.

Communication among populations is vital for population-level adaptation from unicellular organisms to entire mixed ecologies. Understanding inter-cell communication systems has enabled applications in synthetic biology.[160] Mechanisms for cellular sensing and responding are essential for the coordination of spatially separated functional modules. Advancements in programmable cell communication are expected to enable advanced control over synthetic cells. For example, cells with a porous membrane may utilize nucleus-like DNA-hydrogel to express signaling molecules to communicate with neighboring cells. Utilizing this technology it is possible to create mutualisms.[161] Programmable mechanosensitivity in synthetic cells has been accomplished using osmotic pressure through mechanosensitive channels.[162] Further, advancements have been made in the areas of signaling molecules, signal range, active and passive signaling,[163] and information transfer,[164] as discussed previously.[165]

It can also be argued that responsiveness to environmental change is not a necessary property of living cells, at least in captivity. In nature, the bacteria with the smallest genomes are all obligate parasites. Bacteria like mycoplasmas, which are human urogenital pathogens, are

examples of this.[166] They evolved from bacteria like *Bacillus subtilis* through a process of massive gene loss.[167] This was likely possible due to a very stable and nutritionally rich habitat. Human urogenital epithelial cells, which these bacteria parasitize, provide such an environment. One can imagine that over years of completely constant laboratory culture, these mycoplasma species would be able to also discard all the approximately sixty genes they have retained for growth in their natural environment to deal with changes in urine osmolarity or the presence of other parasitic bacteria. A synthetic cell incapable of responding to its physical, chemical, or biological environment could be highly desirable: if it were to be released into the environment, it would presumably not be able to survive, therefore providing intrinsic biocontainment.

Movement

While directed movement, whether on a micro- or macro-scale, is not required for life, many organisms demonstrate some form of mobility.[168] For most, movement is necessary to locate and obtain resources, to avoid living in a buildup of waste-products, to find a mate, and/or avoid predation. In microorganisms, flagella, microvilli, membrane blebbing, and gliding activity enable and facilitate movement.[169] Some organisms are small enough that ambient fluid flow or Brownian motion is sufficient to support their molecular processes. As such, even growth and division can be classified as movement – by that definition all living cells experience microscopic movement.

While directed movement can be thought of as a form of responsiveness, since the movement is often in response to some stimuli, the molecular machinery that enables movement can be distinct from the responsive elements. A greater understanding of the relationship between morphology and motility allows for increased ingenuity in creating artificial movement. The specific strategies for creating cellular protrusions, substrate adhesion, and myosin-dependent actin network contractility in synthetic cells are discussed elsewhere.[170]

Each of the above properties of living organisms have been engineered into synthetic systems to varying degrees, sometimes even in (albeit limited) combinations. In theory, once the criteria of compartmentalization, replication, and metabolism are established simultaneously, a new lifeform should emerge. Table 2 summarizes the state of efforts to date in that regard.

Table 2: Efforts undertaken towards building synthetic cells to date. Classification of approach may be ambiguous in certain cases.

Title	DOI	Year	Lab	Approach	Genetic Replication	Division	Compartmentalization	Metabolism	Movement	Sense & Respond	Evolution
Oparin's Reactions Revisited: Enzymatic Synthesis of Poly(adenylic acid) in Micelles and Self-Reproducing Vesicles	10.1021/ja00096a010	1994	Luisi	Bottom-up	Yes	Yes	Yes	No	No	No	No
Man-made cell-like compartments for molecule evolution	10.1038/nbt0798-652	1998	Griffith	Middle-out	No	No	Yes	No	No	No	No
A vesicle bioreactor as a step toward an artificial cell assembly	https://www.pnas.org/doi/full/10.1073/pnas.0408236101	2004	Libchaber	Bottom-up	No	No	Yes	Yes	No	No	No
Design of artificial cell–cell communication using gene and metabolic networks	10.1073/pnas.0306484101	2004	Liao	Top-down	No	No	Yes	Yes	No	Yes	No
Self-maintained Movements of Droplets with Convection Flow	10.1007/978-3-540-76931-6_16	2007	Ikegami	Bottom-up	No	No	No	No	Yes	Yes	No
Multilevel Selection in Models of Prebiotic Evolution II: A Direct Comparison of Compartmentalization and Spatial Self-Organization	10.1371/journal.pcbi.1000542	2009	Hogeweg	Top-down	Yes	No	Yes	No	No	No	Yes
Creation of a bacterial cell controlled by a chemically synthesized genome	10.1126/science.1190719	2010	Gibson	Top-down	Yes	Yes	No	Yes	No	Yes	Yes
Self-reproduction of supramolecular giant vesicles	10.1038/nchem.1127	2011	Sugawara	Bottom-up	No	Yes	Yes	Yes*	No	No	No

combined with the amplification of encapsulated DNA											
An Open Question on the Origin of Life: The First Forms of Metabolism	10.1002/cbdv.201200281	2012	Luisi	Bottom-up	No	No	Yes	Yes	No	No	No
Spontaneous network formation among cooperative RNA replicators	10.1038/nature11549	2012	Lehman	Top-down	No	No	No	No	No	Yes	Yes
Liposome division by a simple bacterial division machinery	10.1073/pnas.1222254110	2013	Erickson	Bottom-up	No	Yes	Yes	No	No	No	No
Darwinian evolution in a translation-coupled RNA replication system within a cell-like compartment	10.1038/ncomms3494	2013	Yomo	Bottom-up	Yes	No	Yes	No	No	No	Yes
Protein synthesis in artificial cells: using compartmentalisation for spatial organisation in vesicle bioreactors	10.1039/C4CP05933F	2015	Ces	Bottom-up	No	No	Yes	Yes	No	No	No
Self-Guided Supramolecular Cargo-Loaded Nanomotors with Chemotactic Behavior towards Cells	10.1002/anie.201504186	2015	van Hest & Wilson	Bottom-up	No	No	Yes	No	Yes	No	No
Design and synthesis of a minimal bacterial genome	10.1126/science.aad6253	2016	Hutchison	Top-down	Yes	Yes	No	Yes	Yes	Yes	Yes
Growth and division of active droplets provides a model for protocells	10.1038/nphys3984	2016	Jülicher	Bottom-up	Yes	Yes	Yes	No	No	No	Yes
Engineering genetic circuit interactions within and between synthetic minimal cells	10.1038/nchem.2644	2017	Adamala	Middle-out	No	No	Yes	No	No	Yes	No
The origin of heredity in protocells	10.1098/rstb.	2017	Lane	Bottom-up	No	Yes	Yes	No	No	No	Yes

	2016.0419										
Light-Guided Motility of a Minimal Synthetic Cell	10.1021/acs.nanolett.8b03469	2018	Wegner	Bottom-up	No	No	Yes	No	Yes	Yes*	No
Enzyme-powered motility in buoyant organoclay/DNA protocells	10.1038/s41557-018-0119-3	2018	Mann	Middle-out	No	No	Yes	No	Yes	Yes	No
Sustainable replication and coevolution of cooperative RNAs in an artificial cell-like system	10.1038/s41559-018-0650-z	2018	Mizuuchi & Ichihashi	Top-down	Yes	Yes	Yes	No	No	No	Yes
Freeze-thaw cycles induce content exchange between cell-sized lipid vesicles	10.1088/1367-2630/aabb96	2018	Schwille	Bottom-up	No	No	Yes	No	No	No	No
Self-replication of DNA by its encoded proteins in liposome-based synthetic cells	https://www.pnas.org/doi/full/10.1073/pnas.1914656117	2018	Danelon	Bottom-up	Yes	No	Yes	No	No	No	No
Artificial photosynthetic cell producing energy for protein synthesis	10.1038/s41467-019-09147-4	2019	Ueda, Kuruma	Bottom-up	No	No	Yes	Yes	No	Yes	No
Bottom-up Creation of an Artificial Cell Covered with the Adhesive Bacterionanofiber Protein AtaA	10.1021/jacs.9b09340	2019	Matsuura, Hori	Bottom-up	No	No	Yes	No	No	No	No
An Adaptive Synthetic Cell Based on Mechanosensing, Biosensing, and Inducible Gene Circuits	10.1021/acssynbio.9b00204	2019	Noireaux	Middle-out	No	No	Yes	No	No	Yes	No
Motility of Enzyme-Powered Vesicles	10.1021/acs.nanolett.9b01830	2019	Sen	Bottom-up	No	No	Yes	No	Yes	No	No

Mimicking Chemotactic Cell Migration with DNA Programmable Synthetic Vesicles	10.1021/acs.nanolett.9b04428	2019	Choi	Middle-out ?	No	No	Yes	No	Yes	Yes	No
Chemical Signal Communication between Two Protoorganelles in a Lipid-Based Artificial Cell	10.1021/acs.analchem.9b01128	2019	Han	Middle-out ?	No	No	Yes	No	No	Yes	No
Bottom-Up Construction of a Minimal System for Cellular Respiration and Energy Regeneration	10.1021/acssynbio.0c00110	2020	Biner & Hirst	Bottom-up	No	No	Yes	Yes	No	No	No
Self-division of giant vesicles driven by an internal enzymatic reaction	10.1039/C9SC05195C	2020	Lagzi, Rossi	Bottom-up	No	Yes	Yes	No	No	Yes	No
Self-Propelled PLGA Micromotor with Chemotactic Response to Inflammation	10.1002/adhm.201901710	2020	Wilson	Bottom-up	No	No	Yes	No	Yes	No	No
A Step toward Molecular Evolution of RNA: Ribose Binds to Prebiotic Fatty Acid Membranes, and Nucleosides Bind Better than Individual Bases Do	10.1002/cbic.202000260	2020	Keller	Bottom-up	No	No	Yes	No	No	No	No
Hydrodynamic accumulation of small molecules and ions into cell-sized liposomes against a concentration gradient	10.1038/s42004-020-0277-2	2020	Toyota	Bottom-up	No	No	Yes	No	No	No	No
Dissipative self-assembly, competition and inhibition in a self-reproducing protocell model	10.1039/D0SC02768E	2020	Fletcher	Bottom-up	Yes	No	Yes	No	No	No	No
Catalytic processing in ruthenium-based polyoxometalate	10.1038/s41467-019-13759-	2020	Mann	Middle-out ?	No	No	Yes	No	No	Yes	No

coacervate protocells	1										
Membrane molecular crowding enhances MreB polymerization to shape synthetic cells from spheres to rods	https://www.pnas.org/doi/full/10.1073/pnas.1914656117	2020	Noireaux	Bottom-up	No	No	Yes	Yes	No	No	No
Signaling and differentiation in emulsion-based multi-compartmentalized in vitro gene circuits	10.1038/s41557-018-0174-9	2020	Simmel	Bottom-up	No	No	Yes	No	No	Yes	No
Engineering motile aqueous phase-separated droplets via liposome stabilization	10.1038/s41467-021-21832-x	2021	Ces	Bottom-up	No	No	Yes	No	Yes	No	Nod
Light-Powered Reactivation of Flagella and Contraction of Microtubule Networks: Toward Building an Artificial Cell	10.1021/acssynbio.1c00071	2021	Gholami	Middle-out	No	No	Yes	Yes	Yes	Yes	No
Reconstitution of contractile actomyosin rings in vesicles	10.1038/s41467-021-22422-7	2021	Schwille	Bottom-up	No	Yes	Yes	No	No	No	No
Programmable Aggregation of Artificial Cells with DNA Signals	10.1021/acssynbio.0c00550	2021	Choi	Bottom-up	No	No	Yes	No	No	Yes	No
Phase Separation and Protein Partitioning in Compartmentalized Cell-Free Expression Reactions	10.1021/acs.biomac.1c00546	2021	Maeda	Middle-out	No	No	Yes	No	No	No	No
Light-Triggered Cargo Loading and		2021	Göpfrich	Bottom-up	No	Yes	Yes	No	No	No	No

Division of DNA-Containing Giant Unilamellar Lipid Vesicles	10.1021/acs.nanolett.1c00822										
Chromatophores efficiently promote light-driven ATP synthesis and DNA transcription inside hybrid multicompartment artificial cells	10.1073/pnas.2012170118	2021	Mavelli	Middle-out	No	No	Yes	Yes	No	Yes	No
Programmable Fusion and Differentiation of Synthetic Minimal Cells	10.1021/acssynbio.1c00519	2022	Adamala	Bottom-up	No	No	Yes	Yes	No	No	Yes
Signal-processing and adaptive prototissue formation in metabolic DNA protocells	10.1038/s41467-022-31632-6	2022	Walther	Bottom-up	No	No	Yes	No	No	Yes	No
Living material assembly of bacteriogenic protocells	10.1038/s41586-022-05223-w	2022	Mann	Middle-out	No	No	Yes	Yes	No	Yes	No
In vitro assembly, positioning and contraction of a division ring in minimal cells	10.1038/s41467-022-33679-x	2022	Schwille	Bottom-up	No	Yes	Yes	No	No	No	No
Gene silencing and transfection in synthetic cells	http://doi.org/10.1002/bit.28422	2023	Adamala	Bottom-up	No	No	Yes	Yes	No	No	Yes
Synthesizing a minimal cell with artificial metabolic pathways	https://www.nature.com/articles/s42004-023-00856-y	2023	Imai	Middle-out	No	No	Yes	Yes	No	No	No
Clonal Amplification-Enhanced Gene Expression in Synthetic Vesicles	https://pubs.acs.org/doi/full/10.1021/acssynbio.2c006	2023	Danelon	Bottom-up	Yes	No	Yes	Yes	No	No	No

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Engineering cellular communication between light-activated synthetic cells and bacteria	https://www.nature.com/articles/s41589-023-01374-7	2023	Booth	Middle-out	No	No	Yes	No	No	Yes	No
Signal transduction across synthetic cell membranes, with nucleic acid and peptide signals	10.1016/j.cels.2023.12.008	2024	Adamala	Bottom-up	No	No	Yes	Yes	No	Yes	Yes

Technical Challenges

Clearly, building synthetic cells faces significant technical challenges and is still a long way from achieving its declared objective. Box 2 provides a high-level overview of the remaining hurdles and outlines a sensible approach to overcome these. The remainder of this chapter further defines the specific technical challenges, organized by the criteria of life as defined above. Current major research foci are vesicle formation for compartmentalization and genetic circuits for functional metabolism.

Box 2: Steps towards rational bottom-up design and construction of living cells.

To achieve the building of a cell, a series of milestones will have to be reached. While there are likely numerous routes to success, we anticipate the most rational approach to comprise the following six stages, to be accomplished in somewhat chronological order:

1. Accomplish DNA replication in encapsulated cell-free system
2. Develop synthetic translation modules (synthetic ribosome; i.e., ribosomes making ribosomes, and the rest of the translation apparatus)
3. Implement cycling / self-replicating Tx/Tl systems
4. Achieve autonomous division of
    a. synthetic cellular compartment(s) without DNA
    b. machineries that segregate DNA
5. Show adaptation / sense response response (adaptive homeostasis) of synthetic biochemical systems
6. Revisit the definition of biological life to include synthetic biochemistry

Finally, an appropriate bio-safety and -security framework will have to be instituted in parallel to and in correspondence with the progression of steps one to six that is applicable and pertinent to the new lifeform.

Since parallel development and cross-information of the six steps outlined above is highly likely, we have composed an overview of the interdependences in the path towards building of a cell in Figure 4.

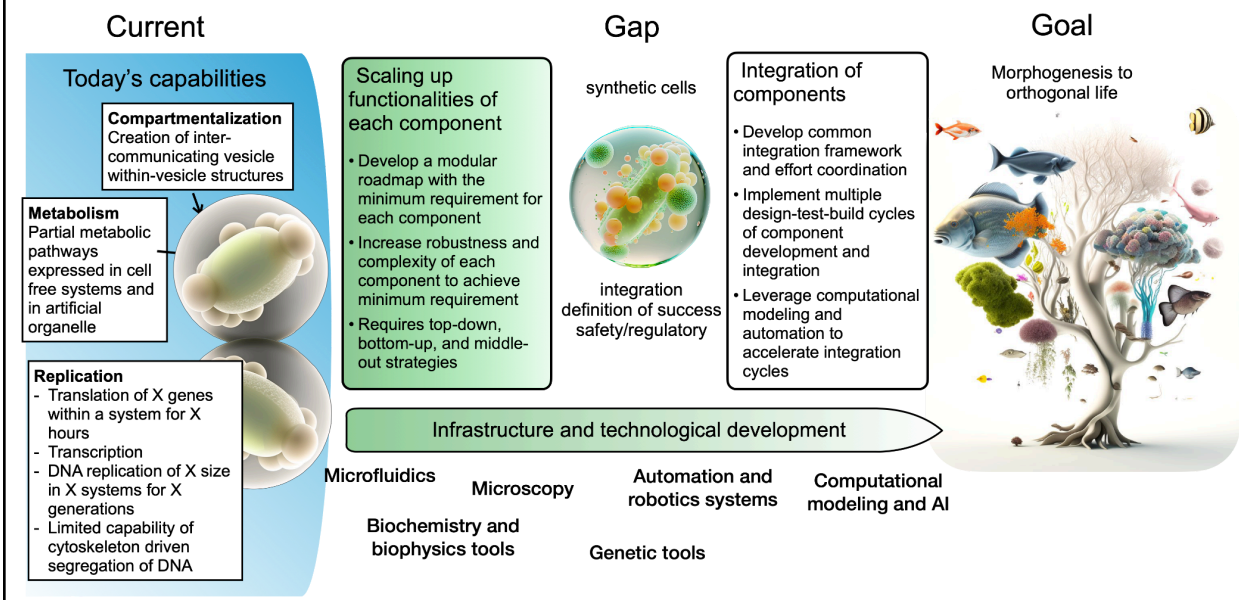

Figure 4: Milestones towards the *de novo* design and construction of a synthetic cell. Many of these steps can be performed in parallel with a mixing and matching approach to integration.

Compartmentalization

To achieve the formation of compartments, reliable methods for vesicular synthesis are required. Traditional means such as thin-film hydration to encapsulate cell-free systems in liposomes lead to a large range in the size and shape of liposomes.[35,171] This leads to variable protein expression among liposomes.[112,172] Another source of variability, especially in small (<10 μm) liposomes, is the random encapsulation of reactants.[173] This randomness leads to some liposomes containing sufficient reactants to produce protein as opposed to nominal content of others. Even with microfluidic encapsulation, which ensures more uniform size and content, macromolecular crowding can lead to large local variability within the same liposome.[174]

Metabolism

Functional metabolism requires the development of robust catabolic and anabolic processes for the mobilization of substrate and energy. This will allow for the biosynthesis of essential cellular compounds, such as carbohydrates, fatty acids, and amino acids that are the building blocks to minimize the reliance on externally supplied nutrients.

Given the large amount of transcription and translation that must occur simultaneously in synthetic cells, temporal control over gene-expression is critical. Whole cells have evolved complex methods to regulate genes in order to allocate limited resources toward the necessary metabolic functions required to maintain life. This control is often maintained via a series of genetic circuits that interact with each other such as feedback and -forward loops, as well as oscillators. Advancements have been made in developing orthogonal gene regulators which

allow for the control of multiple genes simultaneously. However, emulating the complex gene regulation of whole cells in a cell-free environment remains a challenge. Furthermore, as we progress into developing synthetic cells with extended capabilities, e.g. to produce chemicals, allocation of resources becomes an even larger challenge, as there must be a balance between maintenance of cellular functions and formation of the product of interest.[175,176]

Transcription and Translation Systems

Cell-free transcription and translation systems have advanced capabilities of *in vitro* protein expression by creating controllable, tunable systems independent of cell viability. The 'Protein synthesis Using Recombinant Elements' (PURE) system comprises the minimum number of enzymes that are required for Tx/Tl under specific conditions of almost any given gene. The PURE components are expressed *in vivo,* purified individually, and then combined.[40] This artificially reconstituted cell-free translation system allows controlled and high-throughput protein synthesis *in vitro*.[177] As with many novel technologies the cost of supplies is a significant barrier. "PURE to make PURE" is an example of this. Nevertheless, promising advances in generating low-cost methods to produce the PURE cell-free system have been made.[178] Further, while completely tunable, the system consumes many resources and therefore has a lower protein output than crude cell extracts.[179] Crude cell extracts are derived from lysed whole cells where insoluble constituents have been removed via centrifugation, yielding an extract that contains a concentrated mix of cytosolic proteins.[180,181] Further processing by methods, such as dialysis, can improve the performance of such systems. Maintaining transcription and translation over extended periods in either of these cell-free systems remains a challenge, as with no regeneration most transcription and translation processes terminate after a few hours due to depletion of resources. In order to overcome this, long-lasting transcription and translation systems have been developed that continually replenish resources, such as tRNAs, amino acids, and salts, via a feeding solution or exhibit partial endogenous metabolism (e.g., oxidative phosphorylation).[182,180]

Advanced cell-free protein synthesis systems can produce up to 0.5 mg/mL protein in 2 to 4 hours.[183] Nevertheless, these systems pale in comparison to the capabilities of naturally evolved cells as intracellular protein concentrations can be orders of magnitude higher.[184] The protein concentration of the cytoplasm in *E. coli*, for example, can reach 320 mg/mL, depending on the osmotic concentration of the medium.[185,186] If assuming a doubling time of ~20 minutes, a protein production rate of 960 mg/mL cytoplasm per hour is theoretically feasible.

The ribosome is the 'molecular machine' at the core of the translation system. Understanding of the ribosome has been transformed by the determination of three-dimensional structures, single molecule studies, and the construction of ribosomes from *in vitro* synthesized parts.[187,188] Despite this understanding, bottom-up synthesis of the ribosome *in vitro* remains a challenge. To achieve this objective, which is critical to building a true synthetic cell from the bottom-up, several key-achievements are needed, including the synthesis and assembly of rRNA and

rProteins. Recent strides have been made in the construction of semi-synthetic ribosomes using iSAT (integrated ribosomal RNA (rRNA) synthesis, ribosome assembly, and translation technology) in ribosome-free, crude cell lysates.[189] iSAT ribosomes are capable of constructing ribosomes.[190] In contrast to previous methods, this approach mimics the *in vivo* environment, including the co-transcription of rRNA and ribosome assembly, followed by protein synthesis in the same compartment. Similar methods imitate a natural cell's cytoplasmic environment to allow for the *de novo* synthesis of ribosomes.[191]

The translation system is composed of many parts (ribosomes, tRNAs, aaRS, etc.), and synthesizing all of these is an energy-intensive process. Biosynthesis of the *E. coli* ribosome alone requires 7,434 peptide bonds to make a complete set of rProteins.[189] A successfully self-replicating translation system would need to be able to not only replicate components of the translation system but also any essential auxiliary proteins.[192] Any such system would come close to achieving a living synthetic cell.

Replication

No cell is immortal, thus replication is key to the persistence of life, as well as to evolution and the creation of a sufficiently dense population that ensures survival. PCR has been used to replicate genetic material in liposomes since 1995.[193] Although the replication and division of genetic material in liposomes is still not spontaneous, this technology has built a strong foundation for coupling with other technologies that work towards the goal of a synthetic cell. *In vivo* studies of natural cells have helped to elucidate the mechanisms of cell division. The minimal division machinery relies on gene circuits, metabolism, and macromolecular modules that have evolved to function in the environment of a living cell, and thus are not easily transferable to synthetic cells. Nevertheless, major strides have been made to control and observe genome management and separation. Future reconstitution attempts using cellular components may need to create environments that mimic natural crowding and associated electrostatic and excluded volume effects, such as was observed for DNA acting as an exclusion zone for actin fibers encapsulated in beads.[194] Synthetic systems that enable segregation of genomes to complement cell division still need to be established. Minimal-systems, as well as the proto-ring components, must be encapsulated in vesicles or other deformable compartments.[195] Analysis of cell division in JCVI-syn3.0 showed that it does not divide like most normal cells. Because it lacks a set of seven non-essential genes that include the cell-division proteins FtsZ and SepF, the cell appears to divide based on forces of membrane curvature rather than by forming a protein-lipid membrane between daughter cells. A similar process may have existed before the evolution of more sophisticated cell-division mechanisms.[196] Thus construction of synthetic cells with replication systems simpler than those in the vast majority of lifeforms on Earth may be possible.

Emergent Features

Evolution

Evolution relies on the differential selection of variants whose phenotype is hereditary. In other words, evolution should emerge as long as there is heredity coupled to a phenotype and some variability (heterogeneity) in the population. To unleash the potential of evolution in a synthetic cell, genetic material must be passed on through generations. This can be accomplished by designing a synthetic genome, which may utilze either RNA or DNA and could rely on isothermal replication thereof, analogous to existing cells.

The concept of an "RNA world", which suggests that RNA performed as both the genetic information and replication machinery, serves as a common theory for the emergence of life on Earth. Efforts to develop this into a synthetic cellular system could validate the possibility of an RNA origin-of-life and provide a way to bottom-up construct a synthetic cell. However, a fully self-synthesizing ribozyme has yet to be discovered or engineered.

Responsiveness

Designing synthetic cells that can respond to specific stimuli without relying on simply altering gene-expression remains a significant challenge. There are a wide range of chemical reaction systems that are responsive to different stimuli. In biological systems, sometimes similarly responsive reactions take place that employ no genetic elements. Stimuli-responsive compartments could enable dynamic changes in the behavior and functionality of synthetic cells in response to external cues. Achieving this requires the development of responsive cellular components and mechanisms that can detect and transduce signals.[164]

Movement

Many current techniques for the creation of directional movement are focused on single enzyme mediated movement along a limited diversity of concentration gradients. More exotic physics-based techniques using interfacial tension differences could also be incorporated into synthetic cell-like systems. Based on the Marangoni effect, liposome-stabilized cell-sized droplets have demonstrated negative chemotaxis, resulting in movement away from the stimulus.[197] Self-propelled Janus particles have also been tuned to deliver cargo in response to specific obstacle geometries.[198] However, linking of these to complex information-processing pathways inside an artificial system has not yet been shown. While likely challenging, these would nonetheless be a promising route to providing a limited form of directional movement.

Alternatively, complex parts of living cells could be isolated and reconstituted in artificial cell-based systems, for example, cilia and flagella, following a middle-out approach. Methods will need to be developed for their incorporation in a synthetic cell and their linkage to the information processing system for providing more than mere random motion. Knowledge

gained by the development of these techniques can result in a better understanding of the motion-creating machinery that is essential for cell movement, potentially providing information for their bottom-up reconstitution.

A third technique driving directional movement could be the encapsulation of a set of cytoskeletal elements with associated proteins, for example by reconstituting actin network assembly.[199,200] Described as “gliding”, some species of mycoplasmas exhibit this feature, which provides shape transformations but directional movement is limited.

Concurrent Challenges

Integration with Natural Cells

Successful interfacing of artificial and natural cells requires the presence of robust communication pathways. In recent years, synthetic cell-driven quorum-sensing was used not only to achieve signaling responses in bacteria, but also to expand their sensory range via artificial cells acting as chemical translators.[201,202] In another approach, artificial cells encapsulating gene-networks were able to detect, interact and kill bacteria in chemically diverse extracellular-like environments.[203] Lastly, interface with eukaryotic cells has been successfully demonstrated by cuboplex-mediated gene-silencing in Chinese Hamster Ovary (CHO) cells, promoting the differentiation of neural stem cells from an artificially synthesized and released neurotrophic factor.[204]

Integration and Scalability

Overall, challenges of scalability of synthetic cells exist at three levels: scalability of individual components (e.g., scaling the number of genes that can be expressed in a cell-free system), scalability of number of components that can be integrated (e.g., adding a mobility module to membrane protein modules), and the scaling of the replication cycles and culture volume. A standardized workflow can help to establish both vertically- and horizontally-integrated technology stacks for robust, reliable construction of synthetic cells. Such a workflow, may also be helpful to define the components of a synthetic cell at varying levels of complexity and create modular abstraction for prediction and design. One such abstraction, of containers and content, has been proposed and others may emerge.[205,206,207] Scalability of synthetic cells for applications will depend on the success of these efforts at abstraction, coordination, and integration.

Cross-Lab and Larger-Scale Coordination

To be successful in building a cell, the free and open-access flow of data, protocols and probably also people (scientists) will be required. Effective communication and coordination between individual researchers and groups is crucial for establishing and maintaining functioning collaborations. This is especially important as cells require multiple interacting components, which exceeds the capacity of individual labs. Several such groups exist, including the NSF-funded Research Coordination Network (RCN) “Build-a-Cell”.[208] Build-a-Cell is an open, international collaboration supporting the science and engineering of synthetic cells uniquely

designed to address these challenges, experimental as well as social. Members of the Build-a-Cell research community bring expertise from a variety of fields and backgrounds to foster open channels of collaboration, coordinate fundraising efforts, address biosafety and biosecurity concerns, encourage open technology transfer, and organize outreach efforts to increase understanding among researchers, policymakers, and the public. Other groups, such as the German-led MaxSynBio[209], the Dutch-led BasyC[210], and organizations/groups from related fields, exist.[211,212,213]

To realize the full potential of synthetic cells for biomanufacturing, advanced tools and infrastructure for scale-up will be required. U.S. legislation, such as the "Inflation Reduction Act of 2022"[214] and the White House Executive Order 14081 on "Advancing Biotechnology and Biomanufacturing Innovation for a Sustainable, Safe, and Secure American Bioeconomy"[215] as well as U.S. government efforts, such as BioMADE[216] could support infrastructure for manufacturing synthetic cells.

Modular Roadmapping for Individual Components

Developing a modular roadmap for the integration of existing technologies, such as DNA synthesis, nanopore technology, microelectromechanical systems, microfluidics, compact hyperspectral imaging and ML, generative AI and quantum computing, will help to motivate the community toward greater action, focus resources, and accelerate progress. This roadmap must be versatile enough to incorporate new technologies as they emerge. Academic efforts to produce devices for constructing cells are as of yet mostly uncoordinated.

Biosafety and Regulatory Guidelines

The field of synthetic cell research faces not only technical but also social, ethical, and philosophical challenges to define what is life, along with key obstacles requiring prudence for advancing both synthetic cell research specifically and biotechnology as a whole. Future applications, social and political frameworks, and governance for synthetic cell technologies demand thoughtful consideration to ensure benefits while avoiding risks and preventing hazards. Equitable governance and regulatory participation are crucial for biosafety and threat mitigation as synthetic cell technologies become more accessible, reducing the risk of (intentionally or unintentionally) harmful biology. Existing methodologies for risk assessment, especially related to synthetic biology, must be revised or established anew, considering potential hazards through red teaming events and promoting biocontainment strategies.

Ensuring safe market-integration of synthetic cell technologies necessitates efficient regulatory pipelines, boosting private investments and career interests. Standardized definitions are prerequisites for policy protocols, aiding clear communication of standard operating procedures (SOPs) and safety protocols. FDA approval, a lengthy process for drugs, lacks suitable components for assessing synthetic cell safety due to their developmental stage. Classification of synthetic cell laboratories under BioSafety Levels (BSL) provides a standardized foundation for safety measures. BSL ratings, ranging from 1 to 4, determine containment needs based on

pathogen type, a model that is likely also suitable for synthetic cells. Incorporating synthetic cells into existing pathogen definitions simplifies the process, assessing them similarly to naturally-evolved pathogens based on infection capabilities or reproductive rates.

Technology Transfer

To optimize the development of impactful synthetic biology tools for synthetic cells, it is crucial to emphasize knowledge and technology transfer, given the significant expert hours required. Sharing procedures and protocols fosters effective collaboration, reducing redundancy and unnecessary competition among researchers. A coordinated effort to integrate the technology necessary for modeling, making, and measuring synthetic cells will not only enhance collaboration but also facilitate continuous iteration and improvement of underlying technologies. This collaborative approach should include complete reporting of attempted approaches, especially those that do not yield satisfactory results, to prevent researchers from inadvertently duplicating previous failures. Establishing a framework for sharing these non-published results could significantly increase research efficiency.

Research could, for example, follow the lead of software development and create an open-source model for modular "*à la carte*" biological parts to develop a comprehensive synthetic cell chassis. This not only allows for a shared and collaborative understanding of basic biology functions but also avoids limiting the definition of "life" and thus limiting the scope of the field.

Outreach and Education

Synthetic cells offer significant potential for biotechnology, but engaging the public in research and technology development is crucial. Emphasizing education with both policymakers and the public will not only support effective policy frameworks but also enhance the acceptance and impact of new technologies. Fostering a better grasp of and involvement in synthetic cell technologies will aid in cultivating a market. Enhancing science literacy in the realm of synthetic biology will ensure a qualified workforce for this rapidly advancing field.

Comprehensive outreach and knowledge dissemination are essential across all levels to develop research tools, establish clear communication with policymakers, and create a market and workforce conducive to applying new technologies. Notably, BioBits kits have already captured student interest, fostering greater understanding.[217,218,219,220,221] Elevating science literacy through do-it-yourself (DIY) communities not only facilitates ethical discussions but also enhances public awareness and comprehension of ongoing research and eventual technologies.

Leveraging community-based laboratories alongside newly established foundations for global synthetic biology education can effectively drive public engagement and education in synthetic cell technology.[222] These endeavors to broaden STEM education can be interconnected with synthetic cell research.

Accessibility and Equity

Both living and non-living synthetic cells will become a larger and larger part of biotechnology worldwide. The economic impact of this technology could be huge. A sustainable bioeconomy has the potential to enable communities around the world to be self-sufficient, self-determined and resilient. As a highly impactful biotech field still in its nascency, synthetic cell research is well positioned to address historical barriers to access and address equity and inclusion in science more deliberately and democratically. This includes efforts in expanding the reach of education, developing technologies for use across geographic and socioeconomic boundaries, and engaging across disciplines to make decisions about the future of synthetic cell technology.[210]

Conclusions and Steps Ahead

Due to the opportunity to tailor cellular systems for a specific purpose, synthetic cells could have a wide range of potential applications in various fields, such as cell biology, astrobiology and origin-of-life, bioengineering and biomedicine, biomanufacturing and bioprocess engineering. However, achieving a self-sustaining biochemical reaction remains a significant challenge: the development of a fully functional synthetic cell is a complex and ongoing scientific endeavor that requires the integration of multiple disciplines and technologies.

Accomplishing the *de novo* construction of cells will be an enormous achievement, especially if the devised system is capable of reproduction, which will be a key first – but not ultimate – achievement. Any first iteration of a synthetic cell will likely be very basic, lacking substantial versatility in its metabolism and environmental robustness, and hence difficult to keep alive. In summary, the development of synthetic cells is a complex and multifaceted endeavor that will require interdisciplinary collaborations and significant investment in both resources and infrastructure.

When and How to Declare Success

Finally, at what point do we declare success? How do we know if we have created a synthetic cell that is alive rather than a vesicle that just exhibits life-like functions? Following Gánti's Chemoton model of life, at a minimum, the characteristics of compartmentalization, metabolism, and replication must be present.

Is translation, transcription, or cell division required? Mature mammalian red blood cells, for example, do not have a nucleus or ribosomes and thus are not capable of transcription, translation or cell division. How about derived characteristics such as evolution? Clearly, these cells evolved from other kinds of cells, and the evolution of the nucleated cells that give rise to red blood cells will change the red blood cells. The list of exceptions goes on. If we turn to naturally evolved cells, we can easily find counterexamples for each of the current criteria of life, because life is a population-level phenomenon. As it is impossible to account for all possible variations and no "natural definition" of life exists, it may be a matter of creating a cell that is

“generally recognized as alive” or GRAA. This allows the field to progress independently of philosophical agreement on a definition of life. Thus, the definition of success is likely context dependent and will have to be adapted as science progresses.

Abbreviations

BioSafety Levels (BSL); Design-Build-Test-Learn (DBTL); do-it-yourself (DIY); generally recognized as alive (GRAA); integrated synthesis, assembly, and translation (iSAT); non-standard amino acid (NSAA); polymerase chain reaction (PCR); protein synthesis using recombinant elements (PURE); Research Coordination Network (RCN); standard operating procedure (SOP); science, technology, engineering and mathematics (STEM); transcription/translation (Tx/Tl); unnatural base pair (UBP); xeno-nucleic acid (XNA)

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Conflicts of Interest

None of the authors declare competing interests.

Author Contributions

LJR, NJHA, FM, and KPA formulated the concept and structure of the article. LJR, NJHA, EAS, FM, JIG, RCP, IOY, BRM, GARK, FW, JW, IAI, MCJ, APL, VN, CS and KPA wrote portions of the manuscript. LJR, EAS, FW and JW created the figures. All authors edited the manuscript.

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