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Cellular imitations

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Synthetic biologists typically construct new pathways within existing cells. While useful, this approach in many ways ignores the undefined but necessary components of life. A growing number of laboratories have begun to try to remove some of the mysteries of cellular life by building life-like systems from non-living component parts. Some of these attempts rely on purely chemical and physical forces alone without the aid of biological molecules, while others try to build artificial cells from the parts of life, such as nucleic acids, proteins, and lipids. Both bottom-up strategies suffer from the complication of trying to build something that remains undefined. The result has been the development of research programs that try to build systems that mimic in some way recognized living systems. Since it is difficult to quantify the mimicry of life, success often times is evaluated with a degree of subjectivity. Herein we highlight recent advances in mimicking the organization and behavior of cellular life from the bottom-up.

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Introduction

The term synthetic biology was intended simply to denote the assembly of biological parts into larger systems, just as synthetic chemists build larger molecules from smaller molecules [1]. From this perspective, synthetic biology has grown into a wide spectrum of research programs (Figure 1) incorporating elements from engineering, biology, chemistry, physics, design, and art. The predominant way in which synthetic biology is practiced is to engineer subsystems within the larger framework of a cell that was not engineered. Individual, mostly natural, biological parts are thoroughly characterized, that is standardized, so that predictable (sub)systems consisting of these parts can be built. Just as the same set of Lego pieces can be used to build many different structures, standardized biological parts can be put together in many ways giving organisms that manufacture fuel, produce pharmaceuticals, or detect environmental pollutants. The exercise of building biological behavior, in turn, contributes to our understanding of how natural biological systems function. However, the construction of systems that operate within a host that is dependent upon genes with unknown function, as is the case for all known life, leaves many gaps in our knowledge untouched.

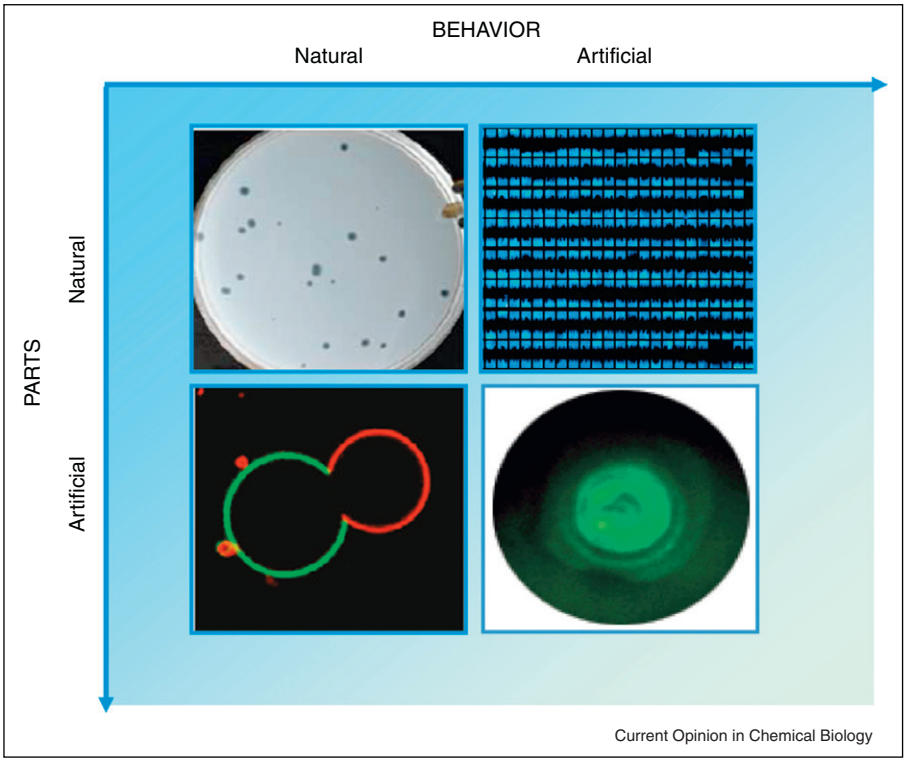
The engineering of life does not solely rely on the use of previously existing natural biological parts. Instead, new cellular pathways can be built with artificial components. Because of the difficulties associated with engineering proteins with new functionality, artificial RNA rather than protein molecules are more commonly exploited. For example, Gallivan and colleagues built a ligand responsive artificial RNA to engineer *Escherichia coli* to swim towards a pollutant molecule [2]. In this case, the artificial RNA was integrated with natural RNA and protein components to elicit the new behavior. Conversely, entire artificial systems can be made to exist within a natural host cell. For instance, orthogonal ribosomes can be engineered to not recognize natural host transcripts and only translate sequences containing orthogonal ribosome binding sites [3].

The *de novo* engineering of cellular life

The examples described above fit broadly within the engineering paradigm. In other words, life is treated as a machine in which characterized parts are assembled in various ways to generate systems with desired function. This is possible because the chassis, that is the host of the engineered genetic elements, is used to provide the ill-understood properties of life. If, however, the desired function is life itself built from non-living component parts, then we begin to move away from traditional engineering. This is because we do not have a clear idea of what is to be built. There is no satisfactory definition of life. Nevertheless, it is generally agreed that biological parts alone are not alive, but the properties that emerge from their cooperation are collectively referred to as living.

Without clear criteria that can be objectively fulfilled for a system to be considered living, the available path forward is simply to build systems that imitate the common features of life. For example, living things generally reproduce, move, adapt to changing environmental conditions, and interact with each other. Of these features of life, reproduction has attracted the most attention, which is understandable since replication and evolution form the foundation of life as we know it. However, a machine, even a machine that is built with natural biological parts,

Figure 1



Different ways in which synthetic biology is practiced. (Top-left) Natural parts can be used to build natural behavior. A refactored T7 genome supports the infection of *E. coli* (adapted with permission from Macmillan Publishers Ltd. [41]). (Top-right) Natural parts can be used to construct unnatural behavior. For example, natural sensory pathways were constructed in such a way as to give synchronous fluorescence of *E. coli* in a microfluidic device (adapted with permission from Macmillan Publishers Ltd. [42]). (Bottom-left) Artificial components can be used to mimic natural behavior. Poly(ethylene glycol) and dextran aqueous phases inside of phospholipid vesicles can divide (adapted with permission from [31]). (Bottom-right) An artificial part can be used to encode unnatural behavior. Here an artificial riboswitch was used to make *E. coli* swim towards a molecule that the bacterium does not naturally swim towards (adapted with permission from Macmillan Publishers Ltd. [2]).

that is programmed to copy DNA and to split into two probably would not be confused with a living system. Perhaps this is because the decision of whether something is alive or not is the result of a subjective comparison between what was previously agreed upon as living with the system in question. The successful mimicking of a single trait when compared against the complexity of a living cell would be perceived as an inadequate representation of cellular life. Additionally, the programming of repetitive behavior in itself misses another aspect of life, which is error. Cellular function is largely based on stochastic processes and even the fundamental event of genomic replication proceeds with error. A system that mimics a trait of life too well, probably would be perceived more as a machine rather than life.

The lack of clearly objective means of evaluating the outcome of experimental efforts in building a cell has slowed progress. A potential solution to this problem would be to shift the responsibility of determining whether something is alive or not away from us and towards natural cells. In this way, the interaction between the interrogator and the artificial system would be mediated by sensory pathways of similar scale. Such an approach is similar to that described by Turing in evaluating artificial intelligence in the absence of an agreed upon definition of intelligence [4]. The translation of such a Turing test to a cellular scale, as previously suggested [5], could allow for a more direct and unbiased way to evaluate success in building cell-like systems with life-like the behavior. A starting point for an artificial system that could pass the cellular Turing test could be the construction of a synthetic quorum pathway between an artificial and a natural cell [6].

The inability to define what is being built poses some problems, but also provides room for a variety of different research avenues. Mimics that morphologically resemble a cell, others that carryout similar chemical transformations as natural cells, and artificial systems engineered to pass a Turing-like test all will deepen our understanding of life. Thus far, most of the progress has been in building bottom-up replication and division mechanisms, but complementary studies are beginning to point to a more

exciting phase of bottom-up synthetic biology that better captures the complexities of life.

Reconstituting the parts and organization of life

To build something that looks like an extant cell, DNA, RNA, protein, and lipids should be assembled in a manner that gives a genetically encoded system with a cytoskeleton and a lipid membrane (Figure 2a). Each of these molecular components can be functionally reconstituted in the laboratory. However, the lack of knowledge concerning the way the biological parts fit together to give life is obvious when one considers that the successful synthesis of an entire genome [7] required genes of unknown function and a recipient host cell to provide additional components with unknown function.

When provided with the required monomeric building blocks, the information stored within a DNA molecule can be used to direct the synthesis of RNA through the activity of a single protein *in vitro*. Although the synthesis of protein from an RNA template is much more complex, after the pioneering work of Ueda and co-workers, it is now rather straightforward to carryout translation *in vitro* [8,9]. Similarly, the construction of a membrane-defined body to house a cell-like system is achieved easily *in vitro*. Many lipids spontaneously form vesicle membranes in aqueous solution that efficiently retain large molecules, allow for the selective exchange of small molecules, and are compatible with growth and division. The interior of a vesicle can be further organized. Polymer solutions, such as polyethylene glycol and dextran, can form distinct aqueous phases to which some molecules preferentially partition depending on their hydrophobicity [10].

Since protein synthesis proceeds efficiently in vesicles [11], vesicle structure and organization can be reinforced by the formation of cytoskeletal mimics (Figure 2b and c). Actin polymer filaments can be anchored to lipid membranes [12] and bacterially derived cytoskeletal elements can be assembled inside of vesicles [13]. It should be noted, however, that while active RNA polymerases can be produced through *in vitro* transcription–translation reactions, the *in vitro* production of translation machinery has not been achieved to date. Therefore, current bottom-up constructions of cellular mimics make use of bacterially derived translation components.

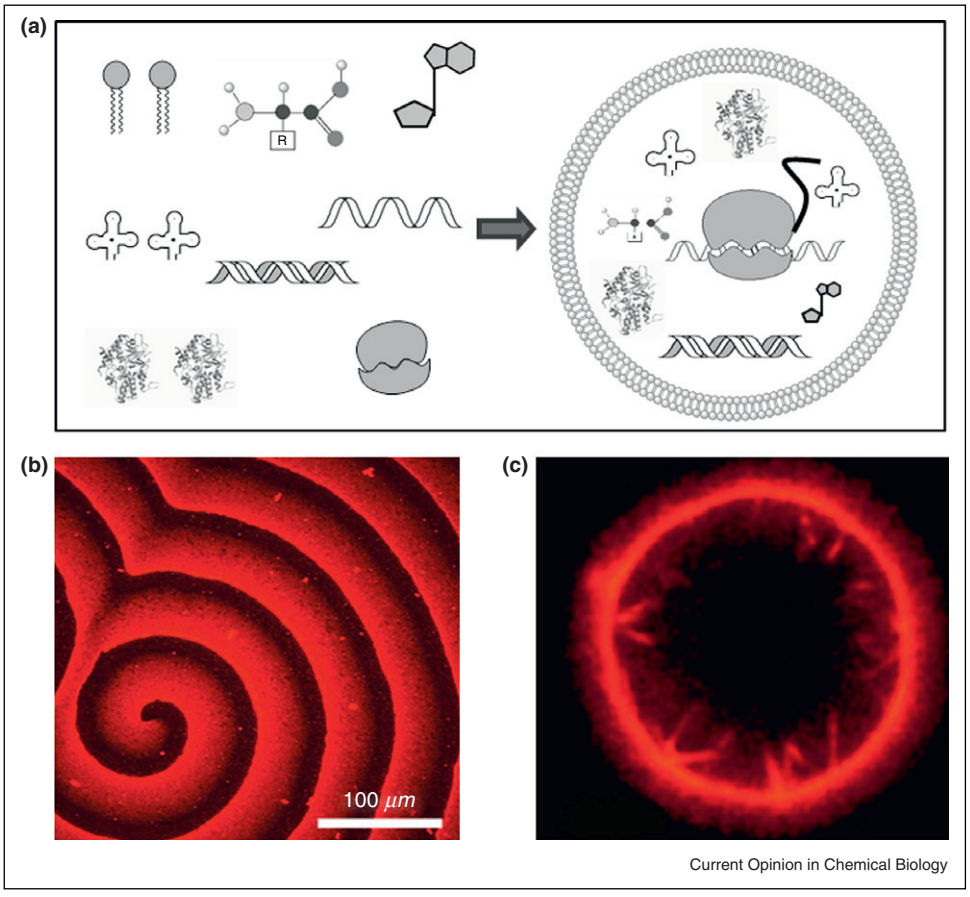
Artificial reproduction

At a minimum, cell-like reproduction consists of genomic replication and the division of the vesicle body [14]. The replication of DNA *in vitro* is easy, but to do so in a fashion amenable to the construction of a cell is challenging. A typical cell uses ten to twenty proteins to synthesize RNA primers, copy the leading and lagging DNA strands, substitute the RNA primer sequences with DNA, and ensure that no regions are left uncopied. Several isothermal DNA replication strategies have been developed that fulfill many of these needed activities [15,16]. However, thus far only the phi29 replication machinery has proven effective in copying entire genomic sequences end-to-end *in vitro* [17•]. Remarkably, only four phi29 proteins are necessary to copy viral genomes *in vitro*. Considering the small size of the phi29 bacteriophage genome, it will be important to determine whether the system in its current form will be capable of copying genomes with greater than 20 encoded genes.

Attempts to further simplify the construction of a cell have sought at times to remove some of the perceived redundancies of the DNA to RNA to protein pathway that pervades life. Since RNA and DNA are both capable of storing information, *in vitro* systems guided by RNA encoded information rather than DNA have been constructed in which the same RNA molecule acts as both the template for replication and the template for protein synthesis [18]. While this apparent simplification does reduce the number of needed components, it is unclear if an artificial, autonomous cell ultimately could be built with an RNA genome. DNA based life, that is all known life, is able to more easily separate genomic replication from the production of protein, whereas an organism that relies on an RNA genome would have to cope with the influences of RNA folding on replication and translation efficiencies [19] and on competition between RNA polymerases and ribosomes for the same template [20]. One potential solution would be to simplify the RNA genome-based organism even further by removing the need for protein function. Not only would this remove complications arising from coordinating replication and translation, it would also greatly simply the genome itself. This is because few genes are required for DNA and RNA synthesis, whereas protein synthesis necessitates over 100 genetically encoded elements [21]. Since RNA can possess catalytic activity and can replicate segments of RNA templates [22•], it is conceivable that a self replicating cell-like system could be built with an RNA genome and without proteins. Nevertheless, significant advances are required in RNA replicase processivity before such a goal can be accomplished.

The lack of a sufficiently processive RNA replicase could be circumvented by building systems that do not depend on catalysts. While the complexities of extant life probably require high activation energy barriers for metabolic processes to ensure proper control and coordination through enzyme activity, simpler cells may not require such regulatory mechanisms. For example, the incorporation of better leaving groups in nucleotides allows for template guided nucleic acid polymerization [23] that is compatible with lipid vesicles [24]. Other non-enzymatic mechanisms exist, too, such as those that exploit intercalators [25] or altered backbone connectivities [26].

Figure 2

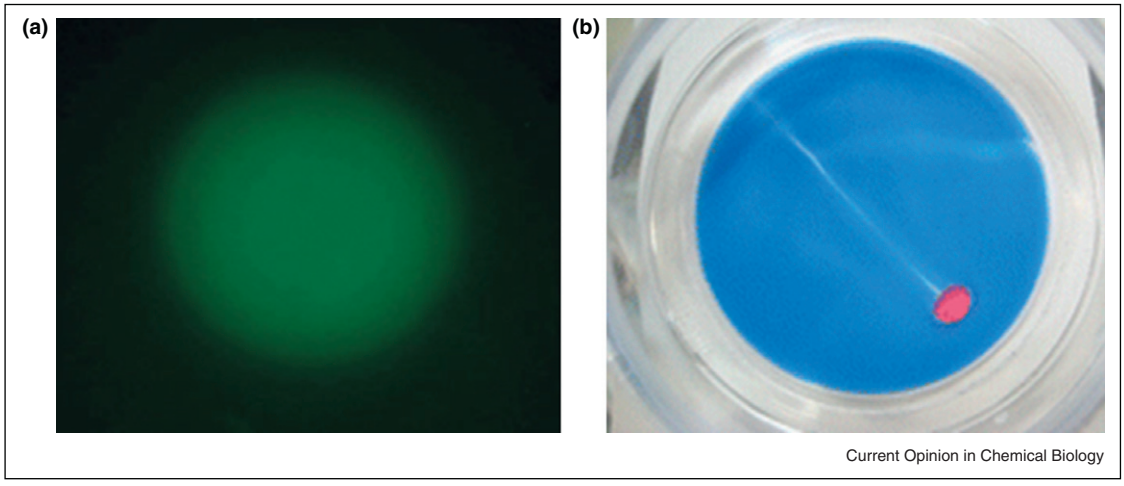


Structural organization of a cell. **(a)** Cellular mimics are often constructed from the basic parts of life, such as DNA, RNA, protein, amino acid, nucleotide, and lipid. **(b)** Some proteins can self-organize on lipid surfaces, such as those of the cell division Min system (adapted with permission from AAAS [28]). **(c)** A synthetic cytoskeleton built with polymerized actin inside of a vesicle (adapted with permission from Macmillan Publishers Ltd. [43]).

Impressively, several advances in *in vitro* vesicle division mechanisms have been reported. One such system relies on the bacterial division pathway consisting of Fts and Min proteins. In particular, focus has been placed on FtsZ, which forms a constricting ring *in vivo* localized to the midcell that divides the cell into two. The Min proteins help guide the placement of the Z-ring by inhibiting FtsZ polymerization at the poles of the cell. Although over fifteen proteins are believed to be involved in bacterial division, much simpler versions have begun to be built in the laboratory. For example, the tubulin homologue FtsZ was engineered to insert directly into membranes by Erickson and colleagues. This engineered protein polymerized into rings within tubular liposomes and generated noticeable indentations within the membrane [27], suggestive of the first steps of division. Although less work has been reported on the Min system, Min proteins self organize into protein waves on supported lipid bilayers consistent with their *in vivo* behavior [28]. To date, the Min and Fts systems have not been integrated into a single *in vitro* system.

Vesicle division mechanisms that do not depend on protein activity have proved easier to build *in vitro*. In fact, membranes consisting of three different lipids that phase separate into liquid ordered and liquid disordered domains can result in membrane curvature, budding, and division facilitated by osmotic pressures [29]. More recently an alternative system that exploits encapsulated aqueous two phase systems was shown to similarly induce budding and division in hypertonic solution [30]. While impressive, both methods only allow for a single cycle of division since the needed asymmetries are not retained in the daughter vesicles. However, when both mechanisms were integrated in such a way as to create a mismatch between the surface area of the two lipid domains with the volume of the two aqueous phases, the daughter vesicles maintained a level of asymmetry sufficient to allow for a second cycle of division [31••]. If this remarkable lipid domain – aqueous two phase system were coupled with a vesicle growth mechanism, then a self sustained growth – division cycle could be envisaged.

Figure 3



Examples of artificial systems that mimic cellular behavior. **(a)** Cell-like systems can be built to sense their surroundings. Here a riboswitch is used to sense the extravesicular addition of theophylline and responds by synthesizing a fluorescent protein (reproduced with permission from the Royal Society of Chemistry [38•]). **(b)** Just as cells move, droplets can be formulated to move down concentration gradients (adapted with permission of the Royal Society [40]).

An unrelated non-protein based system does just that, couples vesicle growth with division. Vesicles composed of single chain fatty acids have a broader range of accessible dynamics than vesicles made from the types of diacyl lipids that are typically found in biological membranes. Although the details of the mechanism are unclear, if fatty acid micelles are added to multilamellar vesicles, the vesicles grow into unstable thread-like filaments [32]. Division into daughter vesicles can be induced either by mild agitation or through the oxidation of thiol containing compounds that interact with the membrane when oxidized [33•]. The fluid shear force division mechanism can go through multiple growth and division cycles through forces imparted by the environment. The latter thiol oxidation mechanism suggests that if a metabolic-like oxidation–reduction cycle were reconstituted within the vesicle, then multiple rounds of growth and division could be mediated by internal processes rather than by external forces.

An alternative pathway developed by the Sugawara laboratory uses DNA replication to drive vesicle division. The lipid composition is more complex, including a mixture of natural and unnatural lipids plus a catalyst that converts precursor molecules into more lipid [34••]. During intravesicular DNA replication through PCR, ionic interactions between DNA and the membrane results in the division of the vesicle. Not only does this system couple two processes crucial for constructing cellular life, that is genomic replication and compartment division, the molecular components used are compatible with biological machinery, suggesting that cellular mimics that more closely resemble life as we know it could be built. However, the lipid composition of the membrane changes over the course of the reaction so that multiple rounds of division are not possible.

Life-like behavior

There are now available many mechanisms for vesicle division that could be exploited for the construction of a cell. However, as noted above, the construction of a self-replicating system in the absence of other distinguishing features of life is unlikely to be perceived as living. A more convincing cellular mimic would sense and respond to internal and external stimuli in order to coordinate different physiological processes and to adapt to changing environmental conditions. For example, natural cells ensure that division only occurs after genomic replication, and natural organisms adapt to fluctuating temperatures by modulating membrane compositions and protein chaperone levels. Interestingly, some of the environmental fluctuations that a cell must cope with arise from the cell itself, since living systems modify their environment by acquiring food and releasing waste. Although examples of *in vitro* constructed sense–response systems are few, recent developments suggest viable routes forward in exploiting sensory pathways for the building of cellular mimics.

In vitro genetic systems can be constructed to sense and respond to the availability of small molecules. An *in vitro* cascading genetic network, for example, was built to control the production of protein in response to IPTG [35]. More recently, *in vitro* negative feedback loops exploiting tetracycline [36] and arabinose transcriptional repressors [37] were built. Rather than using natural

protein transcriptional repressors, protein production can be controlled by the activity of artificial RNA sequences, such as that displayed by the theophylline riboswitch [38•] (Figure 3a). Riboswitches are regulatory elements residing in the untranslated regions of mRNA that control translation through direct ligand binding. The advantage of riboswitches is that they are much simpler to engineer than proteins. Of the systems described above, the arabinose sensing [37] and the theophylline sensing [38•] systems were reconstituted in phospholipid vesicles, thus allowing for the development of cellular mimics capable of responding to the chemical composition of their extravesicular surroundings.

Non-genetically encoded sensing mechanisms are a potential complement to the use of protein and RNA sensors. The aqueous two phase system developed by Keating and colleagues can be used to control the localization of molecules in response to environmental fluctuations. This is because many biological molecules undergo structural changes that affect their surface charge distribution upon shifts in pH or temperature [39•]. Sensing that results in the movement of a chemical system is also possible [40] (Figure 3b). Hanczyc and colleagues built a chemical system that moves away from depleted nutrients and towards molecules that sustain movement.

Now that it possible to build cellular mimics that sense and respond to changing chemical conditions, it seems that the time is right to begin to more deeply probe non-replication aspects of life. Sensory pathways are required for the construction of systems that better represent the complexities of extant life. Unlike life, machines are programmed to act in a very defined manner, performing a designated task regardless of external conditions. Cellular mimics with sense–response capabilities, therefore, probably would come closer to being perceived as living than a machine. Further, the incorporation of sense–response pathways allows for a more objective means of evaluating success through the implementation of a cellular Turing test.

Conclusion

Many of the features of cellular life now can be built in the laboratory. However, the individually reconstituted features of life may not be compatible with each other in their present form. Their integration into a system that better represents the complexity of life poses a significant challenge. It may be that the purely chemical approaches and those that make use of biological molecules will continue to proceed on separate tracks, which would be unfortunate. DNA replication is easier to achieve with the aid of proteins and vesicle division is simpler through purely chemical–physical means. If these two branches of bottom-up synthetic biology found a way to merge, perhaps the synthesis of an artificial cell would be much nearer.

Bottom-up synthetic biology has largely focused on self-replication and in the process has developed a wide variety of ways to copy nucleic acids and divide vesicles. However, life is not simply a machine that divides. Instead, life is integrated with its surroundings, both on a cellular and a chemical level. The recent advances in building cellular mimics capable of sensing and responding to small molecules opens an exciting alternative to the prevalent attempts at building bottom-up cells. Perhaps it is time to allow a bacterium to judge our work.

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