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Review Article

Toward long-lasting artificial cells that better mimic natural living cells

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Chemical communication is ubiquitous in biology, and so efforts in building convincing cellular mimics must consider how cells behave on a population level. Simple model systems have been built in the laboratory that show communication between different artificial cells and artificial cells with natural, living cells. Examples include artificial cells that depend on purely abiological components and artificial cells built from biological components and are driven by biological mechanisms. However, an artificial cell solely built to communicate chemically without carrying the machinery needed for self-preservation cannot remain active for long periods of time. What is needed is to begin integrating the pathways required for chemical communication with metabolic-like chemistry so that robust artificial systems can be built that better inform biology and aid in the generation of new technologies.

Introduction

There is a long tradition of studying biology by either perturbing living organisms or reconstituting *in vitro* systems composed of molecules isolated from living organisms. The biochemical methodologies of the latter approach have allowed us to gain mechanistic and structural insight into the workings of biology that would have been difficult to attain by *in vivo* studies alone. However, there are limitations as well. Clearly, the chemical conditions of a test tube are far from that of the inside of a cell. Discrepancies between *in vitro* and *in vivo* activities further complicate the difficult task of developing a unified computational model of a living cell. One path forward may be to continually increase the complexity of systems assembled *in vitro* until the network approaches the behavior of extant living cells. Such work is, in fact, ongoing even if often motivated by deciphering the chemistry of biology rather than by the goal of synthesizing a cell.

Recent successes in reconstituting biological processes are impressive. The Zerial group exploited 17 purified proteins to successfully reconstitute endosomal membrane fusion [1], and the Musacchio laboratory built functioning kinetochores *in vitro* consisting of 21 [2] and 26 [3] purified protein subunits. Such reconstructions uncovered the crucial role of Rab proteins during membrane fusion and helped reveal mechanistic details of chromosome alignment and segregation during mitosis and meiosis in eukaryotic cells. In other words, by removing complex biochemical systems from their complex biological environments, these laboratories were able to definitively confirm aspects of previously proposed models and additionally refine the models by incorporating new features that were left undetected from work done solely *in vivo* [4].

The above examples, however, reveal the tension between complexity and feasibility. Overly simplified systems may not capture activity representative of biology, but data from overly complex systems may be indecipherable with current technologies. The goal, it seems, is to strike the right balance between the two, so that increasing layers of complexity can be added after individual subsystems are well characterized. Such an approach would be straightforward if biology were modular, but evolution is a messy process that generates webs of interactions between most, if not all, cellular processes. It is,

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therefore, unlikely that individually reconstituted pathways can be put together like the parts of a machine [5]. That is, while it is tempting to search through different organisms for the simplest mechanisms associated with the central features of life, such work may not directly lead to the synthesis of a cell, because the pieces simply may not fit together. For example, mismatched affinities could lead to the ill-regulated activation of pathways or the formation of undesirable, inhibitory complexes that evolution selected against. Perhaps directed evolution methodologies could help alleviate such complications.

Alternatively, it may be simpler to attempt to take apart and put back together a well-characterized organism with a small genome rather than to assemble an artificial cell from parts taken from multiple organisms. Such a bacterium has already been developed by the Venter group [6]. This organism, referred to as JCV-syn3.0, is derived from *Mycoplasma mycoides*, only contains 473 genes, and the *in vitro* synthesized genome has already been shown to function *in vivo*. One significant complication, however, is that despite the small size of the genome, the function of nearly one-third of the genes provide unknown function to the organism [6]. Although it may be less satisfying to put together an existing organism, the endeavor would likely not be easy. Even the partial refactoring of a simple bacteriophage with a much smaller genome than that of a bacterium resulted in the synthesis of much less active viral particles [7]. Attempting to put together an existing organism would also provide for the opportunity to learn design rules from a system that is known to work.

Chemical communication between artificial cells

Since natural selection operates on populations of living organisms, life, as we know it, has evolved in communities. Members of a community interact, and the chemical foundations of these interactions are not different from the molecular organization that exists intracellularly. Compartment growth and division are typically co-ordinated with the replication of DNA, for example, and such coordination is achieved through the triggering of responses to chemical cues [8]. Similarly, the search for food, the avoidance of toxins and waste, and the evasion of predators rely on sensory mechanisms. Therefore, it seems likely that the types of group behavior seen from bacterial biofilms [9] to the differentiation of eukaryotic cells into multicellular organisms [10] emerged from such early forms of primitive chemical communication.

The *in vitro* attempts at mimicking communication channels have made use of a variety of different forms of compartments to house the artificial cell [11]. Most commonly, lipid molecules that self-assemble into vesicles (or liposomes) are used. The lipids are typically diacyl glycerophospholipids of the type found naturally in biology. Simplified versions of lipid-defined compartments are found in water-in-oil emulsion droplets that are usually stabilized by a monolayer of single-chain lipid. In addition to compartments that exploit amphiphiles that structurally resemble lipids found in biological membranes, compartments can be made from structurally distinct components [12]. Examples include compartments formed by the self-assembly of protein–polymer conjugates (proteinosomes) [13], inorganic nanoparticles (colloidosomes) [14], block copolymers (polymersomes) [15], Janus dendrimers (dendrimersomes) [16–18], and peptides [19]. Compartments can even be made without a membrane at all [20]. Neither aqueous two-phase systems [21,22], polyelectrolyte polymer containing coacervates [23], hydrogels [24], microfluidic chip-based compartments [25,26], nor bead-based systems [27] contain a membrane.

Communication between artificial cells can take many forms. For example, the release of small sugar molecules was used to send messages from vesicles to proteinosomes [28]. In this case, an encapsulated chemical message was released from the vesicle through the activity of a pore protein. Expression of the pore protein α-hemolysin was with the transcription–translation machinery provided by the PURE system [29]. Alternatively, if highly permeable compartments are used, then larger chemical messages can be sent. Since proteinosomes are typically highly permeable, proteinosomes were used to build artificial cells that could communicate via the release and uptake of DNA [30]. In this way, Boolean logic gates could be assembled using strand displacement. More recently, protein release was used to send messages to other artificial cells by the Devaraj group. Here, the artificial cells consisted of a clay containing hydrogel nucleus and a highly permeable, polymerized acrylate membrane [31]. Protein signals could then be released after transcription and translation with an *Escherichia coli* extract in the hydrogel nucleus. It should be noted that although not discussed here, chemical communication between nanoparticles has also been demonstrated [32,33].

Communication can be mediated through direct physical contact between artificial cells as opposed to the release of chemical signals to the surrounding environment. An elegant demonstration of such a system was developed by the Mann group, which built an artificial predatory system between coacervates and proteinosomes [34]. Interaction between the oppositely charged coacervates and proteinosomes led to the engulfment

of the proteinosome (prey) by the protease-loaded coarcervates (predator). Furthermore, engulfment led to the acquisition of material (e.g. DNA) carried by the proteinosome. Similar fusion events can be engineered with lipid vesicles. For example, SNARE-mediated fusion between vesicles has been used to regulate genetically encoded networks that would not have been operational if implemented together from the beginning [35]. But direct physical interactions do not require fusion events. Instead, networks of connected artificial cells can be used to generate tissue-like materials. Water-in-oil droplets can be arranged into tissue-like structures through base-pairing of surface-exposed DNA [36] or 3D printing [37]. The latter has been used to build remarkable structures that can exploit electrochemical gradients for communication [38]. These tissue-like structures of the Bayley group exploit lipid bilayers that are formed by the lipid monolayers of two touching water-in-oil droplets to reconstitute α-hemolysin. The droplet-based system can morphologically respond to osmotic gradients [37] and can be encased in hydrogels to allow for persistence in aqueous solution [39]. Similar structures can also be built with proteinosomes. Cross-linked proteinosomes display thermoresponsive, reversible contractile properties and are capable of mechanochemical transduction [40].

Intracellular communication with artificial cells

As noted above, intracellular communication is needed to manage the co-ordinated activities of an artificial cell. Work toward incorporating intracellular communication into artificial cells has relied on various forms of spatial partitioning, including the synthesis of mimics of organelles. The most straightforward way of building organelle-like compartments is to simply encapsulate vesicles inside of vesicles (often referred to as vesosomes). In this way, different reactions can be localized to different regions of the artificial cell [41] in a manner similar to that described above for the clay containing polymerized acrylate artificial cell [31]. Alternatively, separate agarose hydrogels can be encapsulated into a single water-in-oil droplet [42]. If mRNA is synthesized in one hydrogel and if that RNA contains a toehold switch riboregulatory sequence at the 5′-untranslated region, then diffusion to another hydrogel can trigger the synthesis of peptide through base-pairing interactions. Even more complex arrangements of compartments are possible with optical tweezers [43].

Liquid organelles can be generated within compartments similar to that seen with P granules, nuclear bodies, the nucleolus, and stress granules. Although the separation of transcription and translation has yet to be demonstrated with such systems, preferential partitioning of RNA [44,45] and protein [46], including proteins involved in cell division [47], has been observed. Additionally, both transcription and translation are compatible with aqueous phase-separated systems [21]. Similar strategies could be used to exploit encapsulated polymersomes [48,49], and the Huck group has assembled transcriptionally active coacervates within phospholipid vesicles [50]. A different approach was used by the Ces laboratory. Instead of synthesizing an organelle, a living bacterial cell was encapsulated inside a lipid vesicle to aid in the synthesis of a desired product [51]. Here, the artificial cell protected *E. coli* from a potentially toxic environment and the bacterium in turn provided a useful function, such as the production of glucose from lactose or as a biosensor [52].

Chemical communication between artificial and living cells

As the example above demonstrates, living cells and artificial cells can co-operate. The first example of an artificial cell influencing the behavior of a natural cell was with artificial cells that contained the formose reaction [53]. The synthesized sugar molecules functioned as a quorum signal, inducing a luminescent response from *Vibrio harveyi*. Artificial cells with genetically encoded functionality were subsequently built by us that could both sense the environment and in response synthesize and release a chemical signal to bacteria [54]. Since the bacterium could not sense the analyte detected by the artificial cell, the artificial cell essentially expanded the sensing capability of the bacterium without genetic intervention. Stano and colleagues [55] embedded similar artificial cells within agarose to protect against attack from bacteria, and the Tan laboratory built artificial cells that could kill bacteria [56]. More recent advances have shown that artificial cells can be made to synthesize proteins that kill cancer cells [57].

Thus far, most artificial cells that engage in chemical communication do so without a clear sensing mechanism. The Simmel group, however, did produce water-in-oil droplets that could sense *E. coli* engineered to secrete the quorum molecules of *Vibrio fischeri* by reconstituting the response-regulator of the *lux* operon [58]. The same laboratory also separately built water-in-oil droplets that could send chemical signals to the bacterium. Therefore, it was clear that sensing and sending pathways could be put together to generate artificial cells that could engage in two-way chemical communication with bacteria. Such a feat was then accomplished with artificial cells housed within phospholipid vesicles that could both sense and send chemical messages directly to *V. fischeri* [59].

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The authors used the described system to implement a type of cellular Turing test to objectively quantify progress in the synthesis of artificial cells. Furthermore, artificial cells could be built to interfere with the quorum signaling of *Pseudomonas aeruginosa* in response to a biologically released chemical signal [59].

Growth and division

Progress in building artificial cells that engage in chemical communication is important, but what has been built thus far is far from a realistic mimic of a living cell. To build a better mimic, the artificial cell should be capable of replicating its own genome, synthesizing its own transcription–translation machinery, possess the ability to grow and divide, and avoid thermodynamic equilibrium. There has been significant progress in reconstituting many of these processes *in vitro*; however, these studies often exploit components from different organisms. Additionally, studies often rely on purified proteins rather than *in vitro* expressed molecular components from synthetic DNA. One of the more impressive examples of such a biochemical reconstitution was with the purified components of the translation machinery of *E. coli* (i.e. the PURE system) by Ueda and colleagues [29]. Despite the enormous complexity of translation, advances have even been made in synthesizing the parts of the ribosome *in vitro* with the PURE system [60–62]. Transcription is much simpler, as both viral and bacterial RNA polymerases can be expressed in active form from DNA *in vitro* and inside of vesicles [63–65].

Although there are now several viral and bacterial DNA replication systems that have been reconstituted *in vitro*, few have been integrated with *in vitro* transcription–translation machinery or placed inside of vesicles. *Escherichia coli* bacteriophages Φ29 [66–68], T7 [69], and T4 [70] are notable because these bacteriophages replicate double-strand DNA, require few molecular components, and do not require host proteins. Impressively, the Danelon group reconstituted active Φ29 replication machinery by transcription–translation inside of phospholipid vesicles [71]. Other, more complex viral systems that require host components have been reconstituted as well by exploiting purified proteins, e.g. SV40 [72,73], or cellular extracts, e.g. adenovirus Ad5 [74,75]. One of the first viral replication systems to be reconstituted was also used for the very first *in vitro* selection/evolution experiment. The Qβ bacteriophage only needs Qβ RNA polymerase plus two elongation factors to copy the Qβ RNA genome *in vitro* [76,77]. The Yomo group has elegantly reconstituted this Qβ system inside of phospholipid vesicles with the PURE system to investigate the evolution of cellular mimics [78–81].

There have been multiple successes in reconstituting non-viral systems that replicate DNA. The bacterial replisomes of *Bacillus subtilis* [82] and *E. coli* [83] have been reconstituted with 13 and 14 purified proteins, respectively. Similarly, plasmids can be isothermally replicated *in vitro* [84–86]. Impressively, eukaryotic replication has been reconstituted with purified proteins. *Saccharomyces cerevisiae* DNA replication of naked [87] and chromatin DNA templates [88,89] have been recently reported with 24 purified proteins. However, termination of DNA replication was inefficient, indicating that additional factors apart from the 24 purified proteins used in the study are required for efficient termination. Korhonen et al. [90] have also reported the *in vitro* reconstitution of the mammalian mitochondrial replisome.

Cytokinesis is the part of the cell division process during which the cytoplasm of a single cell divides into two daughter cells. Cytoplasmic division begins after the late stages of nuclear division in mitosis and meiosis. During this phase, a complex of several proteins, called the divisome, is assembled. In bacteria, this process is organized by the min system, a network of proteins that control where the divisome is assembled. The Min proteins oscillate from pole to pole ensuring that FtsZ polymerizes into a constricting ring (i.e. the Z ring) at the division plane [91]. Although a robust vesicle division process has not been demonstrated with an Fts-Min system yet *in vitro*, FtsZ does assemble into Z-rings inside of vesicles [92,93] and the oscillatory behavior of MinD and MinE have been reconstituted [94,95].

Division in the absence of growth is not sustainable. An obvious approach to achieve the growth of the compartment would be to reconstitute lipid synthesis. In that way, newly synthesized lipid would naturally partition to the membrane and thus give rise to growth. One complication is that the enzymes that participate in the synthesis of biological phospholipids are membrane-bound enzymes. Membrane proteins are typically more difficult to reconstitute than soluble proteins. Nevertheless, eight different *E. coli* enzymes that mediate acyl transfer and headgroup modification reactions were produced in a cell-free gene expression system in the presence of vesicles [96]. Conversely, the Devaraj group opted for an engineered system that depended on the enzymatic synthesis of fatty acyl adenylates that then reacted chemoselectively with amine-functionalized lysolipids to form phospholipids [97].

Metabolic pathways

Living cells are open systems that use controlled and co-ordinated chemical reaction networks to couple the thermodynamically favorable oxidation of chemical feedstock molecules (or the harnessing of photons from sunlight) to perform active biochemical work. That is, the energy released from catabolic metabolism is used to maintain the existence of the cell through anabolic reactions [98]. The exploitation of metabolic pathways present in cell-free extracts to either decipher catabolic pathways or to generate useful end products are well established [99]. Similarly, strategies to maintain sufficient concentrations of ATP over time in order to extend protein synthesis in cell extracts are commonly employed. Frequently, such strategies recycle inorganic phosphate, an inhibitor of protein synthesis, through the synthesis of ATP via glycolysis or oxidative phosphorylation [100–105]. More controlled systems have been developed with encapsulated purified proteins. For example, a photosynthetic artificial cell that mimicked chloroplast was built with purified proteins. Two light-harvesting complexes plus ATP synthase were reconstituted within lipid vesicles so that the energy from light could be used to synthesize ATP [106]. Impressively, a similar system was built with polymerosomes [107]. However, these artificial cells built with purified metabolic enzymes are incapable of producing their own metabolic machinery. That is one reason why the work by the Kuruma group is important. Berhanu et al. [108] exploited artificial cells with bacteriorhodopsin and ATP synthase that was able to use the energy captured from light to drive the transcription and translation of more bacteriorhodopsin and the F_o subunit of ATP synthase. It should be noted that advances in the engineering of anabolic carbon fixation *in vitro* have also been described [109,110].

Although several enzymes and metabolic pathways have been reconstituted *in vitro* [111] and in vesicles such work did not have to deal with the complexity of the synthesis of the metallocofactors needed for function. This is because the purified proteins already contained the necessary metallocofactors. For simple cofactors, *in vitro* gene expression in the presence of the needed metal ions may lead to the spontaneous generation of the holo state. For example, the addition of iron ions and sodium sulfide to purified [112] and cell-free expressed [113] apo ferredoxin leads to the generation of an iron–sulfur cluster co-ordinated to the protein. More complex metallocofactors would likely need dedicated metallochaperone proteins to sequester, shuttle, and synthesize the desired metallocofactor [114]. For instance, the biosynthesis of the iron and molybdenum cofactor (FeMo-co) of nitrogenase has been reconstituted *in vitro* with purified proteins [115]. More impressively, the synthesis of the H-cluster of [FeFe] hydrogenase has been achieved with cell-free expression [114].

While the reconstitution of extant metabolic pathways that invariably consist of large, complex proteins with inorganic cofactors is impressive, the pathways have not been frequently used *in vitro* in a manner that drives thermodynamically unfavorable chemistry. Instead, there are now several examples of chemically dissipative systems constructed with non-biological molecules. Thus far, such systems typically exploit phase separation to regulate the reactivity of reactants and products. For example, the Fletcher group developed an out-of-equilibrium, self-replicator from a network that both produced and degraded a surfactant molecule [116]. The self-assembly of the surfactant into micelles led to more extensive mixing of the reactants and thus increased the production of micelles. The micelles only persisted in the presence of a fuel source. Similarly, the Boekhoven group showed that liquid phase separation provides a mechanism of selection for non-equilibrium energy dissipating molecular assemblies [117]. The chemical reaction network was composed of a small library of linear carboxylic acids that were condensed into anhydride products with carbodiimides. In water, the anhydrides were hydrolyzed into the carboxylic acid precursors. However, longer, more hydrophobic anhydrides phase separated into oil droplets that provided some protection against hydrolysis.

Of course, building a biological-like cell requires that the chemically dissipative system be composed of biological molecules. van Hest and colleagues [118], for example, have built polymersomes with an encapsulated metabolic reaction network composed of six enzymes capable of converting glucose and phosphoenolpyruvate into molecular oxygen. Importantly, this metabolic system was able to convert chemical energy into movement, since the release of oxygen was capable of propelling the compartment. Although the metabolism was not tied to something useful for the cellular mimic, Beneyton et al. [119] constructed water-in-oil droplets that contained enzymes that produced NADH and bacterially derived inverted vesicles with embedded, native NADH dehydrogenases. That is, NADH was continuously generated and consumed as long as the feedstock (glucose-6-phosphate) was present.

The harnessing of biological and abiological systems that mimic some aspects of cellular life has gone from simple mimicry to attempts at developing technologies that influence the behavior of bacterial [17,18,54,59,120,121]

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and eukaryotic cells [17,121–123]. Therefore, studies focused on chemical communication not only inform our understanding of biology but also help lay the groundwork for future therapies that exploit artificial cells that can interface with and control natural cells. However, the fact that artificial cells cannot regenerate their own component parts and are not capable of sustaining activity over prolonged periods of time may hinder progress in developing some types of technologies. Nevertheless, artificial cells that are active for short periods of time would be advantageous for specific applications [54]. If building robust artificial cells that can chemically communicate is the goal, then it is necessary to begin considering how chemically dissipative systems can be integrated with chemical communication (Figure 1). Thus far, artificial cells that synthesize ATP have been built, but ATP alone is not sufficient to sustain an artificial cell [124]. Similarly, reconstituting metabolic pathways in a way that does not harness thermodynamically favorable chemistry to drive the activity of the artificial cell will not bring the field much closer to building a more convincing cellular mimic or useful biotechnology.

Metabolism is, by and large, mediated by protein enzymes, which means that life, as we know it, is completely reliant on the ribosome. The dominance of protein synthesis in the chemistry of a cell can be seen by the fact that greater than 30% of the dry mass of rapidly growing *E. coli* is attributable to the ribosome [125], and greater than 80% of the cellular pool of RNA is found within the ribosome [126]. Even organisms with genomically reduced genomes, where pressures have led to the loss of dispensable genes, still dedicate over one-third of their genetic content to processes needed for the synthesis of protein. It, therefore, is not surprising that when the activity of the translation machinery of an artificial cell degrades, so does the activity of the artificial cell itself. Cell extracts can typically synthesize protein for up to 8–10 h under batch-like conditions and greater than a day inside of permeabilized vesicles that are continuously fed with a nutrient solution [63,64]. The PURE system is more fragile and typically loses activity in under 2 h in either bulk or inside of liposomes [127–129]. If it were possible to express functional ribosomes *in vitro* [62,130], then this barrier to building long-lasting artificial cells would be removed. However, depending on the desired longevity of the system, a complete ribosomal synthesis pathway may not be necessary. If the more fragile components of the

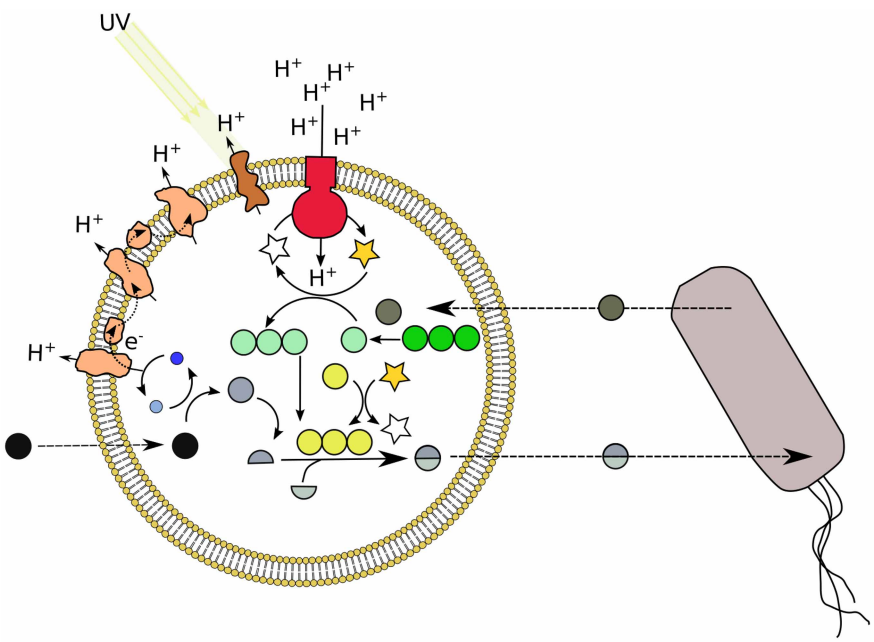

Figure 1. A long-lasting artificial cell that chemically communicates.
A schematic representation of an artificial cell capable of communicating with a natural living cell (bacterium, tilted brown oblong shape). The artificial cell (shown here with a phospholipid membrane) generates a proton gradient by exploiting UV light or by the oxidation of a feedstock molecule (black circle). The energy stored in the proton gradient is then used to drive a series of interconnected anabolic reactions through, in part, the synthesis of ATP (yellow star). Here, ATP is synthesized by ATP synthase (red). ATP is consumed during RNA (light green circles) and protein synthesis (light yellow circles). The machinery for chemical communication and protein synthesis are encoded within the DNA (green circles).

translation machinery were identified and specifically recycled, then it may become easier to increase the longevity of the artificial cells. Perhaps that would be an easier starting point that would help the field advance toward the construction of long-lasting cellular mimics.

Summary

- Communication is a fundamental feature of natural living cells. Often times, communication is accomplished through the exchange of small molecules or through direct physical contact.
- Several groups have built artificial cells that can chemically communicate with other artificial cells or with natural, living cells.
- One major limitation of artificial cells built thus far is their inability to survive for long periods of time.
- Future efforts should try to integrate self-maintenance with the ability to chemically communicate so as to build better cellular mimics.

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Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

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