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Piecing Together Cell-like Systems

Domenica Torino, Laura Martini and Sheref S. Mansy*

CIBIO, University of Trento, via delle Regole 101, 38123 Mattarello (TN), Italy

Abstract: Several laboratories are pursuing the synthesis of cellular systems from different directions, including those that begin with simple chemicals to those that exploit existing cells. The methods that begin with nonliving components tend to focus on mimicking specific features of life, such as genomic replication, protein synthesis, sensory systems, and compartment formation, growth, and division. Conversely, the more prevalent synthetic biology approaches begin with something that is already alive and seek to impart new behavior on existing cells. Here we discuss advances in building cell-like systems that mimic key features of life with defined components.

Keywords: Cell-like, minimal cell, origin of life, protocell, riboswitch, synthetic biology.

BUILDING CELLULAR SYSTEMS FROM THEIR PARTS

Building life from scratch in the laboratory is an old dream with new tools at its disposal. We can now rapidly and affordably synthesize genes [1], assemble genomes [2], evolve new function [3] and make precise changes throughout an existing genome [4]. Most of these technological advances are applied to the modification of existing cells (Fig. **1**), and so the resulting data do not directly address life's beginnings or clearly delineate the required components of cellular function. Any work that either exploits existing cells or cell lysates makes use of a complex, undefined mixture of reaction components that we do not have the tools to fully understand. Here we try to highlight steps forward in building fully defined life-like systems from a minimum number of components.

It is hoped that the process of building cell-like systems in the laboratory will give us insight into what is required to endow a system with the properties of life. There is no currently agreed upon threshold that must be crossed in order to label a chemical system living. However, as progress is made in recreating the functions of life in the laboratory, we may reach a point in which a threshold is crossed, even if it is not recognized until afterwards. Further, since the system would have been built with fully defined components, this systematic approach should give us a much better understanding of the necessary components of life. It is worth noting that this approach does not probe how the molecules of life were built, nor does it directly address the historical path taken between the prebiotic chemistry of Earth [5-11] and the emergence of protocellular structures [12-14].

The central dogma [15] of molecular biology offers one perspective on what is needed to build a cell. Typically, information stored in a replicating DNA genome flows through RNA and then finally to proteins. However, this simple description of cellular life is in fact not very simple, which is born out by analyses of microorganisms. *E. coli*, for example, has well over 4000 genes, and purposeful genetic reductions only reduces the genetic content by 15% [16]. On the smaller end of the spectrum, *Mycoplasma genitalium* has 482 genes, and genetic knockouts have shown that approximately 100 of these genes are not required under laboratory conditions [17]. But even with this small genome, which is small enough to be synthesized [2] approximately one third of the genes provide unknown function to the cell. We have reached a point where our technological ability to synthesize genomes has outpaced our understanding of what we are synthesizing.

By comparing sequences of disparate microorganisms, Moya and others suggest that a minimal cell would contain on the order of 200 genes, of which more than half would be necessary for protein synthesis [18]. Interestingly, a natural symbiotic microorganism, *Carsonella ruddii*, has only 182 genes, a value similar to theoretical predictions of a minimum gene set [19]. Further, over half of the *C. ruddii* genome is dedicated to protein synthesis. In some ways, the impression is given that living systems are nothing more than just a bag of protein synthesizing machinery. Clearly life is more than just protein synthesis, but at least as far back as the last universal common ancestor, protein synthesis has been a crucial aspect of cellular function [20].

One conception of a simplified, laboratory-made cell consists of a vesicle compartment that contains a replicating DNA genome and transcription-translation machinery that responds to changing environmental conditions (Fig. **2**). Much of the needed functions for such a cell-like system appears to depend on protein function. Nevertheless, origins of life research has shown that under specific chemical conditions, several features of life emerge without the participation of proteins. Perhaps future approaches that combine the lessons learned from origins research with those gained from attempts to exploit biological machinery will allow for the synthesis of a simplified cell.

COMPARTMENT TYPES

Compartmentalization is considered to be one of the key steps along the transition from simple chemistry to cellular life [21]. The enclosure of a chemical system within a semipermeable membrane causes several useful features to emerge. For example, encapsulation facilitates evolutionary processes [22, 23, 24], provides for an energy storage mechanism [21-25] and likely influenced accessible chemistry. Although it is possible that prebiotic boundary structures were defined by substances other than lipids, no living systems to date have been identified that are capable of surviving without lipid membranes. Further, several lines of evidence argue for the presence of lipids on prebiotic Earth, including simulated prebiotic syntheses of lipids [5-11] and the identification of lipid

*Address correspondence to this author at the CIBIO, University of Trento, via delle Regole 101, 38123 Mattarello (TN), Italy; Tel: +39 0461-883438; Fax: +39 0461-883937; E-mail: mansy@science.unitn.it

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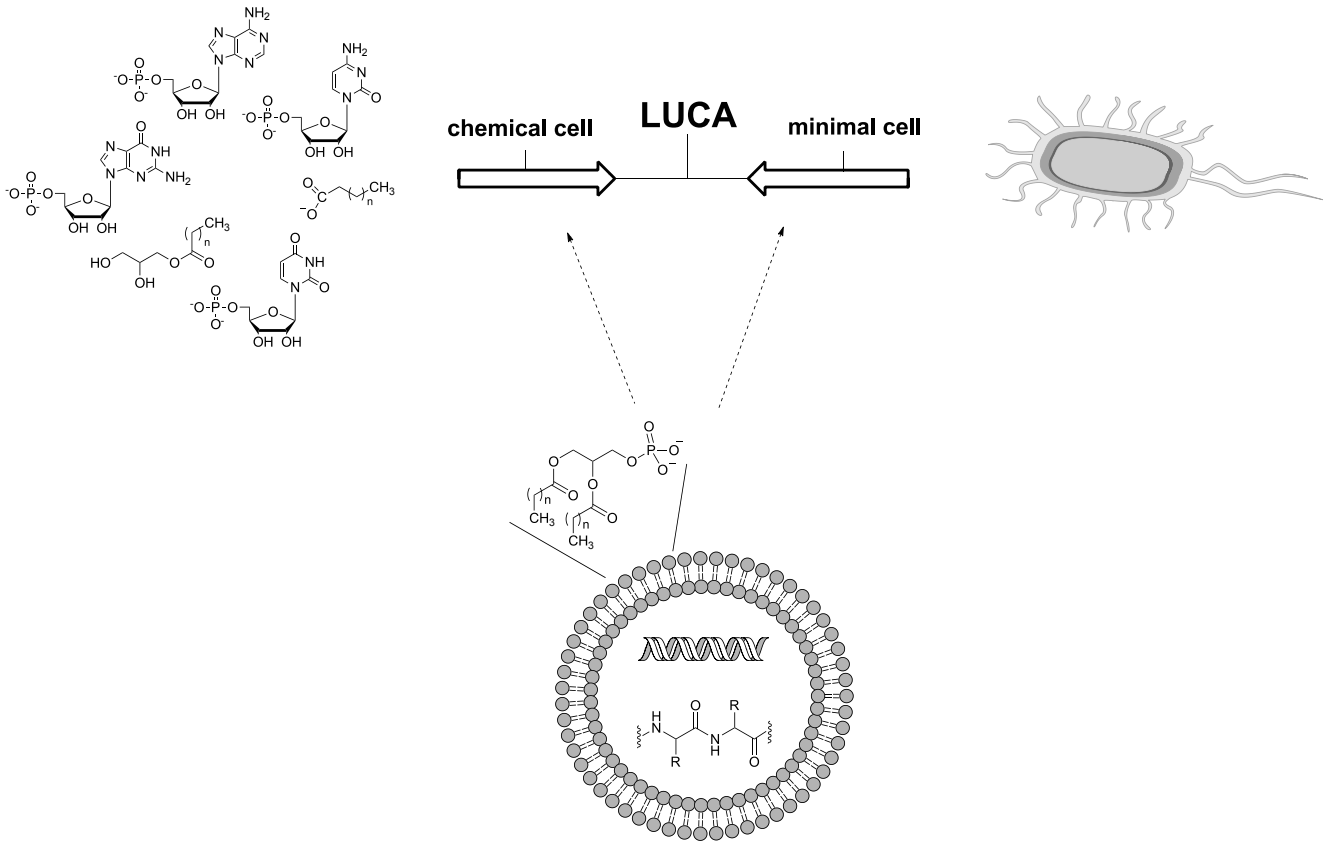

Fig. (1). Different approaches to building new, artificial cells. Typically, laboratories either begin with chemicals (left) or an existing cell (right). Presumably an approximation of the last universal common ancestor (LUCA) exists in between these two extremes. A third approach is to piece together cellular systems from existing biological components (bottom).

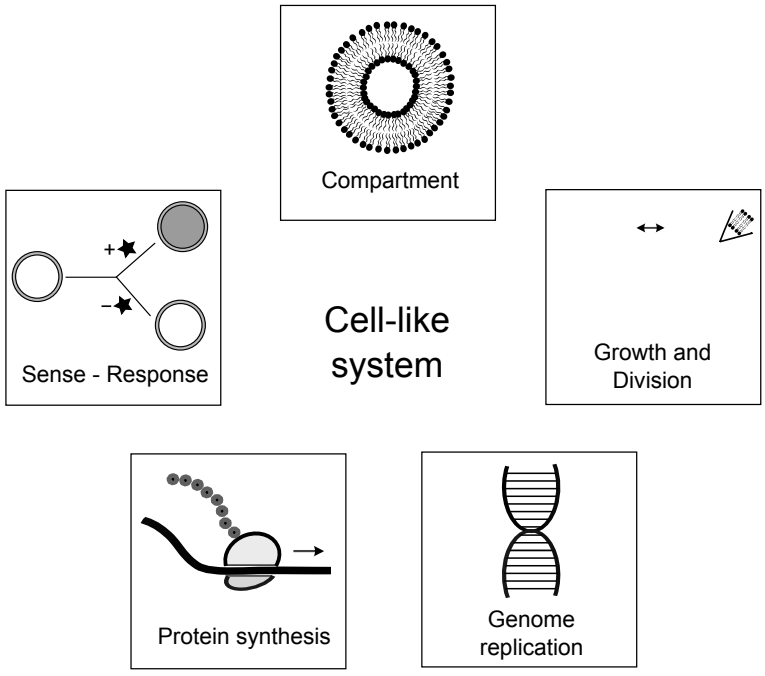

Fig. (2). Features of cellular life that are mimicked by cell-like systems.

molecules within carbonaceous meteorites [8]. Finally, vesicles form easily in aqueous solution, thus suggesting that there were vesicles on Earth even before there was life. The latter point has led some to suggest that there once existed a lipid world in which hereditary was mediated by lipid composition rather than by specific nucleic acid sequences [26].

Prebiotically plausible lipids are generally thought to be saturated, single-chained amphiphiles. In the laboratory, fatty acids and fatty acid derivatives are often used as an approximation of what could have been present. Not only do such lipids form vesicles, they also exhibit many useful features not dependent upon protein function, including the ability to grow and divide, uptake nutrients, and retain macromolecules [23,25,27-34]. The main disadvantages of fatty acid based vesicles is the encountered difficulty in recovering encapsulated enzymatic activity from some enzymes and the vesicle's decreased stability in comparison with phospholipid vesicles. For example, fatty acid vesicles are stable over a narrow pH and salinity range [30,35,36] and difficulty has been encountered in reconstituting DNA polymerase activity within fatty acid vesicles [32-37]. Therefore, fatty acid vesicles are typically used for protocellular research rather than for attempts to build cell-like systems similar to life as we know it.

Contemporary cells exploit membranes of complex composition including monoacyl and diacyl lipids and proteins. Laboratory constructions tend to ignore this complexity and instead rely on the ease in which many lipids alone form vesicles. Of the commonly used vesicle systems, those built with diacylphospholipids are the most robust. However, this robustness comes at a cost. Diacylphospholipids are generally impermeable, thereby posing a difficulty in their use for building cell-like structures. One approach to overcome this limitation is to exploit membrane proteins, such as the bacterial toxin α-hemolysin. This protein expresses as a soluble monomer that then spontaneously oligomerizes into a pore in the presence of a membrane [38]. An alternative approach is to utilize the phase transition temperature of lipid membranes to create packing defects that can be exploited for permeation [39]. Both the α-hemolysin and packing defect mechanisms have been used to feed in substrates for encapsulated RNA [40] and protein synthesis reactions [38]. Interestingly, symbiotic organisms with small genomes, such as *C. ruddii* and *Buchner aphidicola*, are thought to be heavily dependent on passive diffusion mechanisms for nutrient uptake [41]. In short, phospholipid membranes need not be viewed as an impenetrable barrier. Simple passive diffusion based mechanisms exist for nutrient uptake and waste release that may not be much different than what is used by some microorganisms with small genomes.

There is another approach to building compartments with dimensions similar to extant cells. Water-in-oil (w/o) emulsions are easy to make and are extremely efficient in encapsulating hydrophilic molecules. They have been used extensively for molecular evolution experiments [42, 43] and further developments in the technology allow for the delivery of reactants directly to the water droplets without breaking the emulsion [44]. However, w/o emulsions have not proved useful for constructing cell-like systems due the lack of solute exchange across the water-oil interface.

COMPARTMENT GROWTH & DIVISION

A compartment must grow and divide to allow for the cell-like system to replicate. For fatty acid vesicle systems, growth and division can be accomplished simply by adding lipids to preexisting vesicles [27-29,33,34]. Similar mechanisms for phospholipids are not available because of the decreased dynamics of diacyl phospholipids [45]. However, if phospholipid synthesis reactions were reconstituted within vesicles, then once the lipids were produced they would naturally partition into the membrane resulting in vesicle growth. The difficulty with reconstituting such enzymatic activities is that many of the lipid synthesis enzymes are membrane proteins. Nevertheless, Luisi and colleagues have built an encapsulated enzyme system that can produce diacyl glycerophospholipids when provided with fatty acids and glycerol [46]. Although this is an important development, more work is needed to move beyond the use of simple lipids (fatty acids) to build more complex lipids (phospholipids). Also, additional enzymes may be required to flip a fraction of the newly synthesized lipids from the inner- to the outer-leaflet.

Phospholipid vesicles can be coerced into dividing through simple chemical-physical forces. For example, Baumgart *et al.* found that phospholipid vesicles containing liquid ordered (L_o) and liquid disordered (L_d) domains can bud and divide when placed under osmotic stress [47]. However, since the daughter vesicles do not retain the same membrane lipid composition as the parental vesicle, further rounds of division are not possible. Presumably if an additional mechanism were incorporated to restore the original membrane composition then further rounds of division could occur. More recently, Andes-Koback and Keating revealed that osmotic gradients can induce budding and division of phospholipid vesicles that contain an aqueous two-phase system, one enriched in dextran and the other aqueous phase enriched in polyethyleneglycol [11]. Since the resulting daughter vesicles retain the composition of the parental vesicle, they are also capable of dividing. If the Andes-Koback and Keating mechanism were combined with a vesicle growth system, then a growth – division cycle could be built. The system in its current state, however, does require external intervention to adjust the osmolality of the extravesicular space. It is interesting to note that some biological evidence exists suggesting that life can persist in the absence of protein mediated division [48, 49].

Protein based systems have also shown promise in dividing vesicles. Although *in vivo* cell division mechanisms are complex [50], recent studies have shown that parts of the system can be reconstituted *in vitro* [51, 52]. For example, FtsZ is a highly conserved division protein that polymerizes into a ring that interacts with other members of the divisome to split the cell into two. Even if FtsZ is not thought to directly interact with the inner membrane in natural systems, FtsZ constructs can be engineered to anchor into the membrane of synthetic vesicles by the addition of a small helix to the C-terminus of FtsZ [52]. Further, in the presence of GTP this engineered version of FtsZ forms constricting rings that cause visible indentations within the membranes of tubular vesicles [52]. While these vesicles do not divide, the data suggest that a proper mix of Fts proteins could be sufficient to reconstitute functional cell division machinery within vesicles.

REPLICATING GENOMES

There are many ways to copy nucleic acids *in vitro*, but none are currently amenable to the construction of cell-like systems. The use of an RNA genome is attractive for at least two reasons. First, RNA genomes appear to simplify the system by removing the need of a class of molecules, i.e. DNA. Second, RNA polymerases do not require a primer. Although there are no known cells that use an RNA genome, there are viral systems that could be exploited. For example, the bacteriophage phi6 uses a double stranded RNA genome that is fully replicated by a single RNA polymerase [53]. This may seem like a simple system to reconstitute in the laboratory for building cell-like systems, but significant complications may arise when protein synthesis machinery is incorporated. Ribosomes require single stranded RNA as a template. This means that in addition to the double stranded RNA genome, single stranded RNA also

must be present for protein synthesis. In other words, transcription of the genome, even an RNA genome, is always required. Although there are several classes of RNA viral systems with different genome organizations, e.g. viruses that use a circular RNA genome, they all share these same difficulties. An exciting alternative RNA system would exploit an RNA replicase, i.e. an RNA enzyme that copies an RNA genome. Despite the impressive advancements made in building better RNA replicases [54] much more progress is needed before they serve as a practical alternative.

The use of a DNA genome has the advantage of better separating replication and transcription, thus avoiding competition between the two processes [55]. Additionally, methods to replicate DNA *in vitro* have existed for several decades. Some attempts have been made to exploit these technologies for building cell-like systems. PCR based mechanisms for DNA copying inside of phospholipid vesicles have been demonstrated [56]. While useful as a first step, this approach is not practical, because it requires manipulation that is not regulated by the cell-like system (i.e. thermocycling), and it requires the addition of oligonucleotide primers. Another approach uses a more complex mixture of proteins, including a helicase and single strand binding proteins, in addition to a DNA polymerase to replicate DNA inside of vesicles. This system overcomes the thermocycling limitation of PCR, but still requires the addition of oligonucleotides, and it is only capable of replicating short (<100 bp) strands of DNA [37]. An extension of this technique would exploit a primase [57] to remove the need of adding oligonucleotide primers, but this would also add a significant problem. Genomic replication must be complete, which means that there must be a mechanism to ensure that there is no loss of the genomic termini, i.e. telomeres. Since primases add RNA primers that must later be removed, the simplified systems described so far are incapable of fully replicating a genome end-to-end.

The telomere problem is perhaps the biggest challenge to constructing a simple genomic replication mechanism. Viral DNA replication systems could potentially give a simpler solution to this problem than the isothermal bacterial systems described above. For example, the *Bacillus subtilis* bacteriophage phi29 uses only four proteins to replicate its entire genome, including a highly processive DNA polymerase that possesses strong strand displacement activity, a single strand DNA binding protein, a double strand DNA binding protein, and an additional protein that is required to initiate replication (terminal protein) [58]. The natural phi29 genome is linear, contains covalently attached terminal protein at the 5'-termini, and is replicated fully via a protein priming mechanism. Despite the peculiarities of the phi29 system, Salas and colleagues recently demonstrated that DNA can be fully replicated *in vitro* with this four protein component system if the template DNA contains appropriate nucleotide sequences at both termini to define the origins of replication [59]. Thus far the phi29 genomic replication system is the simplest and best characterized isothermal system available.

PROTEIN SYNTHESIS

Much of what has been described is dependent upon the activity of proteins. Cell-like systems that require protein function, therefore, require transcription and translation machinery. Transcription is a very straightforward process to reconstitute *in vitro*, particularly if the commonly used bacteriophage RNA polymerases are exploited. T7 and SP6 RNA polymerases consist of a single protein domain, do not require accessory factors for function, and provide robust activity. Conversely, the synthesis of proteins is a highly complex process requiring over 100 genome encoded components [60]. Nevertheless, this complicated process is understood well enough to be reconstituted *in vitro* from purified, defined components [61]. Moreover, several laboratories have shown that this minimal transcription-translation system functions in water-in-oil emulsion droplets [62] and in vesicles [63, 64] despite the statistical difficulty associated with encapsulating multiple components within a single compartment [65, 66].

ADAPTING TO CHANGING ENVIRONMENTAL CONDITIONS

Not every process within a cell needs to be monitored and coordinated with other cellular functions. For example, mitochondrial genome replication is not thought to be coordinated with division. Nevertheless, no living system simply repeats the same functions over and over again without regard to its surroundings. Cells must constantly adapt to changing intracellular and extracellular conditions. This is largely due to the fact that life and the environment are intimately linked. Life both feeds off of and shapes the environment. Conversely, the environment dictates which living things can and cannot survive. Therefore, if a cell is to survive for an appreciable length of time, the cell must be able to continually adapt to changing environmental conditions, some of which are induced by the cell's own existence.

To adapt to changing environmental conditions, a cell needs to be capable of sensing and responding to stimuli. A straightforward solution to the problem would be to encode a sense-response system that exploits environmentally responsive transcription factors. For example, proteins such as FNR, IscR, and CooA control gene expression in response to oxygen, iron, and carbon monoxide levels, respectively [67,68]. Alternatively, bacterial two component systems [69] could be similarly used to alter gene expression profiles in response to environmental changes. Here a sensor-kinase would control the phosphorylation state and thus the activity of a response-regulator protein, which would then result in changes in gene expression (Fig. **3**). Further layers of control could be built into sensory pathways by exploiting the sigma factors of bacterial polymerases. While bacterial RNA polymerases are more complicated than T7 or SP6 bacteriophage RNA polymerases, their use of initiation factors to guide promoter recognition provide for an opportunity to build in additional control elements. Although thoroughly studied *in vivo*, few attempts have been made to reconstitute any of these protein sensory systems *in vitro* [70].

A disadvantage of relying on existing proteins for sensing capabilities is that the use of existing proteins severely limits the types of cell-like systems achievable. In other words, we are forced to build cell-like systems that sense what natural cells are already capable of sensing if we use existing proteins. Although some examples of building new proteins with desired activities have been reported [71, 72], the present methods are not yet sufficiently developed to be widely applicable. Therefore, other sensing mechanisms that are easier to engineer are desirable.

Natural and synthetic RNA sensors that control protein production exist and are an attractive alternative to protein based sensing mechanisms. One class of RNA sensors is known as riboswitches and exist in the 5'-untranslated regions of bacterial genes. Riboswitches either turn on or off transcription or translation in response to ligand binding directly to the mRNA [73] (Fig. **3**). The distinct advantage of RNA sensors over protein sensors is that we have years of RNA selection and engineering technologies [74] to build on, and so it is possible to build synthetic riboswitches capa-

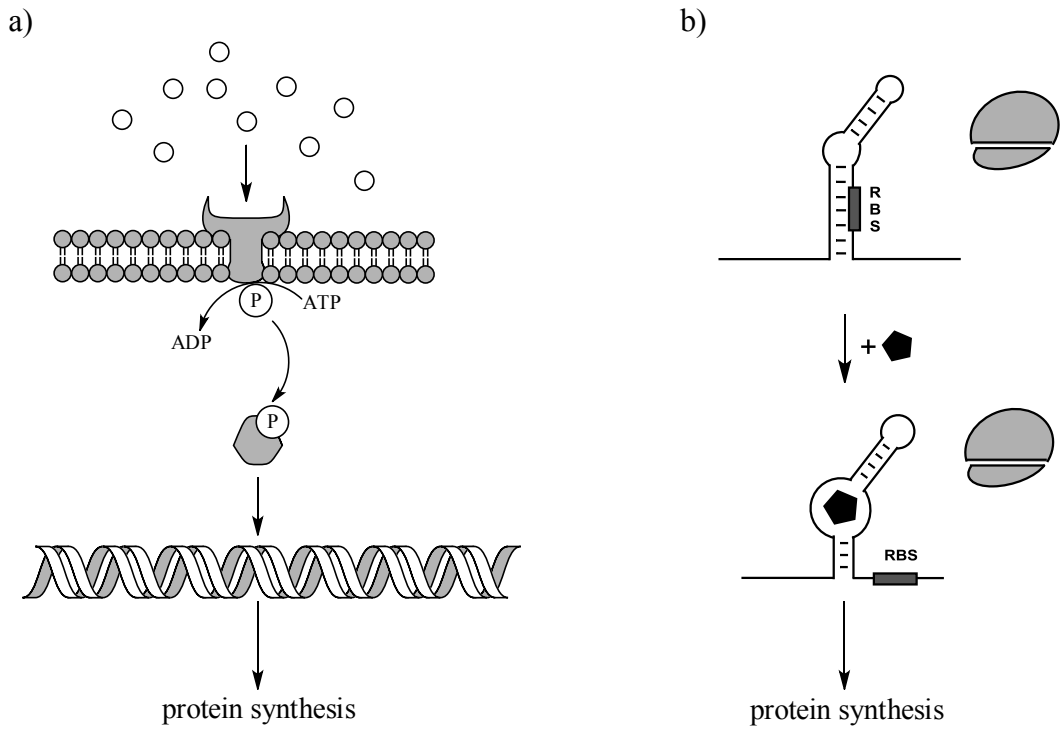

Fig. (3). Simple biological sensors could be built from protein or RNA components. (a) A two component protein system consisting of a sensor-kinase and a response regulator. In this example, a membrane bound sensor-kinase phosphorylates the response regulator only in the presence of ligand. The phosphorylated response regulator then activates gene expression. (b) An example of one type of riboswitch system that directly controls protein production in response to the presence or absence of ligand. Upon the binding of ligand, the ribosome binding site (RBS) is unmasked, thereby allowing for ribosome binding and thus protein synthesis. Both systems shown here illustrate an "on" switch; however, analogous "off" switches also exist.

ble of sensing and responding to molecules beyond those currently sensed by natural cells [75].

The majority of the riboswitch research has focused on measuring sensing activity inside of existing cells, as is the case for the protein based systems described above. However, recent work has shown that a previously selected theophylline riboswitch [76, 77] was capable of turning on gene expression *in vitro*, in water-in-oil emulsions, and in vesicles [78]. Further, this cell-like system could be built to sense molecules outside of the vesicle compartment, thereby demonstrating that artificial RNA sensors could serve as a foundation for the construction of cellular mimics that go beyond what natural biology gives us.

LIMITATIONS

Many challenges still remain. The progress made in mimicking some of the features of life is tempered by the fact that the majority of the experiments have been performed under different conditions. Therefore, it is unclear whether the integration of different cell-like functions in their present form into a single system is possible. There are also fundamental problems associated with the use of purified translation machinery. Although reconstituted transcription-translation systems are able to drive cascading, genetically encoded networks [61,79], none of the described systems are capable of supporting long-term activity. We are currently able to engineer *in vitro* systems that turn gene expression on and then off [38,80,81]. However, we are not able to begin another cycle, because we are not able to regenerate the translation machinery *in vitro*. Currently, the minimal translation system is composed of proteins and RNAs that are isolated from *E. coli*. Until we are able to produce functioning ribosomes from *in vitro* transcription-translation reactions, there will be severe limits on what types of cell-like systems can be manufactured in the laboratory.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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