Experimental perspectives for a chemical communication between synthetic and natural cells

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Abstract. The recent advancements in semi-synthetic minimal cell (SSMC) technology paves the way to several interesting scenarios that span from basic scientific advancements to applications in biotechnology. In this short article we discuss the relevance of establishing chemical communication between synthetic and natural cells as an important conceptual question, and then discuss it as a new bio/chem-information & communication technology (bio/chem-ICT). At this aim, the state-of-the-art of SSMCs technology is shortly reviewed, and a possible experimental approach based on bacteria quorum sensing (QS) mechanisms is proposed and discussed.

Keywords. Synthetic cells, liposome, bio/chem-ICT, quorum sensing, synthetic biology.

1 Chemical communication as a bio/chem-ICT

The most fascinating novelties in modern science consist in using biological paradigms for developing new technologies. One of the most interesting one is the bio/chem-information and communication technology (bio/chem-ICT), which aims at extending the well-known field of ICT, classically based on the transmission of electrical or electromagnetic signals, to the bio/chemical world of molecules.

Recently, Tatsuya Suda and collaborators have highlighted the role of molecular communication in ICT. In their most recent review [1] – which is a good starting point for the discussion we would like to do in this article – these authors describe how biological communication works thanks to chemical signals and provide a rational description of this molecular communication in engineering terms. They suggest that man-made molecular systems, if endowed with proper interfaces, could communicate with living biological systems and discuss the potential impact of developing a new bio/chem-ICT paradigm on the medical field (drug targeting).

Bio/chem-ICT is still in its infancy, but we can already outline here one of the most important goals: the creation of synthetic (artificial) cells that are able to communicate – via molecular mechanisms – with natural cells (Figure 1a). In this scenario, synthetic cells – constructed by assembling separated components (by the so called

"bottom-up" approach) – will be used as a kind of intelligent, *wet-soft robots* capable of communicating with natural cells. As a consequence, the synthetic cells might activate responses in agreement with the "meaning" they perceive by interacting with natural cells (the connection of these arguments with the "bio-semiotic" concepts is a related fascinating facet). As already highlighted by Suda and coworkers, this goal would represent a true way for interfacing synthetic (and possibly programmable) systems to natural ones.

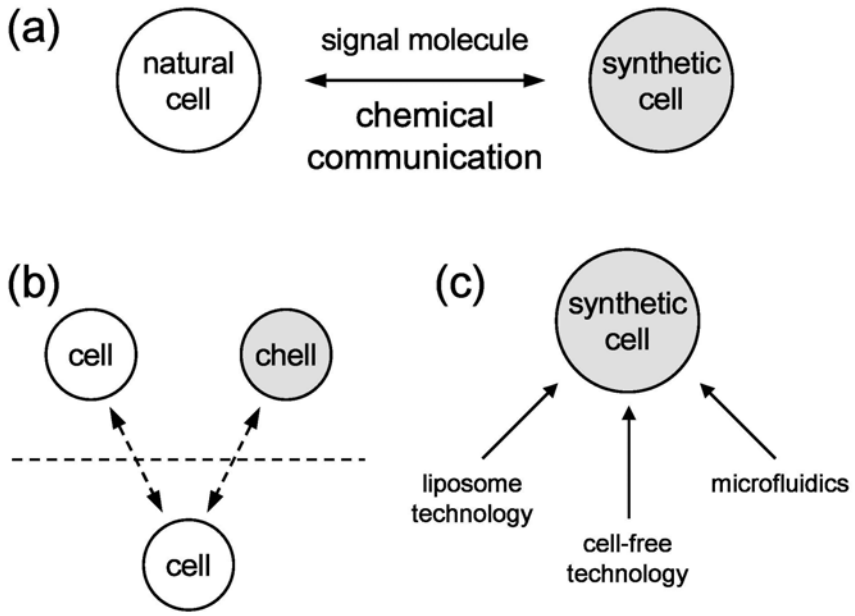

Figure 1. (*a*) Chemical communication between natural and synthetic cells; (*b*) the Turing-like test for chemical cells (*chells*), see the text; (*c*) semi-synthetic minimal cell technology.

With these premises, the questions become: is it possible, according to the current technology, to imagine a research program aimed at developing a chemical communication between synthetic and natural cells? What would be its impact in basic science and ICTs?

In this article, we will try to answer these questions by firstly illustrating the current state-of-the-art on the construction of synthetic cells, and at this aim we will discuss the most successful approach, called *semi-synthetic* [2] (see details below). Then we will examine some possible experimental approaches that derive from combining the semi-synthetic minimal cell (SSMC) technology with the mechanisms of bacterial communications, and in particular those involved in the quorum sensing (QS).

Before turning into technical discussion, however, let us summarize, in the next paragraph, a few remarks on the conceptual interests toward the communication be-

tween synthetic and natural cells. Note also that a more extensive version of this article has been recently published [3].

2 Life and communication

2.1 The autopoietic perspective

The theory of *autopoiesis* (self-production), developed in the 1970s by Humberto Maturana e Francisco Varela [4], deals with the most classical question of biology: "what is life?". The authors developed their theory on the basis of two hypotheses, according to which: (*a*) the distinctive property of living systems is its autopoiesis, that is, the capability of these systems of producing and maintaining their material identity through an endogenous processes of synthesis and destruction of their own components; (*b*) autopoiesis is a global property of living systems, which relies not on their physico-chemical components taken separately, but in the way in which these components are organized within the systems. On these grounds, Maturana and Varela addressed the issue of defining life as the problem of determining what kind of organization supports the biological behavior of self-production. They provided a rigorous solution at the level of the minimal cell. This solution consists in the notion of "autopoietic organization", which aims at characterizing the "fundamental" biological organization.

"[The autopoietic organization is] (…) a network of processes of production (transformation and destruction) of components that produces the components which: (i) through their interactions and transformations continuously regenerate and realize the network of processes (relations) that produced them; and (ii) constitute (…) a concrete unity in the space in which they (the components) exist by specifying the topological domain of its realization as such a network" [5].

This concept is at the basis of the synthetic biology's interest for autopoiesis, as it provides three theoretical tools to ground the production of minimal synthetic living systems able of communicating with natural living systems. Very schematically, they can be described as it follows.

First tool: *an operational definition of life characterizing a mechanism able to generate minimal living systems.* The notion of autopoietic organization proposes a "synthetic" or "constructive" definition of living systems, as it characterizes them not by listing a set of properties, but by specifying a mechanism able to generate these systems and their dynamics of self-production.

Second tool: *a theory of biological systems' interaction with the environment.* According to Maturana and Varela, the systems produced by the mechanism of autopoiesis are not trivial objects, which passively undergo environmental pressures. On the contrary, autopoietic systems can perceive exogenous variations as local alterations of their internal processes of self-production, and can react to them through an activity of self-regulation, that is, a series of changes in their elementary processes that compensate the perturbations [6]. This idea is at the basis of the autopoietic theory of the

interaction between living systems and their environment, grounded in the notion of "structural coupling". This describes the autopoietic unit and its environment as two systems permanently involved in a dynamics of reciprocal perturbations and internal compensations, in which the autopoietic system associates recurrent external perturbations to internal patterns of self-regulation, that is, endogenous patterns of self-production which compensate the external perturbations.

Third tool: *a theory of communication between living systems.* On the basis of the notions of autopoietic organization and structural coupling, Maturana and Varela proposed a theory of communication between autopoietic systems [6], which characterizes it as a dynamics of reciprocal perturbations and compensations in which each system associates the exogenous perturbations produced by the other system to internal operational meaning expressed in patterns of self-regulation.

2.2 The imitation game: a Turing-test like approach

Lee Cronin, Natalio Krasnogor, Ben Davis and coworkers proposed a sort of Turing test for chemical cells (*chells*), as an analogy with the known Turing test for assaying artificial intelligence [7]. The goal was to devise a conceptual test that could help in the field of artificial cellularity. The authors aimed to the recognition of life in general, by means of a "cellular imitation game" setup (Figure 1b). The imitation becomes perfect when a natural cell as interrogator cannot distinguish one of its own kinds from a synthetic cell. In their view, the authors clearly points to the issue of synthetic cell/natural cell recognition and communication, even if not directly referring to molecular communication.

More recently, Ben Davis also published an experimental report on the first attempt to establish a synthetic communication between "chemical cells" (*chells*) and natural living cells [8]. This report, to the best of our knowledge, is the only one on this very new topic. The precursors of the "formose" reaction were encapsulated within liposomes. The product of this reactions are linear and branched carbohydrates that resemble some naturally occurring sugars. One class of products of the intra-liposomal formose reaction escaped from liposomes through a channel (α-hemolysin, see below), and spontaneously reacted with the borate ions present in the external medium. The resulting furanosyl boronates are structurally very similar to a specific class of bacterial signal molecules, and therefore triggered a biological response in a population of the bacterium *Vibrio harveyi.* This work demonstrated the feasibility of generating a synthetic entity that is able to send a signal to a natural receiver, a breakthrough in the field of bio/chem- ITC.

3 The concept and the technology of semi-synthetic minimal cells (SSMCs)

3.1 The concept of minimal cells: from origin of life to synthetic biology

Although already present in the literature on origin of life [9,10], the modern concept of minimal cells was developed in the laboratory of Pier Luigi Luisi at the ETH Zurich, in the 90s. Intrigued by the autopoietic theory, Luisi and coworkers tried to construct in the laboratory the first autopoietic minimal cells by using firstly reverse micelles, then normal micelles, and finally vesicles [11]. All these microcompartments consist in self-assembling structures generally formed by surfactants or lipids. By chemical producing boundary-forming molecules, it was possible to observe the autopoietic growth of these microcompartments. A convenient way for establishing a more complex and recursive autopoietic growth is known as the "semi-synthetic" approach. It consists in the encapsulation of the minimal number of compounds, namely DNA, enzymes, ribosomes and all required macromolecules, inside lipid vesicles. The corresponding structures are known as "semi-synthetic" minimal cells because natural compounds are used for their construction. This differs from the totally "synthetic" approach where not-natural (synthetic) compounds can be employed, at least in principle [12]. The goal of the semi-synthetic approach is setting up a minimal genetic/metabolic dynamics inside such compartments. In this short article there is no space to discuss the most intriguing aspect of the minimal cell construction: When a minimal cell can be defined as "alive"? And what does "alive" mean? The interested reader can find a deeper discussion of these aspects in a recent review [2]. These issues are quite important when minimal cells are intended as model of primitive cells.

More recently, however, after the advent of synthetic biology, there has been a new flourishing interest toward minimal cell research [13]. This is due to the fact that minimal cells, thanks to their minimal complexity, can be built in the laboratory to perform useful functions, without necessarily being related to the origin of life research. In this view, minimal cells are not only a tool for understanding the origin of cells, but can be important tools for diverse applications (as the bio/chem-ICT ones). The last 10 years have been characterized by an intense research activity aimed at understanding and controlling the construction of SSMCs from separated parts. Most of the work has been focused on the production of functional proteins inside liposomes, but also other goals have been achieved. The production of protein is of special relevance because it allows the generation of new functions inside liposomes (i.e., synthesis of enzymes that catalyze a useful reaction). Not many research groups are currently involved in these studies, but the community is indeed growing (for a review on the latest achievements, see [14]). In the next paragraphs, we will shortly review what is the current knowledge on SSMC construction from the viewpoint of two important aspects: liposome technology and cell-free technology (Figure 1c).

3.2 Liposome technology: from classical methods to the "droplet transfer" strategy and beyond (first vesicles from microfluidic devices)

SSMCs are based on liposomes. Liposome technology is a rather well developed technology that progressed mainly for producing drug-containing liposomes, for drug-delivery applications. There are plenty of methods for liposome preparation, but only few of them have been used in the field of SSMCs studies. In particular, because SSMCs are often intended as models for primitive minimal cells, the preparation method should also be – if possible – compatible with allegedly prebiotic conditions.

Two methods have found a widespread application in SSMC studies. The first one consists in hydrating a previously deposited lipid film with a mixture of solutes of interest. Following the swelling of lipid bilayers, a population of liposomes is formed, heterogeneous size and morphology (e.g., unilamellar, or multilamellar, or multi-vesicular vesicles), witnessing that lipid vesicles form according to individual kinetic paths. As expected, also the *entrapment* of solutes in this heterogeneous population of vesicles is rather heterogeneous and not very efficient. A typical way for improving the solute encapsulation and homogenize the liposome suspension consists in repetitive freezing and thawing cycles, possibly followed by the classical extrusion procedure (a typical example is found in [15]). The problem of solute entrapment becomes critical when one considers the low probability of co-entrapping several compounds in the same lipid vesicle. We have recently investigated this aspect during the studies on the construction of SSMCs with minimal physical size [16], and the intriguing effect of "spontaneous crowding" has been reported [17]. Another well-known method for liposome preparation, that partially overcome the issue of poor solute entrapment consists in hydrating the ghosts of previously formed liposomes in form of freeze-dried cake [18]. This method produces also a population of vesicles with a broad distribution, and it has been studied in great details by flow cytometry [19].

Despite the above-mentioned "spontaneous crowding" effect [17], that actually involves about 1% of vesicle preparation, it is clear that the film hydration and freeze-dried cake hydration method lack the control of solute internalization. They are perfectly suitable when SSMCs are studied as a case of self-organizing microcompartment, but are less valid when a technology for producing SSMCs needs to be developed (as for the case of bio/chem-ICT). A new method rapidly emerged in the past few years as the method of choice when a complex mixture of molecules needs to be encapsulated inside liposomes. This method was introduced by Weitz and coworkers in 2003 [20], and it is currently studied and developed in almost all laboratories working in this field. The method consists in transferring a water-in-oil lipid-stabilized droplet, easily filled with the solute of interest, through a lipid-containing interface. In this way it is possible to produce giant lipid vesicles (GVs) in a reproducible way and in good yield (e.g., 5-10,000 GVs/μL).

The recent advances in microfluidic technology might contribute in the near future to the SSMCs technology. It is worth noting, in fact, that in the last 4 years some interesting reports have shown the possibility of producing GVs directly in microfluidic devices. A short review on these methods is available in [14] – see also [21]. If this

technology will become robust and available for most laboratories, it is foreseeable that the next generation of SSMCs could derive from microfluidic controlled assembly – which produces GVs with high reproducibility – rather than from the spontaneous, heterogeneous (yet interesting) self-assembly that has characterized the research done till now.

3.3 Cell-free systems as a typical synthetic biology toolbox

The second ingredient for constructing SSMCs in the laboratory is a cell-free system. This is essentials because the semi-synthetic approach foresees the assembly of a cell from separated components, and cannot therefore relies on components existing in pre-formed cells, as it happens in many other synthetic biology approaches. The choice of the cell-free system to be entrapped inside liposomes clearly depends on the function to reconstitute. For example, there have been studies on RNA synthesis from DNA-template or RNA-template, on DNA amplification via polymerase chain reaction, and, mostly, on the coupled transcription/translation reaction (from DNA to RNA to protein). The interested reader can find technical and more detailed information on these systems in a very recent review [14].

Here it is important to remark that after its introduction in 2001 [22], the so-called PURE system (Protein synthesis Using Recombinant Elements) is considered the "standard" cell-free system for constructing SSMCs in the laboratory. It fits perfectly with the requirement of full-characterized parts/devices/systems in synthetic biology. The PURE system includes 36 purified enzymes, ribosomes, and a tRNAs mixture – as well as low molecular weight compounds – for a total of about 80 macromolecules. It represents the minimal reconstituted system capable of synthesizing a functional protein, and it is therefore suitable for SSMCs studies. The PURE system has replaced the use of cell extracts, with unknown composition. As already highlighted, the production of proteins inside liposomes is a key intermediate step toward the construction of more complex SSMCs.

The analysis of the literature shows that it is possible to synthesize, inside liposomes, water-soluble proteins and enzymes like green fluorescent protein, T7 RNA polymerase, α-hemolysin, Qβ-replicase, β-galactosidase and β-glucuronidase (see [14] for details). In general terms, therefore, it can be said that the synthesis of a water-soluble protein in its folded conformation should not be considered a problem (in absence of important post-translation modifications). Different is the case of membrane associated- or integral membrane proteins, where the only available report [23], which focused on two acyltransferases, shows that the chemical composition of the SSMC membrane strongly affect the synthesis, the structure and the function of this kind of proteins.

Interestingly, it has been also reported that α-hemolysin, when produced inside liposomes, spontaneously forms pores (i.e., channels) in the membrane. Due to its specific size, the pore allows small molecules (< 3 kDa) to freely enter/exit the liposome, whereas enzymes, RNAs and DNA remains entrapped inside [24]. This implies that SSMCs can release/uptake small molecules to/from the environment. It should be also

remarked that some molecules could cross the lipid membrane without the need of a pore.

4 A research program on synthetic cell/natural cell communication

We have seen what is the current state-of-the-art of SSMCs technology. Can we imagine a realistic scenario where a chemical communication can be established between SSMCs and natural cells? The previously reported work of Ben Davis and coworker demonstrates that this is an achievable goal [8]. However, to go beyond the simple case of the Davis' *chells* and use SSMCs, several aspects must be considered. In order to understand how communicating SSMCs must be designed, it is useful to make a short survey on the way natural cells communicate. At this aim we believe that bacterial communication should be taken as a paradigmatic example and model to start this enterprise.

4.1 Bacterial communication and quorum sensing (QS)

Bacteria live preferably as communities. The discovery of bacterial communication via chemical signaling has been one of the most exciting breakthroughs in microbiology. Thanks to chemical communication, bacteria understand the structure of the population and often respond with cooperative behavior, reaching – as a community – goals that are impossible for each single individual [25,26].

The most representative example of bacterial communication is *quorum sensing* (QS), a cell-cell signaling system that allows a coordinate reprogramming of gene expression in response to cell density. QS takes its name from the fact that a response is achieved when a signal compound reaches a certain concentration threshold (corresponding to a certain bacterial cell density, the "quorum") [27]. Bacteria use a very large variety of biochemicals to communicate. This means that bacterial communities, often consisting of different species, communicate thanks to the specificity of the signal production and signal reception pathways.

Among the most well known types of QS mechanisms, it is known that Gram-negative and Gram-positive bacteria usually rely on acylated homoserine lactones (AHLs) and small peptides, respectively, as signal molecules. A third class of QS signals, produced by both Gram-negative and Gram-positive bacteria, is known as autoinducer-2. Can we exploit the simplest of these communication mechanisms to construct SSMCs capable of communicating with bacteria and produce a QS-like response?

4.2 Towards the communication between synthetic and natural cells

It is clear that communicating SSMCs must implement the molecular devices for encoding, sending, receiving, and decoding a chemical signal.

It stems directly from the analysis of how bacteria communicate, that the molecular communication consists in five distinct operational steps: namely, (*i*) encoding a message as a molecule, (*ii*) send/export the molecule, (*iii*) a propagation/transportation step, (*iv*) receive/import the molecule, (*v*) decoding the message. Different physical or (bio)chemical devices can be associated to each step, depending on the kind of communication implemented by living cells. According to the synthetic biology terminology, we might call the biochemical machineries required to accomplish these steps as "devices". Note, however, that not all the operational steps described above need to be associated to a physical device. For instance, the import of a signal molecule can happen by passive diffusion of the molecule into the receiving cell rather than due to the presence of a membrane receptor.

Depending on the devices implemented in SSMCs, one can conceive uni- or bi-directional communicating systems, like "sending" SSMCs, or "receiving" SSMCs, or "sending/receiving" SSMCs. So, we can devise at least three ways of implementing a molecular communication: message sent from the SSMCs to bacteria, message sent from bacteria to SSMCs, or bi-directional communication.

In order to realize the first step (encoding a message as a molecule), SSMCs must be constructed in a way that they can express, under regulatory control, an enzyme that catalyses the formation of the signaling molecule. We have seen that enzymes like β-galactosidase, β-glucuronidase, acyltransferases and even polymerization enzymes like T7 RNA polymerases and Qβ-replicases have been successfully expressed by cell-free systems encapsulated inside liposomes. In order to simplify the experimental approach, the direct precursor(s) of the signal molecule can be added to the protein synthesis mixture when SSMC are prepared.

The second step consists in the exporting phase. This process depends on what kind of signal molecule is used. Some of the compounds used by bacteria for QS are freely membrane permeable (e.g., short-chain AHLs), whereas other might need a protein factor for the export (typically, a membrane protein, that – as we have seen before – is probably difficult to synthesize by cell-free systems). It must be recalled here that α-hemolysin pore might offer a solution for releasing polar/ionic signal molecules, which are not able to cross the lipid membrane.

For realizing a molecular communication it is also need that the signal molecule moves from the sender to the receiver. This occurs by diffusion, but it is required that the molecule is chemically stable.

The fourth step (import) is analogous to the second one (export). Depending on the chemical nature of the signal molecule, an additional molecular device could be needed for completing the communication channel. In the case of reception, it can happen that the signal molecule just trigs a cellular response without physically entering the cell (i.e., via signal transduction across the membrane thanks to a membrane protein). In this case, the presence of the transducing element is mandatory.

The fifth step involves the decoding of the chemical information. In some cases this occurs because the signal molecule binds to a receptor, that ultimately regulates the transcription of a gene. In this case, the "receiving" SSMCs must be able to syn-

thesize the receptor in its active form, so that it can effectively bind the signal molecule and then tune gene expression. In the case of signal transduction across the membrane, the set of proteins required for completing the whole process must be synthesized by the SSMCs.

It is evident that as first approach, the design of molecular communication between natural and synthetic cells should involve:

- a simple enzyme pathway for producing the signal molecule (ideally, a single transformation mediated by an easily produced enzyme);
- a freely diffusible signal molecule that is chemically stable in the medium where the propagation occurs;
- a direct binding of the signal molecule to a receptor/regulator (i.e., avoiding a mechanism based on membrane proteins that mediate a mediating a signal transduction pathway).

Quite probably, short-chain AHLs are the best candidates for setting up an initial experimental program. Indeed, AHLs are produced by a single enzyme, they can freely diffuse outside the sending cell, are stable in the aqueous environment, can freely diffuse inside the receiver, and their decoding is quite simple (intracellular receptor/regulator).

4.3 Conclusions and perspectives

The development of chemical communication between synthetic and natural cells represents one of the next goals of SSMCs research, clearly oriented toward the application of SSMCs in synthetic biology and bio/chem-ICT. As emphasized by Suda and coworkers [1], a technology that can be interfaced with biological systems allows the establishment of a direct communication with cells – in their chemical language – and therefore paves the way to several perspectives for advanced applications, for example biomedical ones.

In this respect, it is interesting to cite here the vision provided by Le Duc and coworkers, who proposed the concept of “pseudo-cell factories” or “nanofactories” [28]. These are liposome based systems (actually a kind of SSMCs) designed for medical applications, namely for being administered to the human body with the aim of targeting toward a specific tissue (by means of surface-bound antibodies, like in “immunoliposomes”). Arrived on their target site, nanofactories would be able to sense their microenvironment, receive input and produce output and ultimately trigger an internal genetic/metabolic network that might produce a drug or any other chemical with biological effect. In other words, LeDuc and collaborators implicitly gave to their nanofactories the capacity of communicating with natural cells. This is indeed the essence of the bio/chem-ICT vision we presented in this article. In more general terms, and in a future perspective, SSMCs, thanks to their modular construction might host regulatory genetic networks that can be made programmable in order to respond to different signals. Remarkably, this is also a form of computing.

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