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# A synthetic biology approach to bio-chem-ICT: first moves towards chemical communication between synthetic and natural cells

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**Abstract** In this article we present novel aspects of the impact that synthetic biology (SB) can express in a field traditionally based on computer science: information and communication technologies (ICTs), an area that we will consider taking into account also possible implications for artificial intelligence (AI) research. In the first part of this article we will shortly introduce some recent theoretical and experimental issues related to our approach in SB, discussing their relevance and potentiality in the field. Next, we define an original SB research programme that aims at contributing to the development of bio-chem-ICTs and AI based on chemical communication between natural and synthetic cells. In particular we present (*i*) a mathematical model that allows us to simulate the main features of the system under construction; and (*ii*) preliminary wet-lab experiments showing the feasibility of our research programme. Based on the bottom-up construction of synthetic cells, the traits of this novel approach and their connections with recent conceptual and technological trends are finally discussed.

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## 1 Introduction

Recent waves of biological research—which combine advancements in genetic/metabolic manipulation, bioengineering and mathematical modeling—have given rise to a new frontier discipline, better defined as an "interdiscipline" (Keller 2009): synthetic biology (SB). The term *synthetic biology*, certainly not new (Leduc 1912; Szybalski 1974), today denotes a plurality of modern and ambitious approaches rapidly emerging as an exciting scientific research area. Likewise many other emergent fields, which are increasingly often called as "the sciences of the artificial" (i.e., sciences which model natural processes through artificial or synthetic systems, *cf.* Damiano et al. 2011), SB integrates both engineering and scientific attitudes, exemplifying one of the most notable changes of academic/industrial research in the last years.

We refer to a reorganization of the classical divide between engineering and science, directed towards supporting a new methodological orientation—"understanding by building" (Pfeifer and Scheier 2001). In order to apply this methodological principle, SB integrates and pursues two main goals. The first is the *engineering goal* of constructing biological devices, able to perform useful functions, by designing and assembling "standard" biological parts (Endy 2005), just like electronic engineering designs and builds tools starting from industry-produced electronic parts. A remarkable example of this kind of SB research has been recently reported by Craig Venter's team (Gibson et al. 2010), which succeeded in transplanting an artificial

genome into a biological cell. The second SB's goal can be characterized as the *genuinely scientific goal* of using these kinds of engineering manipulations of biological parts to better understand life. The aim is that of constructing artificial models of natural biological processes to experimentally explore aspects of life that are difficult or impossible to be studied through the classical analytical approach (i.e., taking apart biological systems), or that are not accessible in natural scenarios of research. The construction of *synthetic cells* from separate parts is a paradigmatic expression of this SB's research line (Luisi et al. 2006; De Lorenzo and Danchin 2008; Chiarabelli et al. 2009), consisting in the "bottom-up" assembly of synthetic cells endowed with a minimal degree of complexity, yet capable of displaying cell-like functions (ultimately: being alive).

The relevance of this approach is manifold. Firstly, it allows synthetic biologists to test the operational power of biological theories, i.e., their capability of guiding the design and the construction of molecular systems behaving in cell-like ways. This approach also allows researchers in SB to establish whether or not a minimal biological complexity can be indeed synthetically produced starting from a limited set of parts. Furthermore, the construction of simplified models of biological cells might help to discover or disprove possible primitive routes leading to the origin of cellular life. More importantly, building minimal synthetic living cells in the lab would demonstrate how, in certain physical and chemical conditions, life can "emerge" from inanimate matter.

Finally, the construction of synthetic cells from separate parts integrates its scientific goals with engineering ones. We refer in particular to useful applications in biotechnology and nanomedicine, such as the definition of new diagnostic or therapeutic methods based on biocompatible "smart" synthetic cells. These developments are realistic, since synthetic cells—due to their embodied molecular computing capabilities—would share with natural cells many abilities, such as chemical communication, molecular recognition, and signal transduction—i.e., the key features for reciprocal interfacing.

This article focuses on this applicative perspective: the development of synthetic cells that are able of communicating with biological cells (Stano et al. 2012; Rampioni et al. 2013). Such a project, in our opinion, represents a starting point not only for future advancement in nanomedicine, but also for relevant innovations in other emergent fields. Indeed, synthetic cells able to produce, manipulate and respond to biochemical signals in a controllable (and possibly programmable) way could find relevant applications in the nascent field of information and communication technologies (ICTs) based on biological and chemical signals (bio-chem-ICTs) (Hiyama and Moritani 2010; Amos et al. 2011; Nakano et al. 2011; Nakano et al. 2013).

With the terms bio-chem-ICTs and bio-chem-ITs one referst to a novel research area with multiple goals and methods, all based on radically new forms of computation, and nano- or micro-scale production (Amos et al. 2011). Instead of simply taking inspiration from biological systems and mechanism, bio-chem-ICTs aim at making use of (and ultimately construct) biological and chemical systems, in order to harness their intrinsic capability of self-organization, adaptability, resilience and flexibility (Amos et al. 2011).

Thus, the connection of our proposal with bio-chem-ICTs is evident if we consider that natural chemical communication is the most effective and sophisticated example of bio-chem-ICT. The mechanisms of molecular communication in biological systems can be utilized to develop minimal synthetic cells that can be interfaced with biological cells, but also for constructing entirely synthetic bio-communication devices that can perform computations. Owing to the above-mentioned capabilities, which are peculiar to all molecular systems (and especially the cell-like ones), our proposal has also deeper implications that can be only outlined in this article. These refer to novel paradigms in (embodied) AI research, as we will briefly point out later, based on bio-chem-ICTs of the type we intend to develop.

## 2 Emerging experimental and theoretical issues about SB and bio-chem-ICTs

Several research lines, emerged in the recent years, emphasize the importance that molecular communication can play in the future of science and engineering. In this section we shortly review those that in our opinion are more relevant to our goal. The choice is necessarily biased by our interest and it should not be intended to represent the plurality of potentially useful technologies and tools. In particular, we will just focus on synthetic cell technology (Sect. 2.1), describing the recent progresses in the field, and to additional selected cases (Sect. 2.2) that inspired us. On their bases, we propose a theoretical framework able to support new advancements in the research on molecular communication, and in particular the new research programme we are going to introduce in the next sections. The engineering of molecular communication between synthetic and natural cells will stem from these backgrounds.

### 2.1 Synthetic cells

Several research groups are currently involved in developing the technology for constructing synthetic cells. These

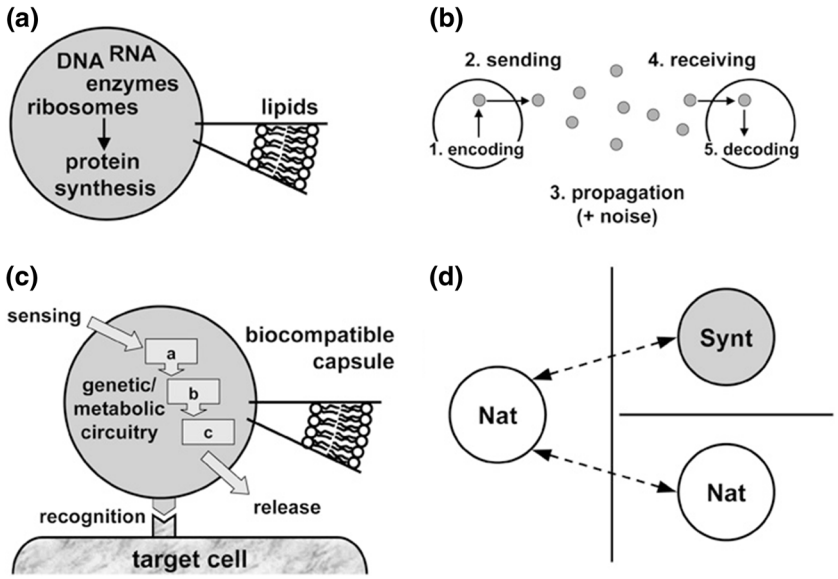



**Fig. 1** Conceptual and experimental tools for developing bio-chem-ICTs in the synthetic biology context. **a** Synthetic cells (Luisi et al. 2006) can be built by encapsulating the minimal number of biomolecules inside lipid vesicles (liposomes). To date, synthetic cells can synthesize functional proteins with good efficiency, but are unable to generate energy, grow/duplicate. **b** Outline of a molecular communication process based on the diffusion of "signal" molecules from a sender cell and a receiver cell, sharing the general scheme of a Shannon communication model (Nakano et al. 2011). **c** Schematic representation of Leduc's "nanofactories" for smart drug delivery/nanomedicine. A biocompatible synthetic capsule recognizes and binds to the target cell (in the human body). By sensing its microenvironment, the nanofactory activates internal genetic-metabolic modules, which produce, for example, a drug or a bioactive compound (Leduc et al. 2007). **d** Extending the Turing test (imitation game) to synthetic cells. The classical interrogation test developed for assessing computer AI is here ideally applied to natural cells (Nat) and a synthetic cell (Synt) as a conceptual tool for assessing wet-lab artificial life (Cronin et al. 2006)

are cell-like compartments based on biochemical machineries encapsulated inside lipid vesicles (liposomes) (Fig. 1a). The liposome semi-permeable membrane acts as a boundary that physically defines the synthetic cell, encloses the bioactive material, and protects it from external perturbations and unwanted reactions.

The chemical nature of synthetic cell components varies considerably depending on the experimental purposes. Together with synthetic cells designed for being used as primitive cell models (which are therefore built with very simple and allegedly primitive molecules), a very promising approach is based on the use of modern biological components, like proteins and nucleic acids encapsulated inside phospholipid vesicles. This is known as the "semi-synthetic" approach, and accordingly the so-produced synthetic cells have been called *semi-synthetic minimal cells* (Luisi et al. 2006); for the sake of simplicity, here we will refer to them simply as synthetic cells.

The main idea of constructing minimal synthetic cells consists in selecting the minimal and sufficient number of chemical species that (once inserted in a lipid vesicle, and fed with the appropriate set of building blocks) are able to constitute a minimal living cell endowed with the capabilities of self-maintenance and self-reproduction, as well as with the potentiality to evolve. A related concept refers to the minimal genome (Mushegian and Koonin 1996; Gil et al. 2004; Forster and Church 2006). In the context of origin of life, the construction of a living synthetic cell intends to allow scientists to observe the emergence of life from inanimate matter in particular physical–chemical conditions. In biotechnology, the construction of synthetic cells paves the way to developing new tools for diverse applications. Irrespective of the purpose of synthetic cell construction, however, a lot of knowledge on how biological systems function can be obtained on the way to build synthetic cells of increasing complexity, and interestingly, this is a genuine outcome of the synthetic (or constructive) methodology—"understanding by building".

Despite the recent advancements, the technology of synthetic cell production is still in its infancy and we are indeed very far from the goal of constructing a living synthetic cell. Current research just started to focus on the reconstitution of simple cellular functions, which are however important and interesting *per se*, and that can also be exploited for useful applications. One of the most well studied functions reconstructed in synthetic cells is the synthesis of proteins, and it will be discussed in detail in Sect. 3.1.

Several review papers have commented in details most of the experimental and theoretical aspects of synthetic cell

construction (Mansy and Szostak 2009; Ichihashi et al. 2010; Stano et al. 2011a, b). Interesting results have been obtained by this approach, which is based on the convergence of two technologies, namely the liposome technology and the cell-free technology. The semi-synthetic approach is attractive in terms of design, programmability, and modular construction of synthetic cells capable of performing biochemical functions. In a certain sense, synthetic cells can be considered as *molecular robots* that can be functionalized, in principle, with sensors and actuators. In contrast with electromechanical robots, however, synthetic cells functions are embodied in the structure of their molecular components, which continuously interact with each other on the basis of diffusion, molecular recognition, and cooperative behaviour.

### 2.2 Molecular communication in the context of ICTs

The general idea of developing artificial chemical communication channels for novel bio-chem-ICTs has been discussed recently by Suda's and Nakano's groups, in in a series of papers summarized in (Nakano et al. 2011, 2013). These authors emphasize how the known mechanism of biological communication, based on exchange of chemicals, can be taken as a paradigm for developing molecular communication in biological machines such as synthetic cells. Their analysis starts from a comparison between the molecular and the electrical communication paradigms (Fig. 1b), showing that they display conceptual similarities, but also practical differences. The potentials of molecular communication technologies are discussed mainly in the context of nanomedicine (synthetic cells that use the "same language" of natural cells), as well as in the context of the ideation of new computing paradigms, based on biological mechanisms and materials.

A fascinating article by LeDuc and coworkers, written in 2007, focuses on the possibility of creating synthetic cells (called "nanofactories") for useful applications in nanomedicine (Leduc et al. 2007). The authors envisage the construction of shell micro-compartments that can be introduced into the body, and that contain a kind of biochemical machinery capable of producing a therapeutic agent when stimulated by the local biochemical environment (Fig. 1c). Since this event has to be generated in a programmable way starting from a chemical signal, the "nanofactory" has to be conceived as a bio-chem-ICT device. Although the design and the construction of such a "nanofactory" is not yet possible, the use of 0.1 micrometer liposomes for drug delivery is already a quite well established result, and numerous studies are currently devoted to specifically target them against a certain type of cells or tissues (Torchilin 2005).

An interesting experiment was recently reported by Davis and collaborators, who developed a simple chemical communication system based on the encapsulation of the formose reaction inside liposomes (Gardner et al. 2009). The products of the formose reaction are sugar-like molecules that react easily with borate, to give adducts that are similar to a signal molecule used by bacteria. When molecules produced by the formose reaction escaped from the liposome thanks to a pore, such molecules could react with external borate to form such adducts. They could then diffuse in the external medium and activate a biological response in a bacterial population.

In addition to these experimental scenarios, synthetic cell/bio-chem-ICTs related issues were discussed in a commentary paper on a possible extension of the classical Turing test for AI to synthetic life (Cronin et al. 2006, on the basis of Harel 2005). The original Turing test was designed within the framework of classical AI, i.e., "cognistivist" or "computational AI" (Pfeifer and Scheier 2001), to circumvent the problem of defining intelligence. An operational test, the *imitation game*, can establish, in principle, if a computer displays an intelligent behavior indistinguishable from that of a human in the context of a natural language conversation and according to the evaluation of a "human judge". Although controversial and still under debate, the Turing test concept, when considered from the viewpoint of SB, leads researchers to wonder what would be the conceptual counterpart of such a test to evaluate the capability of synthetic cells to express complex biological-like behaviors.

Cronin, Krasnogor, Davis and collaborators (Cronin et al. 2006) imagine a chemical version of the Turing test, which would be based on "questions/answers" mediated by (natural) physico-chemical language (e.g., interconversion of chemical potentials, mechanical transduction, signaling), and would involve a synthetic cell and a natural cell communicating with a natural cell "judge" (Fig. 1d). In this *Gedankenexperiment* (thought experiment) direct or mediated molecular communication between synthetic and natural cells plays a key role, and fits well with the research lines in SB and bio-chem-ICT briefly described above.

### 2.3 The notion of autonomy as a conceptual guide for engineering chemical communication between synthetic and natural cells

The research lines briefly described above open the possibility of implementing different relevant uses of chemical communication between synthetic and natural cells. A characteristic aspect of these pioneering lines is that they implicitly refer to synthetic cells as *heteronomous artificial systems*, i.e., systems whose organization and behavior are defined by external agents (the scientists who built these systems). This is evident in the functional analogies

proposed by these lines between synthetic cells and other artificial systems such as computers, "nanofactories", robots, etc. Certainly the qualification of *heteronomous systems* is appropriate to define the kind of synthetic cells that currently we are able to produce. In particular, it is appropriate as it designates a limit of our capability of synthetically re-creating natural cells, that is, more specifically, our incapability of providing synthetic cells with a property typical of natural cells, and absent in devices such as computers, factories and robots. We refer to the *autonomy* of living systems, conceived as their capability of producing and maintaining their material identity (themselves), as well as specifying their own organization and their own behaviors, by themselves, without the intervention of engineers.

We believe that it is crucial for SB not to loose biological autonomy out of sight. Increasingly acknowledged as the distinctive property of (at least) living systems (Damiano 2012), this property has to be considered fundamental not only to synthetically create life, or to test for biological-like complex behaviours. It can play a crucial role also in prevalently engineering research, as the increased autonomy of artificial biological-like systems could imply significant advantages for these systems, such as an augmented capability of coping with highly perturbative environments, a superior behavioral adaptability, and an increased ability of communicating with natural biological systems. It has to be noticed that, of course, the increased autonomy of artificial biological-like systems would contrast their controllability from the outside—i.e., their heteronomous character—and would open the issue of appropriately balancing their autonomous and heteronomous aspects.

Concerning the current research on chemical communication between synthetic and natural cells, it is very important to remark that creating a "natural-like" chemical communication between synthetic and natural cells means to make the former able to communicate with autonomous systems—systems that are not programmed by engineers, as computers or electronic devices are. This makes necessary for SB to programmatically consider the autonomous aspects of natural cells while designing and engineering their communication with synthetic cells, in order to appropriately define ways to engage them in a synthetic/natural communication channel. One of the biological frameworks particularly apt to enable SB to focus on these aspects is autopoiesis (Maturana and Varela 1973), specifically built as a theory of life focusing on biological autonomy.

### 2.4 Autopoiesis as a theoretical framework for a SB approach to engineering chemical communication between synthetic and natural cells

The theory of autopoiesis was developed in the 1970s by Humberto Maturana and Francisco Varela to generate, for the basic question "what is life?", an answer focused on autonomy—an aspect of the living phenomenology that they considered neglected by traditional biology (Maturana and Varela 1973). They grounded the theory of autopoiesis in two main hypotheses, respectively asserting that (a) the distinctive property of living systems is autopoiesis ("self-production"), consisting in the capability of these systems of producing and maintaining themselves—their material identity—through internal processes of synthesis and destruction of their own components; (b) autopoiesis is a global property of living systems, relying not on their physico-chemical components taken separately, but in the way in which these components are organized within the systems. On the basis of these hypotheses, Maturana and Varela faced the issue of defining life as the problem of characterizing what kind of organization supports the biological behaviour of self-production. They elaborated a solution at the level of the minimal living cell. It consists in the notion of "autopoietic organization", directed towards defining the "fundamental" biological organization, i.e., very schematically, the form of the biological organization from which all the other forms derived.

This theoretical concept contains the main reasons of SB's interest for autopoiesis—for the purposes of this article, three main reasons. First of all, this notion expresses a "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. Secondly, it intends to provide the definition of both the minimal *living* organization, and the minimal *cognitive* organization. As Maturana and Varela see, the systems generated by the mechanism of autopoiesis, far from being trivial objects, can perceive some external variations as perturbations of their internal process of self-production (Maturana and Varela 1987). Also, they can react to them through an activity of self-regulation: changes in their elemental processes that compensate the alterations. On this basis, these systems can be conceived able of generating internal operational meanings for the perceived external variations. These meanings are expressed in terms of dynamical schemes of self-regulation, which externally appears as actions oriented to conservation (e.g., absorbing a molecule of sugar, overcoming an obstacle, etc.). This "meaning generation" behaviour—described by Maturana and Varela as the basic "cognitive" behaviour—is the core of the autopoietic notion of "structural coupling", defined to conceptualize the interaction of the autopoietic system with its environment: a dynamic of reciprocal perturbations and compensations, in which the autopoietic system continuously generates and associates to exogenous variations operational meanings of self-regulation that allows it to keep its process of self-production in an ever-changing

environment. Thirdly, the notion of autopoietic organization, by generating that of structural coupling, produces a theory of cognitive interaction between autopoietic systems, and a related theory of communication (Maturana and Varela 1987). The latter describes the communicative process between two or more autopoietic systems as a dynamics of reciprocal perturbations and compensations, during which each system generates and associates internal operational meanings to the exogenous perturbations produced by the other autopoietic systems. The specificity that distinguishes these interactions from those with the environment is that the structural coupling between autopoietic systems leads to "coordinated" or "coupled behavior": the establishment of a coupled dynamics between two or more systems, in which each system influences the behavior of the other(s) by inducing the production of internal operational meanings and, through this, actions. In other words, through coordinated and communicative behaviors, autopoietic systems participate in their respective cognitive activities of facing environmental variations by meaning generation.

In this way, autopoiesis offers to SB three theoretical tools that can be useful to ground the production of minimal synthetic living systems capable of communicating with natural living systems: a definition of life providing the description of a mechanism able to generate minimal living systems, a theoretical characterization of the cognitive interaction of these systems with the environment, and a theory of communication between minimal living systems.

## 3 A synthetic biology approach to chemical communication between synthetic and natural cells

A possible experimental plan for developing chemical communication between synthetic cells and natural cells, intended as a novel paradigm in bio-chem-ICTs, can be based on SB. In particular, the bottom-up synthetic biology approach allows the construction of synthetic cells of minimal complexity (not necessarily alive) that are potentially able to communicate with natural cells by exchanging chemical compounds. It is important to notice that the SB technology we would like to develop for synthetic cells actually derives from the experimental approach to the study of minimal life originally implemented in the 1990s by Luisi's group on the basis of the theory of autopoiesis (Luisi and Varela 1990). Thus, this approach leads to the engineering of molecular communication keeping on sight biological autonomy.

Here we would like to show how currently available elements and know-hows can be combined in an innovative fashion in order to reach this goal.

Firstly, we will describe how it is possible to build synthetic cells capable of synthesizing proteins (enzymes), and therefore capable of performing functions. Next, we will draw a possible experimental strategy for synthetic cell/natural cell communication. According to the typical SB working flow, we will then explore, by numerical modeling, whether such an enterprise is potentially feasible. Finally, preliminary wet-lab experiments will be presented.

### 3.1 The production of protein-synthesizing liposomes

Liposomes can be produced by well-established technologies, and the assembly of solute-containing liposomes can be considered a standard achievement.

As mentioned before, in order to build synthetic cells capable of non-trivial behaviour, several macromolecules (DNA, RNA, proteins) must be inserted inside lipid vesicles for reconstructing one of the most important cellular function: protein synthesis. This is a key function of synthetic cells, because most of the cell "actuators", such as enzymes, receptors, transcription factors, transporters, etc., are proteins. The inclusion of biological macromolecules inside liposomes (the "semi-synthetic" approach) has been pioneered by Luisi and collaborators in the early 1990s (Schmidli et al. 1991; Walde et al. 1994; Oberholzer et al. 1995a, b; Oberholzer et al. 1999), and it is widely used today for producing synthetic cells.

There are several classical methods for preparing lipid vesicles (Walde 2003), and in particular "giant" vesicles (GVs, with diameter $> 1$ micrometer), which are conveniently visualized by optical microscopy. The "thin film hydration" or the "natural swelling" methods have been widely used, but they suffer of limitation due to the nature of employed buffer and lipids, and more importantly, of relatively low encapsulation efficiency. Currently, synthetic cells are conveniently assembled by a method, based on a 2003 report (Pautot et al. 2003), known as the "droplet transfer" method. This is attractive because it allows the preparation of solute-containing GVs in a wide variety of conditions. The method consists in transferring lipid-stabilized solute-containing water-in-oil droplets from an oil solution to an aqueous solution. During this transfer, the droplets become covered by a second lipid layer, producing giant liposomes that—remarkably—contain in principle all solutes initially present in the water-in-oil droplets (Fig. 2a). The droplet transfer method is then perfectly suitable for assembling solute-filled synthetic cells because the capture of solutes is somehow forced by the water-in-oil droplet topology, which implies a facile compartmentation of water-soluble solutes. The method is fast, reproducible, gives a homogenous population of giant liposomes (Fig. 2b), allows high entrapment efficiency, and can be used with high ionic strength buffers. By considering the diffusion of the droplet

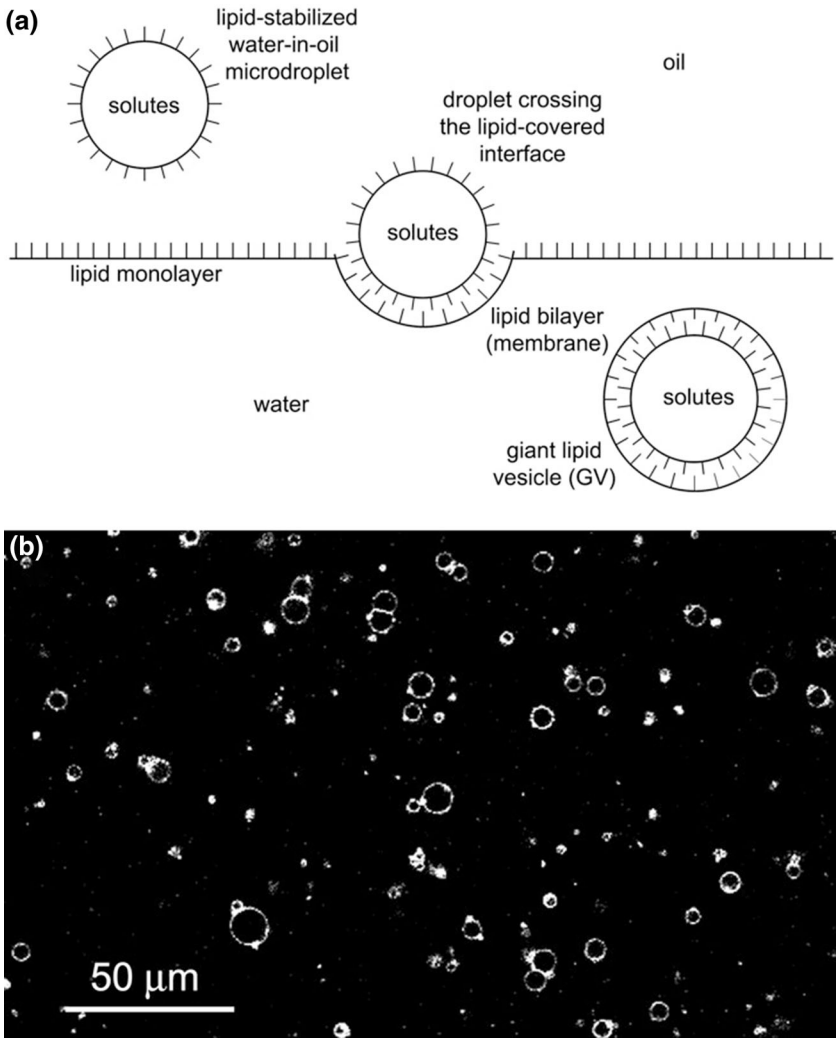



**Fig. 2** Production of solute-filled giant lipid vesicles, to be used as synthetic cells. **a** The method of droplet transfer (Pautot et al. 2003) consists in the transfer of a lipid-stabilized water-in-oil microdroplet through a lipid-covered interface. The water-in-oil droplet, whose surface is covered by a lipid monolayer, becomes covered by a second lipid monolayer so that a lipid bilayer is composed stepwisely. The resultant vesicle contains all solutes initially present in the water-in-oil droplet. The short segments represent schematically the lipid tails. **b** Confocal laser-scanning micrograph showing a population of phospholipid giant vesicles prepared by the droplet transfer method (Trypan Blue staining)

transfer method among the laboratories working with synthetic cells, this method will probably become a kind of synthetic biology "standard tool" for synthetic cell assembly. Interestingly, GVs have been also prepared in a microfluidic device (Matosevic and Paegel 2011) working on the principle of the droplet transfer method.

Protein-producing synthetic cells are obtained by inserting the proper number and type of biomacromolecules inside liposomes. The first report dates back to 2001 (Yu et al. 2001). The set of molecules required for protein synthesis is well known, and it is commonly referred as cell-free transcription-translation (TX-TL) machinery. Starting from a DNA sequence, these cell-free TX-TL machineries are capable of synthesizing proteins. Cell extracts (e.g., from *Escherichia coli*) are typically used as TX-TL machinery. The cell extracts, however, are not well characterized in terms of type and concentration of each single component, i.e., are black-boxes not really appealing for SB approaches. More recently, a well-characterized TX-TL machinery has been obtained: the PURE system (Shimizu et al. 2001). This kit is composed by 36 purified *E. coli* proteins, purified ribosomes and tRNAs, for a total of about 80 different macromolecules, and several low molecular weight compounds (amino acids, nucleotides, ions). The PURE system perfectly fulfils the requirements of synthetic biology in terms of characterized parts (*cf.* with the concept of "standard biological parts", http://partsregistry.org/Main_Page), and it is considered the preferred toolkit for the construction of synthetic cell. Thanks to the PURE system several proteins and enzymes have been synthesized inside liposomes (Stano et al. 2011b), including membrane enzymes (Kuruma et al. 2009). Cell-free protein synthesis proceeds for about 3–4 h, driven by high-energy compounds included in the kit, and it is not significantly affected by the presence of lipids. Thanks to the convergence of liposome technology and cell-free TX-TL systems it is therefore possible to produce functional proteins inside liposomes, and this means that it is potentially feasible the synthesis of enzymes, receptors, transporters and other protein-based actuators (nanomachines) for implementing a desired function in synthetic cells. The next step focuses on addressing the question: according to

which blueprint should synthetic cells be designed in order to encode/send or receive/decode chemical signals?

### 3.2 A possible experimental approach

With the aim of developing a new technology based on molecular communications (Nakano et al. 2011, 2013) a first proof-of-concept experiment is needed to show that the required biotechnological tools are available and that can be combined with each other in a compatible way. Details of molecular communications in biological cells of different types are well know, but in the the context of developing a SB approach based on synthetic cells with minimal complexity, we believe that exploiting the bacterial communication systems will be the most advantageous choice. This is because the bacterial world offers a plethora of communication systems well characterized at the molecular level, hence exploitable for synthetic biology approaches. Indeed, most bacterial genera coordinate their activities at the population level via a widespread intercellular communication system known as quorum sensing (QS). Bacterial QS systems have been extensively revised (Waters and Bassler 2005; West et al. 2006; Williams et al. 2007; Atkinson and Williams 2009). Briefly, QS relies on the synthesis, secretion/diffusion, reception and decodification of signal molecules by members of a bacterial community. QS signal molecules can be small peptides, or small chemicals synthesized by dedicated enzymes. The synthesis of some signal molecules requires complex multi-steps reaction sequences, while in other cases it is achievable via a single step enzymatic reaction from common substrates. The signal molecule is often secreted by living cells through transmembrane protein devices; however, in some cases, the signaling molecule is able to freely cross biological membranes and move from the sender to the receiver by simple diffusion in aqueous solution. For what concern signal reception and decodification, in some cases the receptor protein (which has high affinity for the cognate signal molecule) is a membrane sensor, and the binding of the signal molecule to the receptor causes a conformational change in the protein that transduces the signal to a cytoplasmic effector located inside the cell, which in turn starts an action (response). In this respect, the decodification mechanisms can have different complexity because signal transduction is often a step-wise process mediated by multiple proteins. On the contrary, some receptors are cytoplasmic bifunctional proteins that, upon signal molecule binding, directly activate a response, typically by acting as transcriptional regulators.

In order to design a minimal communication mechanism inspired to cell communication "protocols", a proper design in terms of choice of molecular parts and devices to be implemented in synthetic cells is needed. QS systems based on

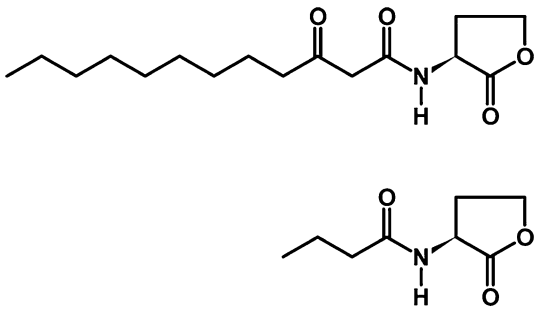



**Fig. 3** Acyl homoserine lactones (AHLs), with long (3-O-$C_{12}$-HSL, top) and short ($C_4$-HSL, bottom) acyl chains

*N*-acyl-homoserine lactones (AHLs, Fig. 3) as signal molecules are promising candidates to establish a synthetic/natural communication system. Indeed, AHLs synthesis requires a single step reaction usually driven by a synthase of the LuxI-like family, and are perceived by a cytoplasmic receptor belonging to the LuxR-family of transcriptional regulators. Moreover, AHLs are considered to be free-diffusible accross biological membranes. For this reason we are exploring the possibility to use *Pseudomonas aeruginosa*, a bacterium endowed with two QS systems based on distinct AHL molecules (reviewed in Williams and Cámara 2009), as natural cell to establish a communication channel with synthetic cells.

In principle, three kinds of practical implementation of chemical communication involving synthetic and natural cells can be envisaged: (*i*) synthetic-to-natural, (*ii*) natural-to-synthetic, and (*iii*) synthetic-to-synthetic. Clearly, different technical and theoretical consequences stem from these three cases and from their combinations, and in each individual case the synthetic partner and the natural partner of the communication should be endowed with specific functions.

Here we will focus on implementation (*i*), namely synthetic cells sending a chemical signal to natural cells (i.e., to bacteria).

### 3.3 Preliminary numerical modeling

The use of mathematical models is a major feature of SB, because a mathematical description of the system under study helps to quantitative thinking and supports a bioengineering approach. The overall strategy consists in a cycle of operations where design, modeling, implementation and experimental testing/validation follow to each other, so that a realistic model is obtained by recursive refinements.

Here, the system under study consists in a synthetic cell sending a chemical signal to a bacterium (Fig. 4). Note that this system can be considered from many viewpoints, hence eliciting a wide range of possible interpretations. In particular, since we refer to sending and receiving a signal molecule, it is important to recognize that it represents, in its essence, the extension of the cybernetic signal theory to

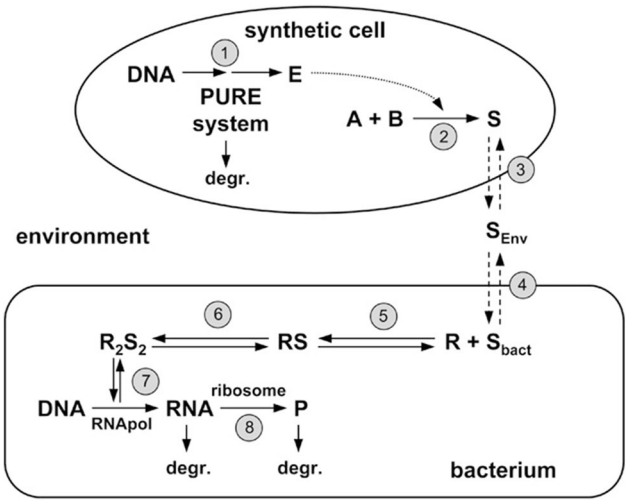



**Fig. 4** Simplified model of synthetic cell sending a freely diffusible chemical signal (S) to a natural cell. The corresponding ODEs set and the parameters used for the simulation are reported in Fig. 10 and Table 1, respectively

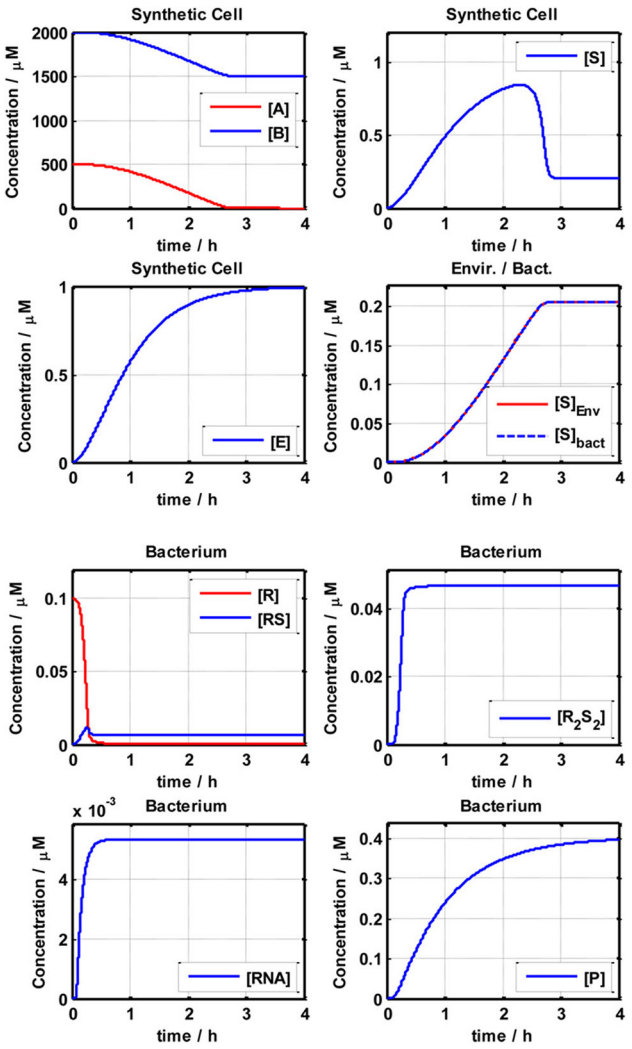



**Fig. 5** Results of numerical integration (the ODEs set is shown in Fig. 10)

biological systems. By reasoning on the analogy between chemical signals and electric signals manipulation, in this context SB often makes use of the concept of "logical gates" to describe such behavior (Baldwin et al. 2012).

The model presented here is based on the Multimedia Box approach that is largely used in the environmental exposure assessment (Van de Meent and De Bruijn 2007). It consists in dividing an ecosystem in different spatially homogenous (zero-dimensional) compartments (boxes) of fixed volume that can exchange chemical compounds by transport process taking place accross the compartment surface boundaries. The time evolution of chemical species present in each single box can be obtained by numerically solving a set of ordinary differential equations (ODEs) according to the supposed kinetic mechanism occurring in each compartment. This is a quite convenient choice for a first modeling attempt, although a more realistic model would instead consider the system as a stochastic one (Mavelli and Ruiz-Mirazo 2010; Mavelli 2012). Therefore, the time evolutions of both bacteria and synthetic cells are described only in average, so neglecting the rule of random fluctuations at this level of description (as an example of a different approach see Mavelli and Stano 2010).

Our model, shown in Fig. 4, has been built to describe a realistic case of synthetic cells producing a short chain AHL (freely permeable), that in turn triggers a response in bacteria. The system we have in mind consists in the synthesis of the QS AHL signal molecule *N*-butyryl homoserine lactone ($C_4$-HSL) catalyzed by a PURE system-produced RhlI enzyme, and an engineered *P. aeruginosa* as biological partner. However, the same model, with minor modifications, can be applied to other analogous cases.

We have modeled our system in a fixed solution volume $V$ (0.2 mL) where a number of bacteria $N_{bact}$ and of synthetic cells $N_{sc}$ coexist together in a precise ratio (320-to-1, as estimated from their concentrations). The synthetic cell diameter (5.4 μm) has been estimated from image analysis. The simulation refers to 4 h.

Inside the synthetic cell it takes place a sequence of two steps (see Fig. 4): (1) production of an enzyme E (a synthase, devoted to the synthesis of the signal molecule S); (2) generation of the signal molecule S from the substrates A and B by the synthase-catalized reaction. Next, S freely diffuses, according to the concentration gradient, from the synthetic cell to the environment (3), and from the environment to the bacterium (4). Inside each bacterium, S binds to a cytosol receptor R, to give the non-covalent complex RS (5), followed by the dimerization of RS to give the transcription factor $R_2S_2$ (6). The transcription of a reporter gene to produce the messenger RNA (mRNA), controlled by the concentration of $R_2S_2$ in a cooperative fashion (7), and the translation of mRNA into the reporter

protein P (8) complete the model, along with the RNA and protein degradation processes (here modeled as pseudo-first order steps). The complete ODE set and the list of all parameters that characterizes this model are reported in the Appendix.

In order to find the values of the thermodynamic and kinetic constants used in the model, we first looked for data referring to the AHL QS system. When not available, the missing values have been estimated as educated guesses. When more than one value was found, we used the geometrical mean.

In particular, step (1) has been modeled on the basis of an experimentally determined intra-liposome protein synthesis profile (Sunami et al. 2010), whereas step (2) is based on measured constants for short chain ($C_4$) AHL in vitro synthesis (Parsek et al. 1999), and modeled by using the integrated form of a Michaelis–Menten mechanism. A simple permeability equation has been used to model steps (3) and (4), by considering a highly diffusive S species (Pearson et al. 1999). The collection of parameters referring to steps (5)-(6)-(7)-(8) contains instead several estimated values, because several thermodynamic or kinetic constants are not known. We have estimated the unknown parameters as educated guess starting from known values for similar systems, and the kinetic equations have been shaped as reported in analogous modeling studies (Stögbauer et al. 2012). For example, starting from published considerations of long chain AHL binding to their receptor (Schuster et al. 2004), we estimated the binding constant of short-chain AHL—i.e., step (5)—by simple thermodynamic considerations (reduction of binding free energy for each missing methylene group) (*cf.* Nakatani et al. 1980). Dimerization constants in step (6) derive from generic consideration on the amount of dimer expected to be found for a sensitive reception system (i.e., 90 % dimer formation after S binding, see Ventre et al. 2003). A fast dimer formation and slow dimer dissociation complete our hypotheses about step (5). Similarly, step (7) constants (affinity of the transcription factor for DNA and the Hill coefficient to describe the binding cooperativity) were estimated on the basis of known values for receptors of AHL families (Schuster et al. 2004), even if it is well known that generalizations should be taken cautiously. Finally, for step (8) we consider a generic reporter protein, about 250 amino acid long, that could be, for example, a fluorescent protein or an enzyme allowing its facile detection. All remaining TX-TL constants and parameters (steps 7–8) were taken from the reported literature values (most of them refers to *E. coli*, see the free web database B10NUMB3R5, http://bionumbers.hms.harvard.edu/), and adapted to our model as detailed in Table 1.

According to our calculations (see Fig. 5), the PURE system would produce, inside liposomes, enough enzyme (1 μM) for an efficient conversion of the substrates A (500 μM) and B (2,000 μM) to the signal molecule S within about 2.5 h (the amount of S is determined by the limiting reactant, A in the present simulation). S diffuses away from the liposome to fill the environment. In the first couple of hours, the production rate of S overcomes its release; consequently, a peak of S concentration (ca. 0.8 μM) is observed inside liposomes. Due to its large volume, the external environment acts as a sink for the S molecules, which is diluted to a final concentration of about 0.2 μM. The signal molecule, however, reaches the bacteria, where it permeates rapidly and binds to the receptor R with high affinity ($10^3$ $\mu M^{-1}$). The complex RS firstly dimerizes—quite efficiently ($10^3$ $\mu M^{-1}$)—to form $R_2S_2$; then the dimer binds cooperatively (with an estimated Hill coefficient = 1.5) to DNA, so activating the transcription of the reporter gene. The mRNA production profile results highly sigmoidal, like a switch, and the stationary state mRNA concentration levels at about 5 nM (due to the concurrent mRNA degradation inside the bacterial cell). Finally, the reporter protein is obtained by ribosomal synthesis, and it follows a sigmoidal profile, but not so steep as the mRNA one, reaching a plateau value of 0.4 μM after 4 h.

The scenario emerging from this simple simulation, that—it is noteworthy to remind—contains several approximations, is quite favourable because it suggests that (*i*) synthetic cells can produce, in principle, an experimentally detectable amount of signal molecule (for instance, measureable by analytical techniques), and (*ii*) that the amount of reporter protein in the bacteria appears to be adequate for its detection and quantitation. In fact, if P is a fluorescent protein, its calculated intra-bacterial concentration (0.4 μM) lies well above the detection limit (around 0.05 μM) and its quantitation can occur also at the level of single bacterial cell. If P is an enzyme, for instance luciferase, its detection also appears feasible by considering typical instrumental detection limits (in samples of about $10^8$ bacteria, luciferase concentrations higher than 0.01 μM inside each bacterium should be detectable).

By keeping constant all thermodynamic and kinetic parameters, and by considering the estimated parameters as reliable, it results that our final observable, the amount of reporter protein P, is a quite robust indicator, being quite insensitive to changes in: (*i*) the $N_{bact}/N_{sc}$ ratio (from 1,000:1 to 16:1); (*ii*) the initial concentration of the limiting reactant ($[A]_0$, from 500 to 2,000 μM); (*iii*) the binding constant between R and S ($k_{on}$ from 1 to $10^3$ $\mu M^{-1}$ $s^{-1}$, by keeping $k_{off} = 0.1$ $s^{-1}$); and (*iv*) the initial concentration of the receptor R inside the bacterium ($[R]_0$ from 1 to 0.001 μM). The latter parameter is a quite important one, because it depends, in bacteria, from several other parameters, typical of the bacterial metabolic state, that are difficult to model in a simple way. It is instead the permeability of the signal molecule S through the liposome and bacterial membrane that play a major role in determining the timing of the biological response (fast response when S is highly permeable, delayed response when S is less permeable). It results,

however, that in all cases after 4 hours enough reporter protein is present in the bacteria, and its detection would be then possible (data not shown).

An interesting question related to the system under study refers to the identification of the most critical factors or rate-determining steps that influence the success of the synthetic-to-natural cell communication dynamics (synthesis of a signal molecule, transmission, reception/decoding of signal). By comparing the rates of the various processes, we conclude that the onset of bacterial RNA synthesis is indeed the most relevant factor. In turn, it depends on the availability of the dimer $R_2S_2$, which—however—forms quite readily in the presented model, thank to high R-to-S affinity. Therefore, a possible way to improve the bacterial response should focus on optimizing the reception circuitry and alternating—if possible—the internal bacterial status.

Let us also quickly discuss what the main assumptions and limitations of the model are. As mentioned, steps (1–2) of our model are considered as quite reliable, because they are based on experimentally determined thermodynamic and kinetic constants. We confirmed that the sigmoidal profile derived from the Yomo's equation (Sunami et al. 2010) fits with data available in our lab (green fluorescent protein synthesized by the PURE system). There are no a priori reasons to doubt that the same equation can also describe the synthesis of the enzyme E. However, the model does not consider that some enzyme molecules could not be produced in a well-folded state, so that the actual S production rate could be less than expected. The model also foresees that all synthetic cells are equally capable of synthesizing E, neglecting the between-synthetic cell diversity (a similar consideration holds also for bacteria, but at a minor extent).

A more general consideration refers to our choice of modeling this system with a set of ODEs. This was done primarily for its simplicity; we are indeed engaged in a long-term project which will approach the modeling of synthetic-to-natural, natural-to-synthetic, and synthetic-to-synthetic communication channels with different analytical tools. In particular, we aim at integrating in our model all sources of noise (intrinsic and extrinsic stochastic effects (Mavelli 2012) in synthetic and natural cells, and environmental noise). This is an important consideration when small-scale systems are modeled, as in the present case.

In conclusion, the simplified model presented here is a useful tool for the construction of a synthetic-to-natural communication system, based on a SB approach. It will be tested, optimized, modified and refined as soon as experimental data become available. It integrates the wet-lab approach with quantitative evaluations and/or order-of-magnitude estimates, which can be very helpful for designing the experiments in the proper way.

### 3.4 Preliminary experimental results

As discussed in the previous chapter, a mathematical modelling supports the feasibility of our research program. However, the proposed in silico simulation takes for granted some assumptions of primary importance that have not been yet supported by experimental wet-lab experiments. In particular, we believe that the main drawbacks of our experimental plan could be: (*1*) the stability of synthetic cells in the aqueous environment used to grow bacteria; (*2*) the ability of bacteria to respond to low levels of signal molecule possibly produced by the synthetic cells; (*3*) the actual diffusibility of the signal molecule across the synthetic cell artificial membrane (a lipid bilayer).

For what concern point (*1*), both the synthetic sender cells and the natural (bacterial) receiver cells must be stable and functional in the same environment to establish a chemical communication. In particular, bacteria should be grown in a medium able to support the metabolic functions necessary to produce a response to the signal sent by the synthetic cell. However, the same medium should fit chemical and physical requirements for synthetic cells formation, stability and functionality. To assess this issue we tried to produce conventional liposomes by idrating a dry lipid film with a conventional bacterial growth medium, LB. As shown in Fig. 6a, after liposome extrusion through a 100 nM pore-size membrane, we obtained a "population" of liposomes with uniform and narrow size range, demonstrating the absence of liposome aggregation or fusion, which could be due to membrane destabilization by some LB components. By using the above-mentioned droplet transfer method (Pautot et al. 2003), we have been able to produce GVs that are stable in LB and that synthesize the green fluorescent protein (GFP), although some GVs were found attached to each other (Fig. 6b).

Another important issue connected to the coexistence of natural and synthetic cells is that bacteria should not jeopardize the physical stability of synthetic cells by misinterpreting them as feedstuff. Many bacterial species can use phospholipids (the main constituents of synthetic cell membranes) as carbon source, by producing enzymes that degrade these molecules (Titball 1998). Moreover, many bacteria synthesize surfactants that could destabilize liposomal membranes, as well as enzymes with AHLs-degrading activity (Amara et al. 2011, Maier 2003). Also the bacterium chosen for this research programme, *P. aeruginosa*, can produce both lipases and surfactants with strong membranelytic properties (i.e. rhamnolipids) (Rosenau and Jaeger 2000, Reis et al. 2011). To explore these stability issues we have performed a liposome stability analysis in the presence of a *P. aeruginosa* culture grown in LB, by means of classic calcein test in conventional vesicles. At high intra-liposome concentration, calcein is self-quenched (low fluorescence).

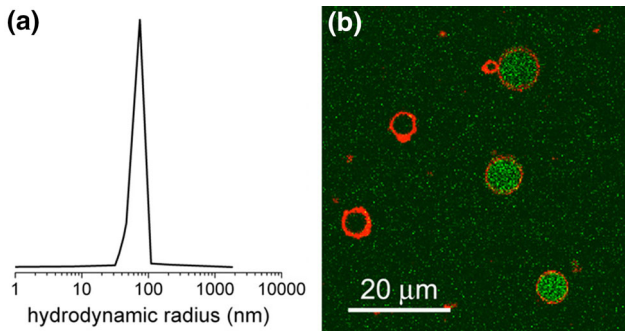



**Fig. 6** Lipid vesicles in LB medium. **a** Dynamic light scattering analysis reveals that extruded vesicles (initial radius 100 nm) maintain their size and do not aggregate in LB medium. **b** PURE-system containing synthetic cells based on giant lipid vesicles produce the green fluorescent protein when suspended in LB medium (membrane staining by Trypan Blue)

If released in the medium, as a consequence of liposome lysis, calcein is diluted and becomes fluorescent (Fig. 7a). As shown in Fig. 7b, low-fluorescence emission indicates that liposomes are stable in a *P. aeruginosa* culture for at least 4 h. The experiments described above provide the first demonstration that synthetic cells can be stable and functional in bacterial culture medium such as the widely used LB, also in the presence of bacteria producing lipases and surfactants (like *P. aeruginosa*). Future analyses will be extended to GVs.

At present we are experimentally validating the feasibility of point (*2*) by generating an engineered bacterial strain able to perceive and AHL signal possibly sent by synthetic cells, and to consequently respond by expressing a gaugeable phenotype. Obviously, in the synthetic-to-natural communication design, the synthetic cell should be the only source of chemical signals to avoid undesired autoactivation of the response, therefore the bacteria must be "signal negative", i.e., deficient/impaired in the synthesis of the signal molecule. Briefly, we generated a *P. aeruginosa* strain mutagenized in the gene *rhlI*, encoding for the AHL molecule $C_4$-HSL synthase RhlI. This strain, named Δ*rhlI*, is deficient in the synthesis of the signal molecule $C_4$-HSL, while normally expressing the cognate receptor RhlR.

Subsequently, we introduced in the Δ*rhlI* strain a genetic cassette P*rhlA*::*lux*, in which the promoter P*rhlA*, known to be activated by the RhlR/$C_4$-HSL complex, controls the expression of the *luxCDABE* operon. This operon contains the genes coding for the enzyme luciferase and for the enzymes required for the synthesis of the luciferase's substrate. Therefore, when grown in the presence of exogenous $C_4$-HSL, the RhlR receptor is active as a RhlR/$C_4$-HSL complex, and drives the expression of the *luxCDABE* operon from the P*rhlA* promoter, ultimately resulting in light emission (Fig. 8a). In this way, the response of the bacterium to the signal molecule $C_4$-HSL, possibly produced by synthetic cells, can be easily detected and quantified by an automated luminometer, also at the microvolumetric scale. We verified this phenomenon by measuring light emission from the bacterial strain Δ*rhlI* P*rhlA*::*lux* grown in the absence and in the presence of different concentrations of the synthetic molecule $C_4$-HSL. As shown in Fig. 8b, a detectable bacterial response was obtained with $C_4$-HSL at a concentration $\geq$30 nM. Notably, this signal molecule concentration is considerably lower with respect to the concentration predicted to be produced by synthetic cells in the presented mathematical modeling (see Sect. 3.3), suggesting that the engineered bacterial strain Δ*rhlI* P*rhlA*::*lux* is suitable for our research programme.

For what concern point *3*), it is to consider that AHL signal molecules, including $C_4$-HSL, are freely diffusible across biological membranes, whose permeability properties are not the same as synthetic membranes. Indeed, the different lipid composition and protein content of natural membranes and liposomes (e.g., liposomes completely lack porin proteins) could impair diffusion of AHL signal molecule possibly produced inside synthetic cells towards the natural receiver cell. To investigate this topic, liposomes loaded with synthetic $C_4$-HSL were produced and incubated with the Δ*rhlI* P*rhlA*::*lux* reporter strain previously described. Gel

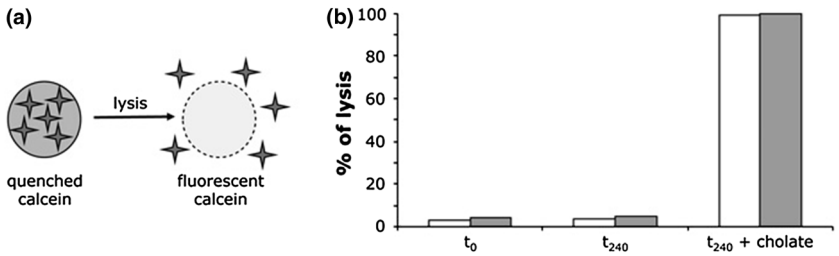



**Fig. 7** **a** Schematic representation of the calcein test used to assess liposome stability. **b** Graph reporting fluorescent emission from calcein-loaded liposomes generated in the bacterial growth medium LB, and incubated for 240 min in LB (*white bars*) or in a *P. aeruginosa* culture grown in LB (*grey bars*). As a control, cholate was added to induce liposome lysis after 240 min incubation ($t_{240}$ + cholate). Maximal fluorescent emission measured is considered as 100 % lysis. Reproduced from (Rampioni et al. 2013)

permeation chromatography of $C_4$-HSL-loaded liposomes was performed to separate the liposomal fraction from not entrapped $C_4$-HSL molecules. The eluted liposome fractions were incubated with the Δ*rhlI* P*rhlA*::*lux* reporter strain for $C_4$-HSL detection; empty liposomes prepared following the same procedure were used as a control. As shown in Fig. 9, the liposomes containing $C_4$-HSL were able to induce light emission in the Δ*rhlI* P*rhlA*::*lux* cells, clearly indicating that this signal molecule can freely diffuse from inside liposomes to the receiving natural cells.

On a whole, the preliminary wet-lab experiments presented in this paper demonstrate the feasibility of our approach for the generation of a synthetic-to-natural communication system in which SSMCs will be able to activate a response in the bacterium *P. aeruginosa* via AHL QS molecules. Experiments are currently in progress with the aim of achieving a fully synthetic bottom-up generation of signal molecules inside synthetic cells, and the consequent activation of a measureable response in the bacterial partner.

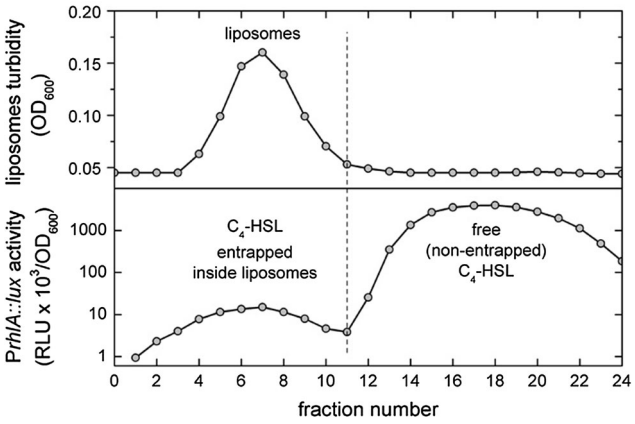


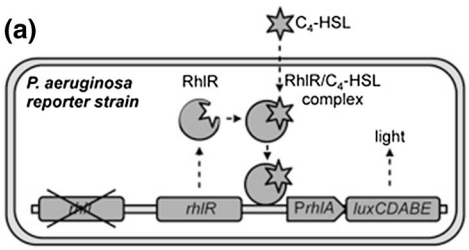

**Fig. 9** Encapsulation of $C_4$-HSL inside conventional liposomes (radius 200 nm) and activation of a bacterial response. Liposomes were prepared by the thin film hydration method in the presence of $C_4$-HSL, which is partially entrapped. In order to remove non-entrapped $C_4$-HSL, liposomes were purified by gel filtration chromatography on Sepharose 4B minicolumn, collecting 24 fractions. The turbidity profile (*top*) reveals that liposomes are present in fractions 4–10. By incubating each fraction with the Δ*rhlI* P*rhlA::lux* bacterial reporter, it is possible to detect its activation (in terms of normalized light emission, *bottom* profile), confirming that $C_4$-HSL was indeed encapsulated inside liposomes and that it is spontaneously released in the medium. Note also that due to the small liposome size, vesicles could not entrap most of the $C_4$-HLS (fractions 12–24). Empty liposomes, used as control, did not activate the reporter (data not shown)

## 4 Theoretical considerations and concluding remarks

In this work we provided the theoretical and preliminary practical bases for the development of a chemical communication system between synthetic and natural cells (bacteria), here intended as a novel approach to the emerging field of bio-chem-ICTs.

Synthetic cell technology, based on a bottom-up SB design, can be adapted and extended for reaching the ambitious goal of interfacing with biological cells, and this is possible by letting synthetic cells share with biological cells a common (chemical) language.

By preliminary numerical and wet-lab explorations, we have first tested whether the scenario we have in mind for a specific research program its realistic, by evaluating quantitatively its potential behavior. Our model, based on realistic estimates of thermodynamic and kinetic parameters, clearly shows that the current state-of-the-art of synthetic cell technology is mature enough for attempting such an approach. Moreover, we have proved that conventional liposomes are sufficiently stable in culture medium such as the widely used LB, also in the presence of bacteria producing lipases and surfactants. Functional proteins (GFP) can be produced in GVs prepared in LB, and further experiments are currently carried out in our laboratory. Moreover, by an optimization procedure, we have constructed a



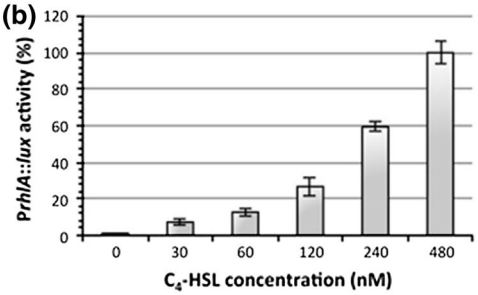




**Fig. 8** Schematic representations of (**a**) an engineered *P. aeruginosa* strain unable to synthesize $C_4$-HSL as a consequence of a mutation introduced in the *rhlI* gene, but able to respond to exogenous $C_4$-HSL via the RhlR receptor. P*rhlA*, promoter activated by the RhlR/$C_4$-HSL complex; *luxCDABE*, operon coding for the enzymes required for light emission. **b** Histogram reporting the activity of the Δ*rhlI* P*rhlA::lux* reporter strain grown in LB for 3 h in the presence of different stock (10×) concentrations of synthetic $C_4$-HSL. The Relative Light Units (RLU) were normalized by the cell density of the bacterial culture ($A_{600}$), and the resulting values were reported as % of activity with respect to the highest (100 %) and the lowest (0 %) promoter activities measured

reporter bacterial strain able to detect a signal molecule in a range of concentration that fits well with the calculated amount of signal molecule produced by a synthetic cell, as inferred by our mathematical model. In the near future we will report the outcome of experiments aimed at testing whether synthetic cells are indeed able to send chemical messages to these engineered bacteria.

We also believe that grounding these experimental scenarios on an accurately chosen theoretical framework will allow them to be relevant not only for applications in biotechnological fields, but also for further progress in fundamental science.

As mentioned above, the bottom up SB approach, on which the semi-synthetic minimal cells are based, relies on autopoiesis, and the autopoietic approach is allowing experimental research on minimal life and the origins of life since the early 1990s (Luisi and Varela 1990; Luisi 1996). We are convinced that the experimental scenarios proposed here can allow the extension of this research to the fascinating issue of *minimal cognition*, and, in this way, can enable SB to contribute to AI research. In particular, this can happen with regard to the investigation of (minimal/chemical) communication, which is the biological basis of the development of minimal forms of cognition in higher forms. On this basis, we think that the SB approach we present here might generate a SB program in AI based on autopoiesis. Further work (both experimental and theoretical) is required to fully develop and expand these preliminary ideas, which promise to be very fecund.

**Acknowledgments** Parts of this work have been published in the proceedings of the Italian Workshop on Artificial Life and Evolutive Computation (Rampioni et al. 2013). This study was supported by grants from the Ministry of University and Research of Italy (Futuro in Ricerca 2010—RBFR10LHD1) and from the University Roma Tre (Internationalization Project). Luisa Damiano is grateful to the EU-FP7-FET COBRA project (Coordination of Biological and Chemical IT Research Activities), for supporting a research visit to the University Roma Tre (Rome). The authors thank Pier Luigi Luisi (University Roma Tre) for inspiring discussions on synthetic cells and autopoiesis. Hagen Lehmann is acknowledged for his help in preparing the manuscript final version.

## Appendix: Details about the mathematical model

The ODE set (Fig. 10) has been numerically integrated by writing a homemade program based on the Rosenbrock method suitable for stiff differential equations (Hairer and Wanner 1996). The parameters involved in the model are shown in detail in Table 1.

**I. Synthetic cell**

1 $$\frac{d[E]}{dt} = (k_{TXPS}t)\cdot k_{TLPS}\exp(-k_{inactPS}t)$$

2 $$\frac{d[A]}{dt} = \frac{d[B]}{dt} = -k_{cat}[E]\frac{[A]}{K_{MA}+[A]}\frac{[B]}{K_{MB}+[B]}$$

3 $$\frac{d[S]_{sc}}{dt} = k_{cat}[E]\frac{[A]}{K_{MA}+[A]}\frac{[B]}{K_{MB}+[B]} - \frac{\sigma_{sc}\wp}{V_{sc}}([S]_{sc}-[S]_{env})$$

**II. Environment**

3,4 $$\frac{d[S]_{env}}{dt} = N_{sc}\frac{\sigma_{sc}\wp}{V_{sc}}([S]_{sc}-[S]_{env}) + N_{bact}\frac{\sigma_{bact}\wp}{V_{bact}}([S]_{bact}-[S]_{env})$$

**III. Bacterium**

4,5 $$\frac{d[S]_{bact}}{dt} = -\frac{\sigma_{bact}\wp}{V_{bact}}([S]_{bact}-[S]_{env}) - k_{on}[R][S]_{bact} + k_{off}[RS]$$

5,6 $$\frac{d[R]}{dt} = -k_{on}[R][S]_{bact} + k_{off}[RS]$$

5,6 $$\frac{d[RS]}{dt} = k_{on}[R][S] - k_{off}[RS] - 2k_{\dim}[RS]^2 + 2k_{diss}[R_2S_2]$$

6 $$\frac{d[R_2S_2]}{dt} = k_{\dim}[RS]^2 - k_{diss}[R_2S_2]$$

7 $$\frac{d[mRNA]}{dt} = \frac{1}{3L}k_{TX}C_{RNApol}\frac{C_{DNA}}{K_{MTX}+C_{DNA}}\cdot\frac{[R_2S_2]^n}{K^n_{MR_2S_2}+[R_2S_2]^n} - k_{\deg,mRNA}[mRNA]$$

8 $$\frac{d[P]}{dt} = \frac{1}{L}k_{TL}C_{rib}\frac{[mRNA]}{K_{MTL}+[mRNA]} - k_{\deg,P}[P]$$

**Fig. 10** Complete set of the kinetic differential equations used in the model. The time evolution of the internal species concentration for synthetic cells and bacteria represents the average over the two populations, assuming that each compartment behaves similarly, that is, by neglecting stochastic fluctuations

**Table 1** Physical parameters, thermodynamic and kinetic constants used in the model

| Step | Symbol | Meaning | Value | Units | Note |
|---|---|---|---|---|---|
| | V | Reaction volume | $2\ 10^5$ | $\mu m^3$ | 1 |
| | $N_{sc}$ | Number of synthetic cell in V | 1 | | 2 |
| | $N_{bact}$ | Number of bacteria in V | 320 | | 3 |
| | | Synthetic cell radius | 2.7 | μm | 4 |
| | $\sigma_{sc}$ | Synthetic cell surface | 91.6 | $\mu m^2$ | 5 |
| | $V_{sc}$ | Synthetic cell volume | 84.2 | $\mu m^3$ | 6 |
| | $V_{bact}$ | Bacterium volume | 1 | $\mu m^3$ | 7 |
| | $\sigma_{bact}$ | Bacterium surface | 4.8 | $\mu m^2$ | 8 |
| 1 | $k_{TXPS}$ | Transcription rate (PURE system) | | | 9 |
| | $k_{TLPS}$ | Translation rate (PURE system) | | | 10 |
| | $k_{TXPS}\ k_{TLPS}$ | Product of TX-TL rates (PURE system) | $2.8\ 10^{-7}$ | $\mu M\ s^{-2}$ | 11 |
| | $k_{inactPS}$ | Translation inactivation constant (PURE system) | $5.3\ 10^{-4}$ | $s^{-1}$ | 12 |
| 2 | $k_{cat}$ | Catalytic constant of the enzyme E | 0.1 | $s^{-1}$ | 13 |
| | $K_{MA}$ | Michaelis–Menten constant for A | 10 | μM | 14 |
| | $K_{MB}$ | Michaelis–Menten constant for B | 200 | μM | 15 |
| | $[A]_0$ | Initial concentration of A | 500 | μM | 16 |
| | $[B]_0$ | Initial concentration of B | 2,000 | μM | 17 |
| 3,4 | $\wp$ | Permeability coefficient | 0.1 | $\mu m\ s^{-1}$ | 18 |
| 5 | $K_{binding}$ | RS thermodynamic binding constant | $10^3$ | $\mu M^{-1}$ | 19 |
| | $k_{on}$ | RS binding rate constant | $10^2$ | $\mu M^{-1}\ s^{-1}$ | 20 |
| | $k_{off}$ | RS dissociation rate constant | 0.1 | $s^{-1}$ | 21 |
| | $[R]_0$ | Initial concentration of the receptor | 0.1 | μM | 22 |
| 6 | $K_{dim}$ | $R_2S_2$ thermodynamic dimerization constant | $10^3$ | $\mu M^{-1}$ | 23 |
| | $k_{dim}$ | $R_2S_2$ dimerization rate constant | 1 | $\mu M^{-1}\ s^{-1}$ | 24 |
| | $k_{diss}$ | $R_2S_2$ dissociation of the dimer rate constant | $10^{-3}$ | $s^{-1}$ | 25 |
| 7 | $3L$ | Length of the mRNA | 750 | Bases | 26 |
| | $k_{TX}$ | Transcription rate | 50 | NTP $s^{-1}$ | 27 |
| | $K_{MTX}$ | RNA polymerase/DNA binding constant | 0.5 | μM | 28 |
| | $K_{MR2S2}$ | Hill affinity constant of $R_2S_2$/DNA promoter | $2.5\ 10^{-5}$ | μM | 29 |
| | $n$ | Hill cooperative coefficient | 1.5 | | 30 |
| | $k_{deg\text{-}mRNA}$ | mRNA degradation rate constant | $3\ 10^{-3}$ | $s^{-1}$ | 31 |
| | $C_{RNApol}$ | RNA polymerase concentration | $6\ 10^{-2}$ | μM | 32 |
| | $C_{DNA}$ | Promoter/reporter gene concentration | $2\ 10^{-3}$ | μM | 33 |
| 8 | $L$ | Length of the reporter protein P | 250 | aa | 34 |
| | $k_{TL}$ | Translation rate | 15 | aa $s^{-1}$ | 35 |
| | $K_{MTL}$ | Ribosome/mRNA binding constant | 0.1 | μM | 36 |
| | $k_{deg\text{-}P}$ | Protein degradation rate constant | $3\ 10^{-4}$ | $s^{-1}$ | 37 |
| | $C_{rib}$ | Ribosome concentration | 0.04 | μM | 38 |

*1*, Calculated by considering the number of GVs typically produced in one experiment (about 20,000) dispersed in a sample volume of 200 μL; *2*, See 1; *3*, Calculated by considering typical bacterial $OD_{600}$ of about 0.8, in 200 μL (ca. $8\ 10^8$ *E. coli* cells/mL gives $OD_{600} \sim 1$); *4*, Rough average of GVs radius; *5*, –; *6*, –; *7*, Rough estimate of single bacterium volume; *8*, From 7., and by considering a spherical shape; *9*, –; *10*, –; *11*, From (Sunami et al. 2010) by considering a working DNA concentration (in synthetic cells) of 20 nM; *12*, See 11; *13*, Referred to the in vitro synthesis of short-chain ($C_4$) AHL, from (Parsek et al. 1999); *14*, See 13; *15*, See 13; *16*, Estimated by considering realistic experimental conditions; *17*, See 16; *18*, Referred to short-chain ($C_4$) AHL diffusion (equilibration time <30 s), from (Pearson et al. 1999); *19*, A rough estimate of the binding constant of long-chain (3-O-$C_{12}$) AHLs to their receptor (see also Schuster et al. 2004) could be $10^6$ μM ($\Delta G_{binding} \sim -8.3$ kcal $mol^{-1}$). From this value, an educated guess of short chain ($C_4$) AHLs binding to their receptor can be calculated under the simplifying hypothesis that—other things being equal—the binding energy is reduced due to missing hydrophobic interactions (minor number of $CH_2$ groups). By considering a contribution of $\sim$0.6 kcal $mol^{-1}$ per each methylene group (Nakatani et al. 1980), an estimate of −4.1 kcal $mol^{-1}$ is obtained (i.e. $K_{binding} \sim 10^3$ μM); *20*, Estimated in order to consider a rapid binding kinetics; *21*, See 20; *22*, Estimated; *23*, Estimated by considering a significant ($\sim$90 %) dimerization (Ventre et al. 2003); *24*, Estimated; *25*, Estimated; *26*, Referred to a reporter protein 250 amino acid long; *27*, Geometric mean from values in B10NUMB3R5 database; *28*, Estimated; *29*, Estimated as the geometric mean of values referred to similar transcription factors (Schuster et al. 2004); *30*, Estimated as a value between 1 and 2 in order to consider a cooperative binding between the dimer $R_2S_2$ and possibly palindromic promoters; *31*, From B10NUMB3R5 database; *32*, Estimated as the fraction (1/100) of the total RNA polymerase pool in *E. coli* (6 μM from B10NUMB3RS database, geometric mean) involved in the transcription of the reporter protein; *33*, Calculated by considering one promoter region (one reporter gene) in the *E. coli* chromosome (1 molecule/cell); *34*, See 26; *35*, Geometric mean from values in B10NUMB3R5 database; *36*, Estimated; *37*, From B10NUMB3R5 database; *38*, Estimated as the fraction (1/100) of the total ribosome pool in *E. coli* (40 μM from B10NUMB3R5 database, geometric mean) involved in the translation of the reporter protein

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