RESEARCH ARTICLE

Biomachines for Medical Diagnosis

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Current evolutions in medical practices induce a change of paradigm with the convergence of diagnosis and therapy, going to precision medicine and "theranostics". One can observe the new role of biomarkers in biomedical and therapeutic applications, for instance in the development of molecular multiplex biosensors (nucleic acid, proteins, and metabolites). In addition, there is an increasing interest for point-of-care (POC) and of home monitoring/testing technologies devoted to probe patient parameters in his direct environment. The obvious constraints for such a kind of new clinical practices are simplification, drastic cost reduction while keeping high performances. Within this context, synthetic biology provides new opportunities to develop a novel generation of biological biosensors able to perform multiplexed biomarkers detection, simple computation and returning simplified relevant results. In order to design robust synthetic biological biosensor systems reliable in a clinical context and based on biochemical circuits, we developed an original methodology ensuring biochemical implementation of logical tasks within nonliving artificial cells. This methodology covers *in silico* design, simulation, microfluidics production and clinical validation on human samples. It ends up in very simple assay like for instance the new insulin-resistance assay, which is also quick and easy to run out of a laboratory and at low cost.

Introduction

Medical diagnosis is an evolving field. In general, the diagnosis is based on the detection of biomarkers (objective and measurable parameters that signify a pathological state or a risk) from dedicated technologies. This detection of biomarkers must lead to decisions in very different terms: risk assessment, characterization of pathological states or clinical stages. An international study has shown that diagnostic tests account for less than 5% of hospital costs and around 1.6% of health costs. On the other hand, their results contribute in 60 to 70% of the medical decision (Source = The Lewin Group, Inc., The Value of Diagnostics Innovation, Adoption and Diffusion into Health Care July 2005).

Recently we have witnessed a paradigm shift with the convergence of diagnosis and therapy leading to new directions towards personalized medicine, precision and theranostics. This is characterized by the development of companion diagnostic tests of targeted therapies (for example in the case of resistance to treatments). These tests allow stratification of patients or monitoring of treatment. Similarly, the concept of biomarkers has been considerably broadened to be very variable in nature (nucleic acids, proteins, metabolites, etc.) and the consideration of multi-scale complexity is finally recognized as a necessity. Diagnosis has always directly benefited from technological advances that can improve performance in terms of sensitivity, specificity, and throughputs. For instance, bioelectronics devices brought tangible improvements in term of cost effectiveness and usability. The diagnostic sector is culturally adapted to technological innovation thanks to the interface with basic research, and foster by the fact that the "time to market" for diagnosis innovation is faster than in the context of pharmacy (drug discovery).

Consequently, new clinical practices are emerging with the desire to open up on POC (Point of care), self-test by the patient, or home monitoring and this generates new interfaces between patients, ICT (Information and Communication Technologies) or electronics.

Thus, to address the new medical requirements for advance alert, frequent use, disease identification, personalized treatment prescription and patient follow up, it is required to simplify the tests, to drastically diminish their cost and response-time. All of this must be performed while keeping high performances to ensure patient benefit and to be compliant with the current regulatory constraints. Thus, a classical diagnostic test involving sample collection, assay running in a laboratory, with complex machines and educated operator will be less relevant in the future. Portable electronic devices emerge to fill the current needs with increasing success. However, one can observe that this new kind of simplified and portable devices show degraded performance compared with existing heavy technologies in laboratories. It is mainly because these solutions involve electronics-biochemistry interfaces that suffer from lack of sensitivity, specificity to detect tiny bio molecular events in a very noisy and wet environment (blood, serum, urine, etc.). Given these observations, we

decided to mimic the most performant system able to sense and interpret tiny biological parameters: the biological cells. Our objective was to design artificial cell as we design machines in order to run sensitive and specific diagnostic assays into biological noisy environments. Synthetic biology allows reprogramming biological cells for many purposes including medical diagnosis [**1**,**2**]. Living cell such as bactosensors were already designed to run medical diagnosis assays directly into blood or urine [**3**]. But living cells are difficult to controls in terms of composition and behaviors at the individual scale [**12**]. This lead to unexpected artificial cell behaviors among time. To overcomes all these difficulties and keep the performances of biological systems we developed and describe in this article a methodology to design, produce and validate simplified artificial cells. This artificial cells are little biomachines programmed to run specific medical diagnosis assays showing equivalent performances compared to heavy laboratory solutions.

Biomachines principles

To design our biomachine we used the basic principles of synthetic biology. This means designing artificial cells by combining elements or modules from existing biology. In our case, instead of designing alive cell by reprogramming it, we decided to design very simplified artificial cells from scratch. As well to avoid any unexpected variation in composition and modification in time our artificial cells are not alive. Although 100% made of biological compounds they are more biochemical constructions than alive systems.

Our biomachines can be divided into 3 modules: biomarkers identification of different biomarkers, integration of these signal into a decision algorithm (mimicking clinical decision), readout of the result under various possible form (visible dying, fluorescence, electrons etc.). The specific biomarkers quantitative detection is performed by biochemical reactions (enzymatic) designed in purpose. These reactions are organized in network to implement a logic response with given threshold and kinetics. The final qualitative response is then provided (**Fig. 1**). The membrane of our systems (here bilayer vesicle) provides a selective barrier controlling the exchange between inner et outer compartments of the vesicles. Thus, it prevents functional alteration of our artificial biochemical network due to the complexity of tested sample.

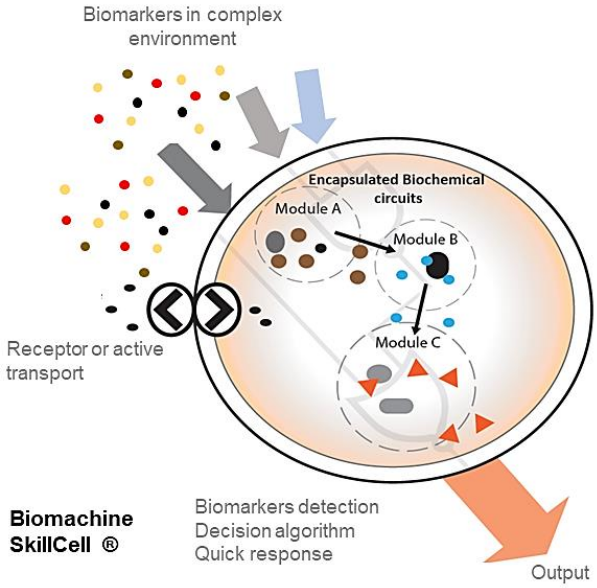


Fig. 1. Biomachine principle or medical diagnosis. Biomarkers such as metabolites, proteins, etc. are captured by our biomachine thanks to passive or active mechanisms. The artificial biochemical network is encapsulated into a bilayer membrane vesicle. When the right combination of biomarkers quantities is identified then a qualitative response is provided.

The overall conception of our biomachines combines *in silico* and experimental steps (**Fig. 2**). After having identified a precise clinical question targeting a specific human sample (blood, serum, urine, saliva, etc.), we formalize the clinical decision algorithm into a logical function. Then using our design software suite we build various biochemical networks potentially satisfying the logic function with the desired specific biomarkers as input parameters. Then these networks are evaluated *in silico* for their robustness and performances. The next step is the real fabrication of vesicles encapsulating the designed network. Finally, these artificial cells can be tested under real experimental conditions.

In silico design

To design formal models of bio-sensing and bio-computing problems we had to identify precise biochemical implementations satisfying Boolean Logic, molecular input/output, dynamic range and kinetic specifications within a large multidimensional design space. The first step was to develop a systematic *in silico* framework to almost

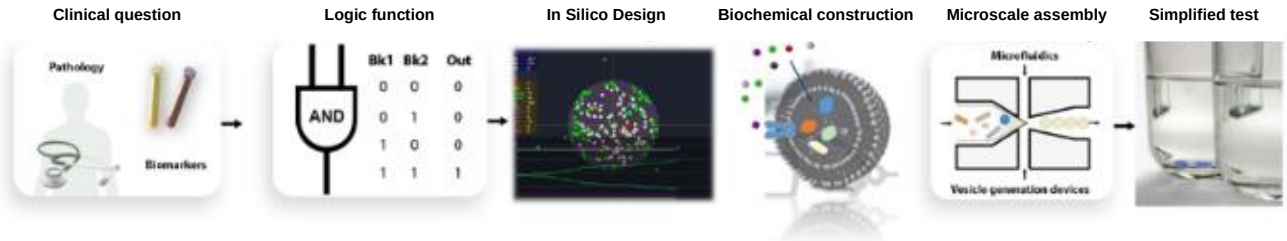


Fig 2. Design schedule of Skill Cell design for diagnosis.

automatically design synthetic biochemical logic circuits. We first developed the computational tool, SkillCell Maker, which automatically proposes bio-chemical implementations of circuits performing a given Boolean function, with specific biochemical inputs and output. Second, we developed and refined HSIM (Hyperstructure Simulator), a hybrid SSA and entity based stochastic and ODEs simulator, which enables fast and accurate model prediction and allows performing large assessment of kinetically and functionally suitable logic devices and circuits (**Fig. 3**). Third, we use BIOCHAM (Biochemical Abstract Machine) to optimize the initial concentrations of enzymes in order to maximize the robustness of the design with respect to its functional specification expressed in quantitative temporal logic [**4**].

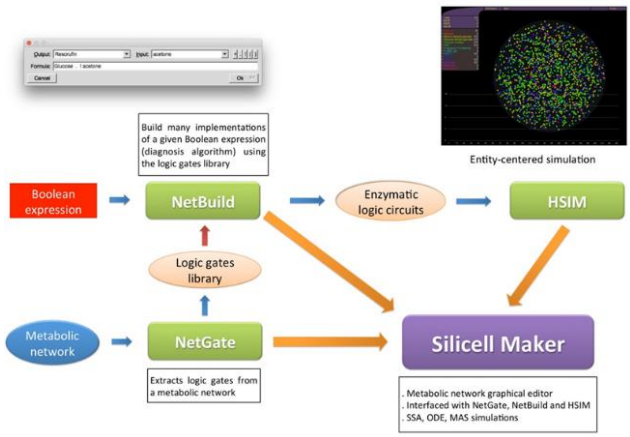


Fig. 3. General principle of SkillCell maker.

Silicell Maker was initially made to be a SBGN compliant graphical bio-network editor. Then we added specific *plugins* to use it to design our artificial cells. The first one, NetGate, builds a bank of enzymatic logical gates from an initial metabolic network, and the set of truth tables of the desired logical gates. The second *plugin*, NetBuild, uses a bank of logical gates made by NetGate to propose enzymatic circuits that implements a given Boolean function with specific input and output metabolites. The third *plugin*, HSIM, is a biochemical reaction network simulator that implements the three most known solving methods (SSA, Entity-centered, and ODE). HSIM is used to make fine grain simulations to ensure that the enzymatic networks made by NetBuild actually compute the Boolean function correctly, using real enzyme kinetics. Then, we use BIOCHAM and HSIM to find the best concentrations of enzymes and internal metabolites, that gives the more reliable and robust results, before the *in vitro* validations.

Control the biomachine contents using microfluidics

Our Biomachines are made of artificially designed biochemical networks encapsulated into liposomes, a type of vesicle. They are constituted of lipids bilayer containing an aqueous core. Because of their similarity with cell architecture regarding cellular membrane and size, as well as their capability of creating out-of-equilibrium compartments, they have been used as models for artificial cells' studies and drug delivery systems. Bulk methods present limited control of membrane formation process and liposomes produced by these methods are often polydisperse and multilamelar. Low encapsulation yield and identical inner and outer material are problems faced by bulk methods. Some examples of such methods are extrusion through porous membrane, freeze-drying, electroformation and thin-film hydration [**5,6**].

Microfluidics handles with fluids inside geometrically constrained channels in low Reynolds Numbers scale. Laminar flow, on the contrary to bulk method, allows precise control of lipid hydration process and thus production of micro/nano sized liposomes presenting monodispersity, membrane unilamelarity and high yield of encapsulation. Pulsed jetting and flow focusing methods are widely used and developed amongst various methods for liposomes production. In our case, we used a specific two flow-focusing method to produce our "biomachines" (**Fig. 4**).

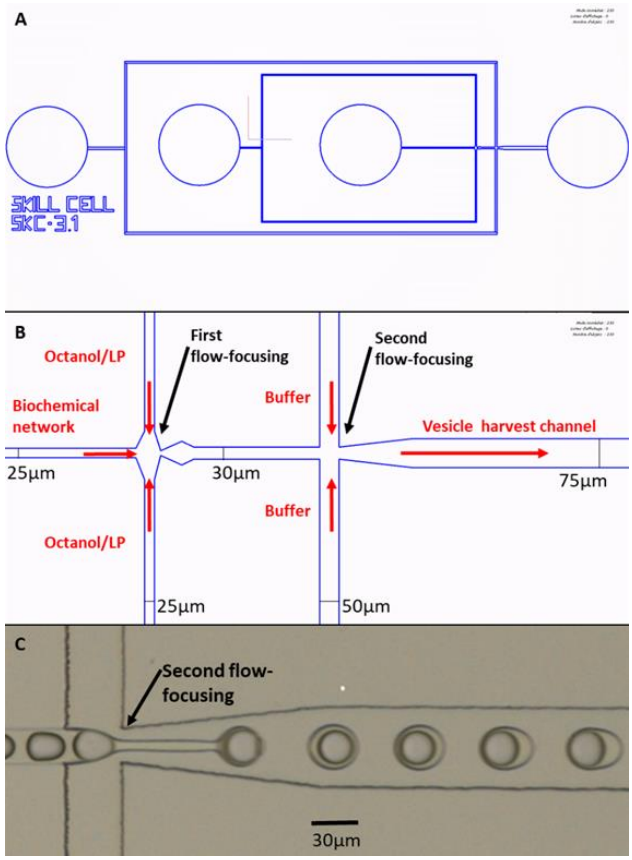


Fig. 4. Microfluidic method for Giant Unilamelar Vesicle (GUV) production containing biochemical networks. (A) Overview of SKC3.1 design. (B) GUV are produced by two consecutives flow focusing pointed by black arrows. Width dimensions of channels are shown and flow directions are indicated by red arrows. (C) Flow focusing showing the formation of double emulsion vesicles. W/O/W vesicles are formed with an excess of 2-octanol that are released spontaneously in a post-production step.

At the end of the process solvent excess elimination is performed and the bilayers are stabilized.

Biomachines applied to medical diagnosis: Example of simplified insulin-resistance test

Insulin acts mainly on liver, muscle and adipose tissues to regulate glucose homeostasis. Disturbed insulin-signaling leads to a state of insulin resistance (IR) which may be silently present several years before the development of Type 2 diabetes (TD2). TD2 is stablished when no compensatory response to IR is achieved [**7,8**].

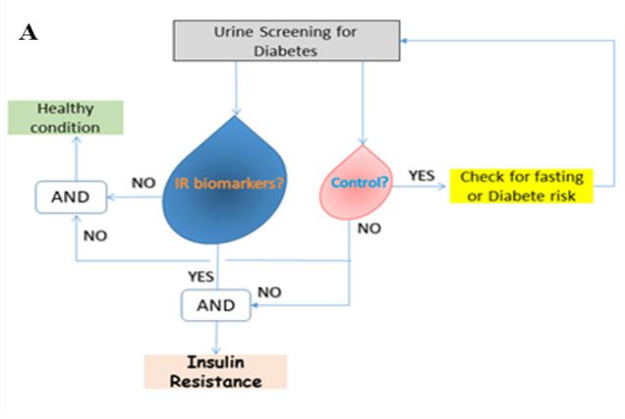
Epidemiology of insulin resistance is not well stablished and is mainly due to the difficulty to identify insulin-resistant subjects. Hyperinsulinemic-euglycemic clamp (HEC) is the gold standard method for diagnosis of insulin resistance. It is based on a constant perfusion of insulin (100µU/mL) in parallel to a variable glucose perfusion into the blood of the patient. Achievement of glucose steady-state allows to measure the glucose uptake by the tissues [**5**]. Because this method needs hospitalization of the patient, it is impossible to apply this diagnostic method for the screening of large populations. Thus HOMA-IR blood assay has been developed but it still requires blood sampling and laboratory facilities. We then developed an insulin-resistance test that does not require laboratory facilities and gives result within a few minutes.
Recent studies identified new biomarkers for IR. High levels of branched-chain amino acids (BCAA, i.e. leucine, isoleucine and valine) were detected in plasma from patients at risk at developing a TD2. Sedentary lifestyle and overfeeding promote the impairment of BCAA catabolism in adipose and muscular tissues via phosphorylation and inactivation of BCKD (Branched-Chain α-Ketoacid Dehydrogenase). Elevated BCAA plasma levels might promote 1) insulin secretion; 2) lipogenesis via stimulation of insulin secretion; 3) incomplete β-oxidation and accumulation of triacylglycerols (TAG) and diacylglycerols (DAG) and 4) chronic over-activation of mTOR, S6K-1, JNK and IRS1 (insulin receptor substrate 1) [**9-11**].

Moreover, the branched-chain amino acid metabolite 3-Hydroxyisobutyrate was shown to be involved in the stimulation of endothelial free-fat acids transport related to intracellular accumulation of TAG and DAG rendering glucose utilization superfluous. Altogether, these conditions could result in insulin resistance [**14**].

Principle of our test

We used synthetic biology principles to design and construct diagnostic Biomachines (so called SkillCell®) based on an artificial biochemical network able to detect and respond to BCAA and glucose in human sample (blood, saliva or urine).

Diabetic but not insulin resistant subjects' present elevated glycosuria. Thus we used our test to access urinary BCAA and glucose to correlate our measurements to different clinical profiles of insulin resistance and diabetes. The principle of the test is that diabetic subjects would present high-BCAA and high-glucose concentrations in blood (possibly urine and saliva) while IR subjects present high-BCAA and low-glucose levels. Our biomachines contain an artificial biochemical network design (programmed) to differentiate them thank to a decision algorithm (**Fig. 5A and B**).



B

BCAA	Glucose	Output
0	0	-
1	0	Red MTT
0	1	AmplexRed
1	1	AmplexRed

Fig. 5. Biomachine for diagnostic assay of insulin resistance. (A) Schematic and (B) Truth table of medical algorithm decision used for biochemical network implementation.

In order to verify the capacity of our biomachines to measure the three amino acids (BCAAs) in complex row human sample we performed analytical validation. To this end we compared the measured biomarkers in patient samples using our biomachines with standard metabolic analysis using mass-spectrometry coupled to liquid chromatography (**Fig. 6**).

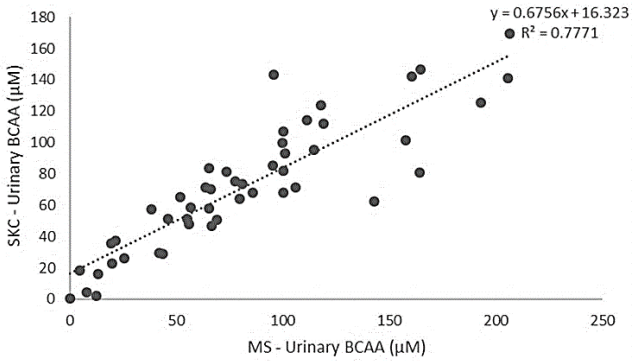


Fig. 6. Mass-spectrometry validation of measurement performed by implemented biochemical network for insulin resistance.

As most of enzymes are sensitive to pH variations, salt concentration and temperature, we produced cell-like vesicles containing BCAA and glucose-detection network to reduce this effect. Confinement of our biochemical networks inside phospholipid bilayers of liposomes conferred good protection to such unpredictable conditions found in human sample (data not shown).

Using *in silico* simulation and experimental validation we adapted the biomachine compound contents and kinetics of response in order to ensure a final readout with a visible dying corresponding to relevant clinical threshold. We basically kept the clinical reference stating that a patient with HOMA-IR >2.4 can be considered as insulin-resistant.

In order to facilitate the micro biomachines handling we trapped them into macro beads (balls of 5mm to 10mm diameter on demand) of alginate (**Fig. 7**). The insulin-resistance test is then a single bead that can be dropped directly into the liquid sample (blood serum, urine, saliva, etc.). After 15min the result can be visually observed. If blue you are IR, white no IR. If the bead turns red then you have too much glucose. You then should either redo the test while fasting for 10h or you may be diabetic and this test is not relevant.

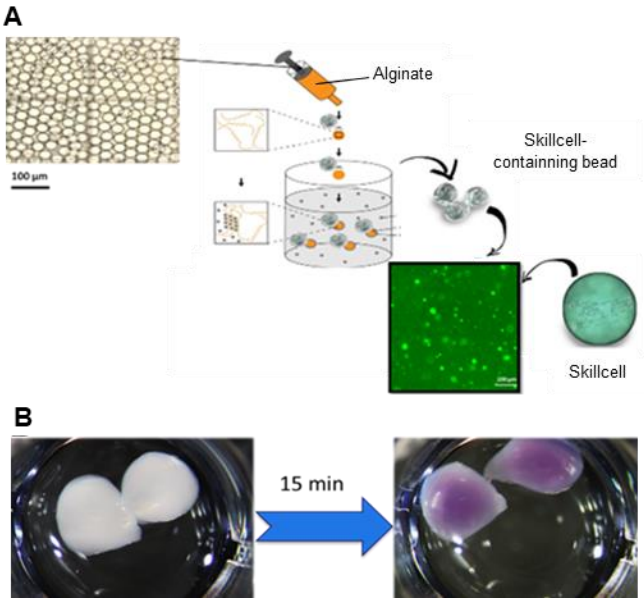


Fig. 7. Biomachine handling format. Schematic representation of micro biomachines encapsulation process within alginate hydrogel macrobeads. (**A**) Microscopic image of the micro biomachine systems (Top-left). The alginate-micro biomachines mixture is flowed through the needle to pull a polymeric droplet stabilized by ionic cross-linking (Central-middle) and fluorescence micrograph of the alginate macrobeads after micro biomachines encapsulation. Scale bars: 100 µm. (**B**) View of alginate beads containing biomachines before (left) and 15 after incubation with BCAA. Micromachines sense and respond to the presence of BCAA.

Conclusion

Current and future developments in medical diagnosis request diagnostics test that remains clinically relevant with high performances but easy to perform, low cost and fast responding. We developed an original methodology to design, simulate, produce and validate a new kind diagnostic device based on biomachines. These biomachines are made of artificially design and *in silico* optimized biochemical networks, encapsulated in microfluidics made vesicles. While trapped into alginate balls it became a simplified but highly performing diagnostic device. We showed a quick example of insulin-resistance screening assay very simple to use, performing a simplified decision algorithm and return the insulin-resistance status of a patient within 15min. This is not requiring any laboratory facilities and then can be used in various contexts. This specific example is already under clinical evaluation at large scale and available under the name of SkillCell IdIR ®. Using our designing tools, the conception of a new biomachine (SkillCell) measuring different biomarkers, performing a programmed decision algorithm and returning a simplified status (final answer) within minutes takes from 2 to 5 months. It is very short compared to classical diagnosis assay design which often requires year to be designed. We think that our biomachine methodology opens a new technological alternative in medical diagnosis.

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Author's contributions

Conceived the plan: FM; Performed the expeirments: all; Data analysis: all; Wrote the paper: FM FS PA Authors have no competing financial interests.

Conflicts of interest

Partnership between CNRS and SkillCell Company has been established to develop our innovation.

Keywords

Biomachine, synthetic biology, medical diagnosis, biomarkers, skillcell.

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