3D-PRINTING ELECTRICITY-PRODUCING BACTERIA : A NEW PARADIGM FOR DEVELOPING GRAPHENE-BASED BIOSENSORS

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PRINTBIO aims a new paradigm of biosensors following a 3D printing strategy of electricity-producing bacteria (Geobacter) to convert the presence of specific chemicals into a electrical current without any external or artificial transducers. Our unique 3D printing approach will create a bioelectrochemical functional material by using a electroconductive and biocompatible hidrogel. The 3D-embedded bacteria will be printed on a graphene-based electrode and a synthetic biology approach will design genetically engineered Geobacter strains able to switch on-off the capacity of electricity production conditioned to the presence of a specific chemical in water.

Keywords: Biosensor, electroactive bacteria, Microbial Electochemical Technology, 3D-printing Graphene, Geobacter, Water quality

1. INTRODUCTION

- **Relevance of the project.** A current need in the modern society is to be accurately and constantly informed about the quality and composition of water and products that we consume or encounter in our daily life. The need for controlling our water resources and sources of drinking water is a question of safety where water suppliers, adminitrative bodies and even Army corps are fully involved. At the EU level, directives such as the Drinking Water Directive (DWD) and the Water Framework Directive (WFD) determine regular monitoring to ensure the quality of water. Many Member States (MS) have included the security of water supply in their national security plans but the nature and extent of their vulnerability assessments is still limited In that context PRINTBIO can be the first step into developing a new generation of Biochips for high-through-put analysis for detecting ANY pollutant in water.
- **Breakthrough character of the project.** PRINTBIO explore the fascinating concept of using microorganisms, so-called electroactive, for converting the metabolism into a electron transfer process where electrical current can be harvested. In contrast, a biosensor shows plenty potential just by detecting a small electric current like the microAmpere range ones already obtained in all lab scale devices. Due to these above features, electroactive based biosensor has received attentions in the last few years. So far, they seem to work mainly for a rough estimation of organic matter content in wastewater. However, we believe that we can "domesticate" and 3D-print *Geobacter sulfurreducens* via Synthetic Biology to convert it in à-la- carte bioelectrochemical reporters ready-to-use in a nanoelectrochemical platform based on graphene as conductive material. Never the use of the word *switch* was more accurate than in this engineered strain able to turn on-off the production of an electrical current by activating the oxidation of acetate in presence of analytes of environmental relevance.
- **Main results.**
  - i) *Geobacter sulfurreducens* was be successfully 3D-printed in an electroconductive hydrogel.
  - ii) *Geobacter sulfurreducens* was capable generating electrical current using graphene as electrode using a screen-printed platform while embedding in electroconductive hydrogel in presence of acetate.
  - iii) *Geobacter sulfurreducens* was genetically engineered to have an on-off switch (gltA gene) for controlling the oxidation of acetate and consequently the production of electricity as part of the biosensor signal.

2. STATE OF THE ART

Electrochemical Biosensors have gained great interest due to the high specificity, the ability to carry out many chemical reactions at room temperature minimizing kinetics limitations and the advantage of direct transduction of sensor response into an electrical current [1]. Measuring electrical current as the transduced response provides high sensitivity and fast response for the Biosensor performance. This performance is assessed

in terms of electrochemical response through cyclic voltammetry and chronoamperometry techniques [2].

Electrochemical Biosensors are developed based on screen printing technology [3]. Screen printed electrodes (SPE) are a very versatile electrochemical platform to develop Electrochemical Biosensors enabling mass productions of devices at significant low cost and many different electrode materials [4]. Graphene is one of the most recent materials available with the most outstanding and promising properties for biosensing applications [5].

One of the main drawbacks of biosensors is the requirement to purify, concentrate and attach enough biological catalyst on the SPE. In contrast to enzyme biosensors, whole cell microorganisms possess many of these enzymes and the procedures required to culture, growth and attach to electrodes are less expensive and time consuming compared to enzymes. These steps can be even more simplified when dealing with electroactive microorganisms, because whole cell performs as a direct transducer from the metabolic activity into the electrical response [6,7].

In principle, the metabolic activity for exocellular electron transfer only takes place in presence of very specific substrates according to every electroactive microorganism. However, genetic engineering provides tools to construct whole cell biosensors for specific target molecules (analytes) based on coupling the expression of a gene in presence of a reporter gene. This strategy uses the analyte as a switch to trigger the production of electrical current by the electroactive microorganism [8].
3D printing of living cells allows to produce three-dimensional structures where spatial distribution of microorganisms can be controlled precisely and on demand [9,10]. This is a promising field that has been developed in recent few years.
So far, this technology has been used only with model microorganisms such as *E. coli*. One of the most relevant aspects is the matrix for printing. Biocompatible polymers are regularly employed, but further efforts are required in order to develop complex and sophisticated formulations of the inks in order to fulfil conditions for an efficient electrical connection between microorganisms in the printing ink and the SPE.
To the best of our knowledge, 3D printing of electroactive bacteria has not been reported yet, and it will be an unprecedented work the combination with genetic modification of *Geobacter sulfurreducens* and assembled in a graphene SPE platform in order to develop a whole cell electrochemical biosensor for detection of pollutants.

BREAKTHROUGH CHARACTER OF THE PROJECT

Breakthrough in the sensor field. Detection of pollutants in the environment is of social concern specially when new xenobiotics products are constantly generated by chemical industry. One of the emerging domains in sensing technology is the use of living cells (bacteria) as biosensors. Synthetic Biology has been the major driving force for this development due to the relative ease to redesign hardware components in microbial cells and to assemble synthetic genetic circuitry for sensing and producing robust output signals. However, achievements do not correlate with an equal increase in the commercial use of biosensors, and the market for field tests is dominated by chemical test kits instead. Those limitations will be overcome by the use electroactive bacteria, like those from the genus *Geobacter*, with capacity for coupling metabolism with the exocelullar electron transfer to an electrode so an electric current can be harvested [6]. Such EET mechanism is a natural process in Geobacter so the electrical current can be harvested without any artificial transducer. On top of that PRINTBIO also followed a Synthetic Biology approach since *Geobacter* can be genetically engineered to condition the production of current to the presence of a specific analyte. This attractive concept open the door to designing “a-la-carte” biosensors using *Geobacter* as biological chassis hosting the on-off switch for connecting the microbial metabolism with a graphene electrode.

Breakthrough in the 3D field. The use of living cells for 3D printing is strongly exploited in medical industry for production of artificial skin, patches, tissue engineering to name just a few. In contrast, the use of bacteria in such 3D immobilization structures is still in its infancy. Among that biotech applications, printing artificial biosensors has been suggested to have a high potential due to the structural and compositional control of living microrganims. Our 3D printing approach is innovative since it merges electrochemistry with the classical biocompatible hidrogel for providing mechanical stability. The result will be a new generation of hydrogels hosting bacteria with capacity for transferring electrons beyond the hydrogel, expanding the applications to electrochemical platorms that already available.

3. PROJECT RESULTS

The project was focused on the achievement of three main objectives.
First objective was focused on developing a suitable bioink to provide the most favourable conditions for printing electroactive bacteria. This bioink was made of an electroconductive hydrogel matrix to provide electrical connection between bacteria and the Screen-printed Electrode (SPE). This was one of the challenges for achieving of a proper biosensor performance. As starting point, conventional hydrogel formulation was used, but

further modifications were explored to improve the electrochemical response of bacteria. Typical hydrogel formulation is based on using biocompatible organic molecules with specific rheological properties suitable for 3D printing. Addition of redox mediators as quinone-like compounds, carbon nanotubes, graphene and graphene oxide were used as modifiers and their effects were assessed.

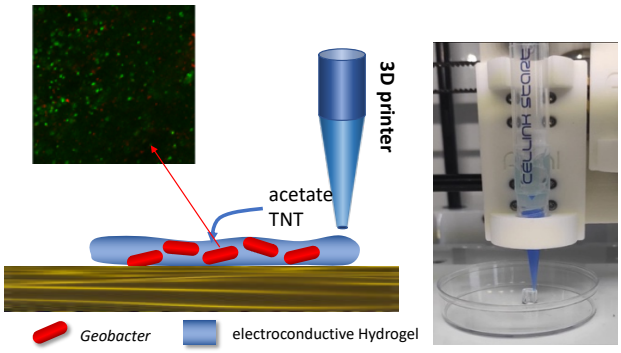

Fig. 1. Scheme for BIOPRINT concept. Image captured during 3D printing of the bioink (hydrogel matrix and bacteria) during viability essays.

Factors like toxicity became relevant at that point, Fig. 1, indicating that a proper balance between toxicity and electrical conductivity is required.
In order to carry out 3D printing with electroactive bacteria, hydrogel should contain high density of bacteria, 100-fold higher than standard concentrations in growing culture ($7*10^8$ cell/ml). Essays with lower concentration of bacteria were performed but no significant activity was detected. Due to the low electrical conductivity of the hydrogel matrix, the signal was dependent on cell density.

The second objective was to develop an electrochemical biosensor platform based on 3D printing electroactive bacteria from *Geobacter* genus on graphene SPE. The electrochemical response through Cyclic voltammetry analysis proved an effective electrical connection is between graphene SPE and the 3D printed bacteria. Moreover, the electrochemical response of bacteria was correlated to the concentration of acetate, a carbon source metabolically converted into electrical current by *Geobacter*

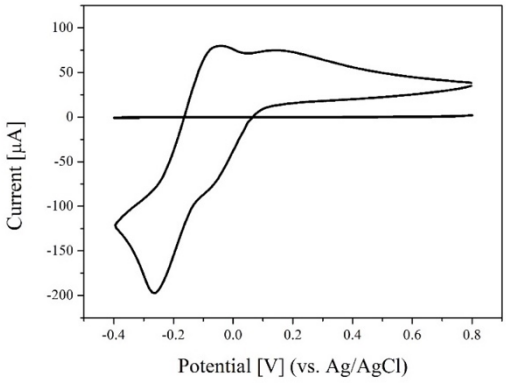

Fig. 2. Cyclic Voltammogram showing the electrochemical response of 3D printed *Geobacter sulfurreducens* on graphene SPE with a solution containg sodium acetate as electron donor.

The third objective was to follow a synthetic biology approach for conditioning the electricity production in *Geobacter* to the present of an specific analyte.
Our rationale is based in the fact that acetate is the sole electron donor for *Geobacter sulfurreducens* to be converted in electricity. So, a genetic control of its oxidation may be the ideal switch to turn on&off the electrical generation and, indeed, the biosensor response. Among the different enzymes participating in acetate oxidation, citrate synthase (gltA) was to found to be key since its abundance was directly linked to rates of *Geobacter* growth [10].

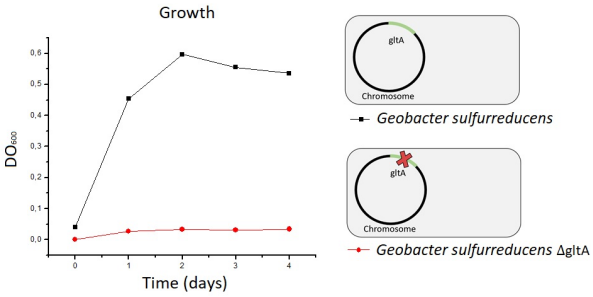

Fig. 3. Growth curve *Geobacter sulfurreducens* wild type and constructed mutant (ΔgltA) after knocking-out the gene *gltA*. Cultures were grown on acetate as sole carbon and energy source.

To evaluate this possibility, a *Geobacter sulfurreducens* strain mutant was constructed by removing the citrate synthase gene from the chromosome (Figure 3). A copy of this gene was cloned under the control of an expression system. IPTG was chosen as a key molecule for the expression system. It was successfully achieved that the G.s. mutant was able to growth and perform exocellular respiration process only in presence of IPTG.

4. FUTURE PROJECT VISION

4.1. Technology Scaling

Phase 1 revealed the feasibility of the rationale after PRINTBIO. However, our innovative concept should grow reaching the following steps at Phase2:

- To assure a medium-long term viability of cells inside the 3D-printed structure.
- To develop a robust electrochemical platform to operate under demo conditions at different water composition.
- To optimize the material composition of the hydrogel for maximizing the relation between electrical signal and concentration of analyte.
- To explore further genetic systems to lock-unlock the production of electrical current just in presence of the analyte.

4.2. Project Synergies and Outreach

PRINTBIO team (already two partners) have identified a number of technological collaborators to a 7 partner consortium able to reach 5-7 during Phase 2:

- A SME able to synthesize riboswitches *a-la-carte* as regulator tools for binding analytes and switiching on-off the production of electrical current in Geobacter.
- Research institution expert in electroconductive graphene-based materials
- Software&App developers (preferably SME) to make portable devices controlled by smartphones.
- A Communication Agency (eg. YOURIS) devoted to disseminate activity products.

Additonally, thanks to Phase 1 we have identified at least 3 independent ATTRACT projects developing tools or materials that can be integrated in our Phase2. Thus, some partners in Phase 2 will be recruited from the following projects

- EU-RAINS
- 3D-MIPS
- PTMsms TM

Printbio had its own specialized personnel devoted to communication&dissemination during Phase 1. However, we plan to recruit a we will mainly focus on i) Social Media (twitter, Instagram, linkdn), and ii) elaborating video products for different stakeholders.

4.3. Technology application and demonstration cases

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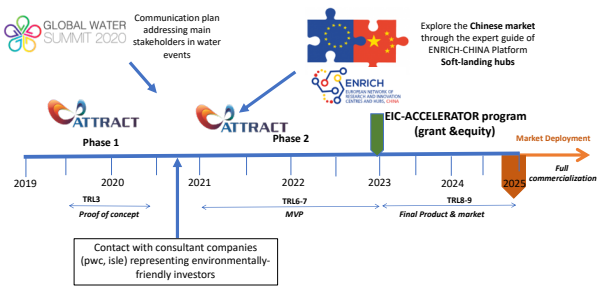
Demo	Science impact	Industry impact	Social challenge
Biosensing acetate in brewery wastewater plant (anaerobic digestor)	Knowledge in microbial electron transfer in a medium with complex microbial community	Better control for treating wastewater in food&beverage sector. Support letter from Mahou brewery (Madrid, Spain	*Environment, resource efficiency*
Biosensing groundwater i) oil spill and ii) arsenic	New tools for genetic control of electron transfer for detecting specific analytes	Detection of spills from oil companies (Eni), and monitoring water quality in drinking water suppliers (India).	*Environment, resource efficiency* *Secure societies*
Biosensing explosives (TNT, DNTs)	New approaches for converting nitro into amino groups	Tool for counter-terrorism companies. Will be tested at Army facilites from Spain and Switzerland	*Secure societies*

You should also explain how your technology application cases will bring benefit to the Research Infrastructure communities in Europe and if you envision a concrete action and/or partnership in this direction.



Technology commercialization

We have developed a route map (Figure 4) that illustrate main steps from Phase 1 to full commercialization. Universities and Research Institutions are key for generating disruptive concepts; however, market uptake of solutions generated by PRINTBIO will be surely benefited from having the SME Nanoelectra as coordinator. During the course of the project Nanoelectra has been contacted by different consultancy companies (PWC, ISLE) representing environmentally-friendly investors, as well as water stake holders including international companies (China). In this context Nanoelectra will submit a proposal to INNOWIDE program in order to launch market activities in the Chinese hub co-supported by EU. through the expert guide of ENRICH EU-CHINA. We anticipate interest in be funded by EIC-accelerator program to achieve **market deployment** for our solutions in **2025**.

4.4. Envisioned risks

Issue	Contingency	Mitigation
Demo site	Expected Demo site not available suddenly	Every demo will have a mirrored location to immediately replace the failed one
Cellular activity	Limited survival of cells due to moisture lost	Expression of water-retaining extracellular polymeric substances (EPS) to survive dryness inside hydrogel
Electrical current production	Not high enough to verify the presence of analyte	To add biocompatible electron mediators (quinones) inside hydrogel.

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4.5. Liaison with Student Teams and Socio-Economic Study

During Phase 1 Printbio has liased activities with MSc students from local universities at Madrid due to covid restrictions. On top of that, during phase 2 we will organize an internal team fully devoted to integrate international MSc students as part of a co-creation strategy similar to the one from Living Labs. Thus, the student will be i) testing and validating our biosensor prototypes, and ii) suggesting upgrades, and iii) exploring applications to meet social challenges. In order to offer the MSc students a broad and educative perspective we are planning to have an internal committee formed by a business developer, a scientist and a journalist specialized in Science dissemination.

Moreover, Nanoelectra as SME coordinating Printbio is developing already its own **business model** including market analysis, financial need, scaling up potential, that will surely contribute the socioeconomic analysis required in Phase 2.

5. ACKNOWLEDGEMENT

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7. REFERENCES

[1] Kimmel, D.W., LeBlanc, G., Meschievitz, M.E. & Cliffel, D.E., 2011, Electrochemical sensors and biosensors, Analytical Chemistry, 84: pp. 685-707.

[2] Bard, A.J & Faulkner, L.R., 2001, Electrochemical Methods. Fundamentals and Applications, John Wiley & Sons Inc., New York, NY, USA.

[3] Thiyagarajan, N., Chang, J-L., Senthilkumar, K. & Zen, J-M., 2014, Disposable electrochemical sensors: A mini review, Electrochemistry Communications, 38: pp. 86-90.

[4] Chu, Z., Peng, J. & Jin, W, 2017, Advanced nanomaterials inks for screen-printed chemical sensors, Sensors and Actuators B: Chemical, 243: pp. 919-926.

[5] Brownson, D.A.C., Kampouris, D.K. & Banks, C.E., 2012, Graphene electrochemistry: fundamental concepts through to prominent applications, Chemical Society Review, 41(21): pp. 6944-6976.

[6] Estevez-Canales, M., Berná, A., Borjas, Z. & Esteve-Núñez, A. 2016 Screen-Printed electrodes: New tools for developing microbial electrochemistry at microscale level, Energies, 8: pp. 13211-13221.

[7] Prévoteau, A., Rabaey, K., 2017, Electroactive biofilms for sensing: Reflections and perspectives, ACS Sensors, 2(8): pp. 1072-1085.

[8] Ueki, T., Nevin, K.P., Woodard, T.L. & Lovly, D.R., 2016, Genetic switches and related tools for controlling gene expression and electrical outputs of *Geobacter sulfurreducens*, Journal of Industrial Microbiology and Biotechnology, 43: pp. 1561-1575.

[9] Kyle, S., 2018, 3D Printing of bacteria: the next frontier in biofabrication, Trends in Biotechnology, 36(4): pp. 340-341.

[10] Schaffner, M., Rühs, P.A., Coulter, F., Kilcher, S. & Studart, A.R., 2017, 3D Printing of bacteria into functional complex materials, Science Advances, 3(12): eaao6804