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

Carlos Manchón¹, Cristina Villar², Antonio Berná¹, Sara Mozo², Abraham Esteve-Núñez²*

¹ IMDEA WATER, Campus Tecnológico de la Universidad de Alcalá, Alcalá de Henares, Madrid, Spain; ² University of Alcalá, Campus Externo, Alcalá de Henares, Madrid, Spain; ³ Nanoelectra, Campus Tecnológico de la Universidad de Alcalá, Alcalá de Henares, Madrid, Spain
*Corresponding author: ciencia@nanoelectra.com

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 an 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 hydrogel. 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 Electrochemical 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, administrative 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 chronamperometry 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 performances 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 fulfill 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 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 exocellular 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 microorganisms. 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 platforms 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.

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*10⁸ 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.

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].

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 Phase 2:

- 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 switching 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 preferably controlled by smartphones.
- A Communication Agency (eg. YOURIS) devoted to disseminate activity products.

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

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

Printbio has 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

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

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

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

5. ACKNOWLEDGEMENT

This project has received funding from the ATTRACT project funded by the EC under Grant Agreement 981.

7. REFERENCES