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► **To cite this version:**

Gabriel Lemerrier, Fadhila Sekli-Belaidi, Venkata Suresh Vajrala, Emeline Descamps, Julie Foncy, et al.. Towards the analysis of mitochondria isolated from leukemic cells with electrochemical instrumented microwell arrays. EUROSENSORS XXXI 31th European Conference on Solid-State Transducers, Sep 2017, Paris, France. <hal-01687843>

HAL Id: hal-01687843

<https://laas.hal.science/hal-01687843v1>

Submitted on 7 Feb 2018

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Toward the analysis of mitochondria isolated from leukemic cells with electrochemically instrumented microwell arrays

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Presented at the **EuroSensors 2017 Conference**, Paris, France, September 3–6, 2017.

Abstract: This work deals with the development of electrochemical transducers for the analysis of the metabolic status of mitochondria isolated from leukemic cells. It proposes the use of ring nanoelectrodes (RNE) integrated into microwell arrays for the simultaneous monitoring of the oxygen (O₂) consumption and the hydrogen peroxide (H₂O₂) production. The sensor enabled the real-time recording of the oxygen consumption of approximately 10,000 isolated mitochondria. Solutions are now proposed to detect H₂O₂ production and to reduce the number of mitochondria under test, targeting the single mitochondrion analysis.

Keywords: Bioelectrochemical nanosensor, microwell arrays, isolated mitochondria, acute myeloid leukemia

1. Introduction

1.1. Motivation

Recent studies introduced a direct relationship between the metabolic status of a sub-population of leukemic cells and its aptitude to overcome the chemotherapy [1]. Indeed, it has been shown that in acute myeloid leukemia (AML), the leukemic cells that exhibit the higher basal level of oxygen consumption rate (OCR) and mitochondrial respiratory capacity, are resistant to chemotherapy whereas glycolytic cells respond positively *in vivo*. These resistant leukemic cells are considered as the origin of the frequent relapses observed during treatment of AML and the cause of the patient death. Mitochondria are organelles typically responsible of 90% of the oxygen consumption by the cell and directly involved in the mechanisms leading to production of H₂O₂, a member of the reactive oxygen species (ROS) family participating to cell signaling and oxidative stress. By taking into account that mitochondrial heterogeneity exists within a single cell, innovative devices permitting the single mitochondrion analysis will offer the observation of biological processes at a new scale, to go further in the understanding of the mechanisms leading to chemoresistance in leukemia and cancer, but also of those involved in many diseases linked to mitochondrial dysfunction.

1.2. Concept

The concept presented here consists in the integration of ring nanoelectrodes (RNE) into microwell array. Considering the location of mitochondria at the bottom surface of the microwell, the confinement provided by the geometric structure maximize the collection ratio of the sensor since the diffusion layers related to the RNE polarization almost totally close the microwell. The electrochemical microwells (ElecWell) are built on a transparent wafer in order to provide an optoelectrochemical system (Figure 1), firstly to be able to evaluate the fill rate of the microwells by mitochondria during an experiment but also to offer the possibility to simultaneously perform electrochemical and optical studies. Long-term experiments can be envisaged since the microscopic platform is temperature-controlled.

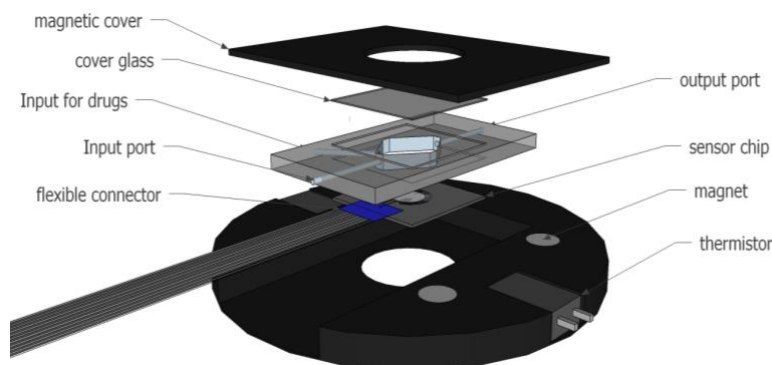


Figure 1. 3D drawing of the whole system comprising a temperature-controlled microscopic platform, the electrochemical sensor chip and a fluidic chamber

2. Materials and Methods

2.1. Device manufacturing

Previous works reported the design by multiphysics simulation, the manufacturing process and the electrochemical characterization of the first generation of the ElecWell [2].

In the second generation of the ElecWell device, the platinum constituting the optional disc microelectrode (DME) is replaced by indium tin oxide (ITO) to be able to use it for surface functionalization by electrochemical technics while conserving transparency. 370 nm of ITO is deposited onto the whole wafer surface for such a purpose. The microwell radius is decreased to 1.5 μm to limit the number of mitochondria susceptible to enter the microwell by taking into account that the value of their average diameter is 1 μm . The last major difference is the presence of planar counter (CE) and reference electrodes (RE) defined by lift-off to obtain an all-integrated three electrodes electrochemical system. The passivation step is performed by inductively coupled plasma enhanced chemical vapor deposition (ICPECD) of 100 nm of silicon nitride (Si_3N_4).

After the cutting step of the chips and the connector assembly using silver paste, polypyrrole is electropolymerized onto platinum RE by following the procedure described by Bard et al. [3] to form an undissolvable quasi-reference electrode. Then, black platinum (BIPt) is electrodeposited at the RNE surface by chronoamperometry in order to enhance the sensitivity to H_2O_2 [4].

2.2. Functionalization of surfaces

Mitochondria as well as many biological elements have the tendency to sediment onto surfaces and form a biofilm. During a typical experiment the normalization is permitted by optical observation through the rear face of the chip. If some mitochondria are located at the inter-wells surface, they won't be counted so the normalization and the measurement will be distorted. Thus, solutions are needed to render the chip surface non-adhesive to mitochondria while keeping the microwells inside intact. The chosen solution is the deposition of poly(L-lysine)-*graft*-poly(ethylene-glycol) (PLL-g-PEG) by the technique of microcontact printing (μCP). To do so, polydimethylsiloxane (PDMS) is

polymerized onto a flat silicon wafer previously treated with perfluorodecyltrichlorosilane (FDTS) to facilitate the demolding. Then, both the PDMS stamp and the sensor chip undergo an oxygen plasma to generate negative charges at their surface. The PDMS stamp is incubated for two minutes in PBS containing $1 \text{ mg}\cdot\text{ml}^{-1}$ of PLL-g-PEG. Then, the PLL-g-PEG layer is transferred onto the chip by applying a soft pressure for two minutes.

Several washings are performed to evacuate mitochondria located outside microwells, however those are also susceptible to provoke their escape from microwells. In order to avoid this possibility, antibodies directed against the most abundant protein of the mitochondrial outer membrane, are directly incorporated in electropolymerized polypyrrole (Ppy) on the ITO DME surface. A very thin layer is formed so that the bottom of the microwell remains transparent. To do this, the pyrrole monomer is diluted in aqueous solution at 20 mM in presence of anti-VDAC antibody at $25 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$. When the electropolymerization occurs, the antibody is believed to act as the counteranion so it is incorporated into the polymer matrix to ensure electroneutrality.

2.3. Fluidics

Instead of using polymers to build a permanent fluidic on the chip, a commercial solution ensuring laminar flow was selected. This solution provided by Warner Instrument is originally designed for long-term studies of cell cultures by microscopy. Two microscope slides on both sides of a RC-21B fluidic chamber are inserted in a temperature-controlled microscope platform. Within the context of our application the microscope slide constituting the bottom of the fluidic chamber is replaced by the sensor chip.

3. Results

3.1. Measurements of mitochondrial activity

Mitochondria isolated from *saccharomyces cerevisiae* were used in this development phase for practical reasons. The OCR is measured by cyclic voltammetry at the RNE. Voltammetric scans are performed at 4 V/s in the interval $[-1 ; -0.1] \text{ V}$ with a delay of 20s between scans in order to avoid the complete consumption of the oxygen within the microwells by the RNEs. The current value corresponding to the oxygen concentration in solution is taken at -0.7 V vs Ag/AgCl at each cycle.

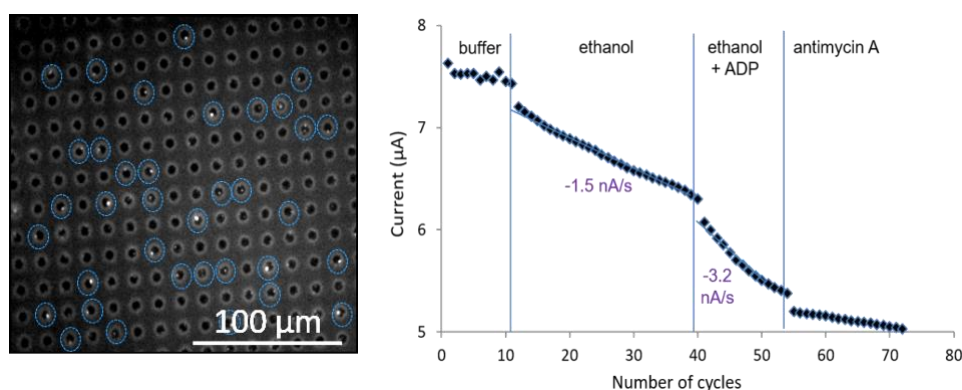


Figure 3. Simultaneous fluorescence microscopy (rear view through the chip) of mitochondria in microwells (a) and OCR measurement by cyclic voltammetry at the RNE (b). Blue circles on A indicate the microwells filled by one mitochondrion.

Measurements of OCR by cyclic voltammetry at the RNE were obtained with 100×100 microwell networks (Figure 3). We notice that mitochondrial respiration starts after the addition of ethanol (1%), a twofold increase is observed after addition of 1 mM of adenosine diphosphate (ADP), then the respiration is inhibited after addition of $1.8 \text{ }\mu\text{M}$ of antimycin A. These results validated the ability of the system to analyze the metabolic features of mitochondria but they also evidenced the need to

develop solutions to enhance the filling rate of the microwells and to avoid the sedimentation of interfering mitochondria at the inter-wells surface, what the next generation of the device should offer.

3.2. Functionalization of surfaces

Anti-VDAC antibodies were successfully immobilized at the Pt DME surface and ITO slides by following the procedure previously described. A secondary fluorescent antibody was used to check the presence and the accessibility of the primary one. A control experiment has been performed to verify the absence of non-specific interactions between the secondary antibody and Ppy electropolymerized in absence of the primary antibody. No non-specific interactions were detected, provided a preincubation with bovine serum albumin (BSA).

The efficacy of the anti-biofouling layer deposited by μ CP was evaluated by optical observations conducted with isolated mitochondria. Firstly, 100 μ l of mitochondrial solution is deposited on the chip. Nothing is done for 3 minutes to allow mitochondrial sedimentation. Then, the solution is withdrawn by pipetting and a first observation is done to automatically count the number of particles stuck on top of the chip for a given surface (18.75 mm²). After that, the chip is washed thoroughly with PBS and mitochondrial particles are counted a second time. A reduction of 90% of the number of sedimented mitochondria is observed in presence of PLL-g-PEG compared to bare SiO₂.

4. Discussion

The first generation of the ElecWell permitted to validate the approach experimentally with the measurement of the OCR of approximately 10,000 isolated mitochondria in response to the additions of metabolic substrates and antimycin A. Nevertheless, interfering mitochondria prevented the rigorous normalization and the production of hydrogen peroxide has not been detected.

The second generation of the ElecWell device is expected to improve the manipulation of biological elements thanks to the addition of the microscope platform and the fluidic. The fill rate and the sensor sensitivity should also be enhanced thanks to the presence of anti-VDAC antibodies at the microwell bottoms and the BIPt at the RNE surface. Finally, the anti-biofouling layer should allow rigorous normalization of the measurements making the data obtained useful for biologists. All those developments merged herein allow us to believe in the final goal consisting in the analysis of mitochondria isolated from leukemic cells, at the single mitochondrion level.

Conflicts of Interest: The authors declare no conflict of interest.

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