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***Abstract*— We report here the fabrication and the electrochemical characterization of recessed disk microelectrodes (rDME) and ring nanoelectrodes (rRNE) integrated into microwell arrays. The technological process based on the reactive ion etching of a SiO_2/Ti/Pt/Ti/SiO_2 stack is optimized in order to realize functional electrochemical microdevices on glass substrate and so, enable the coupling of amperometric measurements with optical analysis. Multiphysic simulations and electrochemical characterizations are carried out to study and enhance the amperometric performance of recessed ring nanoelectrodes arrays (rRNEA) according to their geometry. Finally, all these results demonstrate that rRNEA are fitted for the detection of bio-electrochemical species at the microscale and consequently, for the analysis of the metabolic status of isolated mitochondria through the measurement of dissolved oxygen and hydrogen peroxide.**

I. INTRODUCTION

The Acute Myeloid Leukemia (AML) is a cancer related to abnormal proliferation of myeloid progenitors in blood and bone marrow. This severe pathology concerns about 20,000 new persons per year (1.2% of all new cancer cases) and provoke the death of about 10,500 patients (1.8% of all cancer deaths) in United States of America [1]. The poor overall survival, approximately 25% at five years after diagnostic, is due to the common relapses observed after current treatment based on the coupled use of cytarabin and daunorubicin (antiproliferative agents). Our working hypothesis rests on Otto Heinrich Warburg observation in 1923. Actually he has put forward the fact that tumor cells exhibit aerobic glycolysis instead of oxidative phosphorylation to produce adenosine triphosphate (ATP) which is essential in the metabolic process of whole living organisms [2]. In addition, some studies highlight a relationship between metabolic features of leukemic cells and their ability to overcome current treatment. Those few residual leukemic cells (RLC) are considered as the starting point of relapses [3].

The aim of this project is to design, manufacture, and characterize microwell arrays integrating ring nanoelectrodes to enable the opto-electrochemical analysis of the mitochondrial function in vitro to go further in the understanding of the link between chemoresistance and cellular metabolism.

II. DESIGN BY MULTIPHYSICS SIMULATION

A. Maximization of the collection ratio

The first step of the design work consists in the determination of the optimal geometrical parameters in order to maximize the collection ratio defined as the ratio between the flow of species produced at the microwell bottom (φ_{in}) and (φ_{out}) the flow of species reacting at the ring nanoelectrode (Fig.1).

Simulations results demonstrate that in order to maximize the collection ratio, we have to maximize the microwell aspect ratio H/R, to maximize the electrode thickness T and to locate the rRNE at the microwell mid-depth. After matching the simulation recommendations with the technical limitations these geometrical parameters have been defined: H1 = H2 = 2.5µm, T = 200nm and R = 3µm. By the way, the average diameter of a mitochondrion is 1µm [4].

B. Determination of the optimal interwell distance

In the case of ultra-microelectrode arrays (UMEA) it is crucial to ensure the absence of the crosstalk phenomenon that occurs when an overlap of the diffusion layers of two or more neighbouring UME is observed. The objective here is to determine the minimal center-to-center distance (d) ensuring the absence of crosstalk. The cyclic voltammetry curves obtained by simulation indicate that the absence of crosstalk phenomenon occurs while $d \geq 20R$.

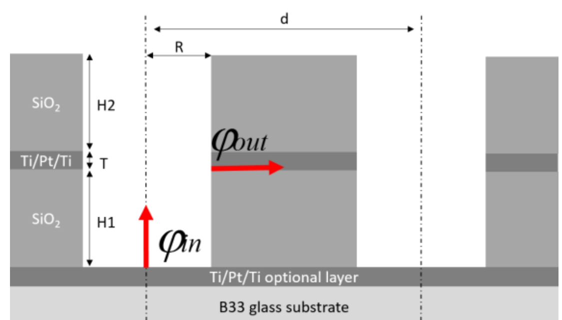

Figure 1: geometrical parameters of the instrumented microwell network

III. MANUFACTURING PROCESS

A. *Formation of a stack of insulator and conductive materials*

The integration of platinum rRNE into microwells is achieved using silicon-based technologies. Starting from a B33 glass substrate, an initial facultative step consists in the deposition of a titanium/platinum/titanium (Ti/Pt/Ti) stack patterned by "lift off" technique. This allows the fabrication of rDME at the bottom of the microwells which are required for the generator/collector mode characterization (see hereafter). Then, the technological process involves three deposition steps performed in a row. First, a 2.5µm silicon oxide (SiO_2) layer is deposited by plasma-enhanced chemical vapor deposition (PECVD). Then, a 200nm platinum layer is deposited by evaporation while using two 20nm titanium interfacial layers to ensure platinum adhesion on SiO_2. Finally, another 2.5µm PECVD silicon oxide layer is deposited to form a SiO_2/Ti/Pt/Ti/SiO_2 stack.

B. *Opening of the electrical contacts and definition of the microwells*

Once the stack is defined, a wet etching step is performed to open the contacts of the rRNE. Then, the final and critical step consists in the inductively coupled plasma reactive ion etching (ICP-RIE) of the whole stack to define the microwells and eventually open the electrical contacts of the rDME. To do so, a 13µm AZ40XT photoresist layer is patterned by photolithography. Then, successive steps of ICP-RIE are performed with different gas mixtures in function of the material: CF_4/Ar to etch silicon dioxide, SF_6 to etch titanium oxide and Cl_2 to etch platinum.

IV. ELECTROCHEMICAL CHARACTERIZATION

Every electrochemical characterization is performed with a PARSTAT MC potentiostat (Princeton Applied Research) considering a three electrodes setup: rRNE and/or rDME as working electrode, external platinum wire as counter electrode and external silver wire as pseudo-reference electrode.

A. *Characterization of a single microwell*

The limiting current of a single rRNE is measured by cyclic voltammetry in a drop of 1 mM ferrocene dimethanol (Fc) aqueous solution supplemented with 0.1M potassium chloride as support electrolyte. An oxidation current of 400pA is measured for a 3µm radius microwell whereas simulation result is 390pA. To determine the effective collection ratio, Fc is oxidized on the rDME to form ferrocenium (Fc^+) that is then reduced at the rRNE to regenerate Fc. This experimental configuration is what we call generator/collector mode and the rDME is up to now dedicated to this use. The ratio between those two limiting currents is 0.89 against 0.85 by simulation.

B. *Characterization of microwell arrays*

The same cyclic voltammetry is performed in ferrocene solution with rRNEA in order to confront experimental results with simulation. Four different center-to-center distance are investigated: d = 5R, 10R, 15R and 20R (10x10 networks). The crosstalk phenomenon is reflected by a bird beak shape observed on the voltammogram instead of a plateau. Experimental results confirm the simulation results. In other words, in this configuration d must be at least equal to 20R.

V. FIRST EXPERIMENTS WITH ISOLATED MITOCHONDRIA

The first experiments carried out with isolated mitochondria put forward the fact that the device is not yet sufficiently adapted to the manipulation of biological material (fig.2). The rRNEA device (100x100 network) is able to measure the oxygen consumption rate (OCR) of mitochondria but the fill rate of the microwells only reaches 20% and some of them located on the interwell surface interfere in the measurement and so, forbid the normalization and complicate the biological interpretation. The works are currently focused on this point and solutions including microcontact printing (µCP) of Poly-L-lysine grafted Polyethylene-Glycol (PLL-g-PEG) and direct incorporation of antibodies in electropolymerized polypyrrole at the rDME surface are being developed. Furthermore, electrodeposition of platinum black at the rRNE surface is also considered to improve the sensor sensitivity to hydrogen peroxide [5].

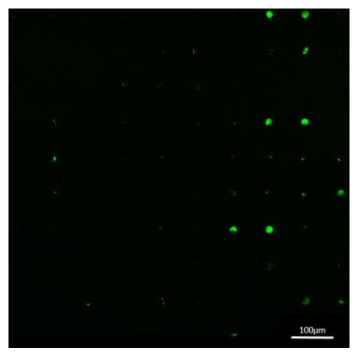

Figure 2: fluorescence imaging of mitochondria isolated form MOLM14 cells and labelled with MitoTracker Green in microwells (rear view)

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