Angiogenin induces nitric oxide release independently from its RNase activity

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We present here some additional data and background on:

- **Sensor fabrication**
- **Experimental procedures and methods**

1 Sensor fabrication

The multiple microelectrode array was produced by stereolithography. This is a widely used method in microengineering, particularly adapted to mass production of microdevices. Most of the modern electrical devices and components (processor, transistors) are built using this technique. In brief, this process involves deposition, by vapor or spin coating, of a thin layer of material. A layer of photoresist is then deposited and patterned through a mask with light polymerization. The unpolymerized resin is then washed away and the material is etched, for instance chemically. Repeating this protocol for each layer of the system enables production of rather complex and intrigated patterns. However, when a large number of layers are deposited, an additional step of grinding and polishing is usually required to guarantee even and flat layers.

Our sensor is made of a glass substrate, gold connections

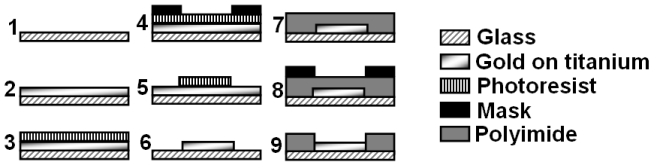

Fig. 1 Fabrication of an electrode microarray using stereolithography.

on titanium and a polyimide insulating layer.[1] The fabrication process is detailed on Fig.1: (1) a 1 mm thick cleaned glass wafer was (2) coated first with gold on titanium. (3) Photoresist was spin coated and (4) patterned through a chromed mask. (5) The resin was then developed. (6) The gold electrodes were etched. A polyimide layer was (7) deposited, (8) patterned using a chrome mask and developed to obtain (9) a completed device. The working electrodes are 35 μm in diameter, and are 230 μm apart (Fig.2). They also present a 2 μm recess. The sensing part of the sensor was made by INNOS Ltd (Southampton, UK) and were encapsulated in an eight pin package (First Level, US).

In our experiments, we have not used NO calibrations, as calibrating a sensor for *in situ* measurements raises several issues. In particular, the background conditions are radically different between calibration scans run in PBS and real measurements carried out in biological samples. Chemical interactions with the analytes generated by the cells or the tissue samples are expected to affect the measured signal. In addition, cells growing on the sensor can hinder diffusion or locally increase the current generated, compared to free homogeneous solution. For all these reasons, we have decided to report the more robust relative changes in measured DPV peak currents, to minimize the electrochemical stress on the cells and to avoid the uncertainety due to absolute measurements.[2]

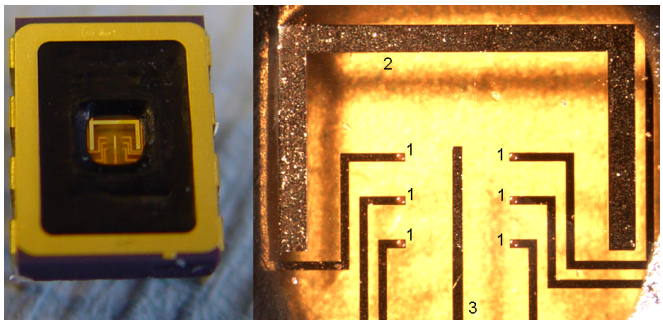

Fig. 2 Picture of the sensor (left) with a focus (right) on the microengineered part featuring 1) the recessed working electrodes, 2) the counter electrode and 3) the pseudo reference.

2 Experimental

2.1 Chemicals

All the chemicals used in this study were purchased from Sigma, unless stated otherwise, and were used without fur-

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ther purification. Deionized water purified through a Millipore system was used throughout all the experiments. Angiogenin was purified as previously described.[3] RNase inhibitor was purchased from Intron Biotechnology.

2.2 Cell culture

HUVEC were purchased from Clonetics (San Diego, USA) and cultivated in EGM-2 medium (Clonetics) on gelatin coated 75 ml flasks, at 37°C, 95 % O_2, 5 % CO_2. Cells from passage 6 to 9 were used.
ESC-derived endothelial cells were a gift from Prof. HM Chung (CHA Stem Cell Institute, Pochon University, Pochon, Republic of Korea) and were prepared by mechanical isolation and cell sorting[4] and cultivated in the same media.

2.3 Differentiation of human embryonic stem cells into endothelial cells

In brief, ESC were cultured in suspension in basic fibroblast growth factor-free hESC culture medium for 9 days until formation of embryoid bodies.[4] These embryoid bodies were cultured in DMEM for 9 days. Their center was then isolated mechanically with pipettes and cultured in EGM-2, a specific endothelial media. The differentiated cells were selected using expression of Von Willebrand factor, a large glycoprotein present in endothelium. This sorting was achieved using mouse monoclonal anti Von Willebrand factor antibody and a flow cytometer. The cells were then cultured in EGM-2.
These cells exhibited typical endothelial behaviour, such as capillary formation.[4]

2.4 Modification and preparation of the sensors

The MMA were modified using a Sylgard (Dow Corning) custom-made reaction cell to allow deposition of a 500 μl volume on the sensor. The experimental setup used for the electrochemical measurements is presented on Fig.3. A lid made from a Petri dish was also fitted on the cell to minimise evaporation and avoid contamination when the sensor had to be placed in the humid incubator. Prior to any experiment, biological debris was removed using trypsin solution. Trypsin was deposited into the cell and incubated at 37°C for one hour. They were then rinsed with 70 % ethanol followed by water. The gold electrodes were electrochemically cleaned by performing cyclic voltammograms between 1.6 and -0.3 V vs Ag|AgCl at 0.5 $V.s^{-1}$ in 0.1 M sulphuric acid until stability of the graphs. The electrodes potential was then held at 0.2 V vs Ag|AgCl to reduce gold oxides.

The sensor was then sterilized with 70 % ethanol and UV light and rinsed with PBS (pH = 7.4). 100 μl of human fibronectin (20 $\mu g.ml^{-1}$, in water) was deposited on the sensor and let to dry. The excess of fibronectin was rinsed with PBS.

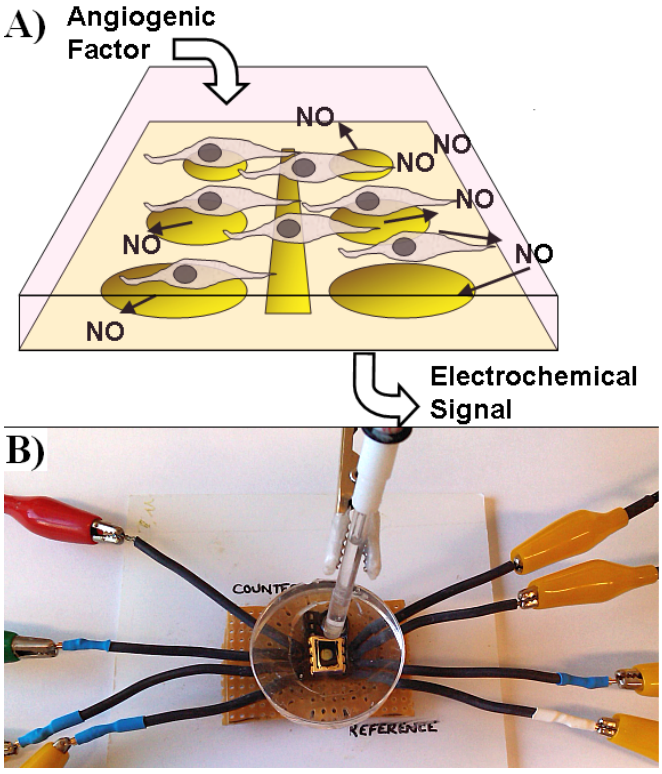

Fig. 3 A) Scheme of our experimental setup showing the cells cultivated directly on the surface of the device; B) Experimental setup showing the modified MMA and the connector

2.5 Electrochemical measurements

Cells were harvested using trypsin and counted with a cytometer and Trypan blue. 1000 cells were suspended in 500 μl of EBM- 2 medium and deposited on the sensor. The chip was incubated for at least 2 hours. A DPV was then recorded between 0 V and 1.5 V vs Ag|AgCl. 2 μl of angiogenin (5.26 $mg.ml^{-1}$ in deionized water) were added with the relevant inhibitor, if required (protocol described below) to reach a final concentration of 21 $\mu g.ml^{-1}$. The MMA was placed in the incubator for one hour and another DPV was recorded.

2.6 Inhibitors

For the L-NAME study, the cells were maintained in culture medium containing 100 μM L-NAME during at least one hour before any measurement.
RNase inhibitor was mixed with angiogenin (1:2 mass ratio) and incubated at 37°C for one hour. This mixture was then added to the cell medium to reach a final angiogenin concentration of 21 $\mu g.ml^{-1}$.

2.7 Data processing

The DPV results were processed by normalizing the peak current obtained after angiogenin injection by the peak current measured before injection:

$$current\ ratio = \frac{peak\ current\,(1\ hour\ after\ addition)}{peak\ current\,(before\ addition)} \quad (1)$$

This normalization guaranteed consistency between the different measurements by minimizing the effect of variations in size, background species, heterogeneity in cell population, etc.[2] The results obtained for each experimental condition were then averaged, the standard deviation was calculated and the means compared using Student's t-tests.

References

1 B. Patel, M. Arundell, R. Quek, S. Harvey, I. Ellis, M. Florence, A. Cass, A. Schor and D. OHare, *Analytical and Bioanalytical Chemistry*, 2008, **390**, 1379–1387.

2 R. Trouillon, C. Cheung, B. Patel and D. O'Hare, *Biochimica et Biophysica Acta (BBA)-General Subjects*, 2010, **1800**, 929–936.

3 S. Jang, D. Kang, S. Chang, H. Scheraga and H. Shin, *Biotech. Lett.*, 2005, **26**, 1501–1504.

4 S. Cho, S. Moon, S. Lee, S. Kang, J. Kim, J. Lim, H. Kim, B. Kim and H. Chung, *Circulation*, 2007, **116**, 2409–2419.