

An electrochemical functional assay for the sensing of nitric oxide release induced by angiogenic factors

Raphaël Trouillon¹, Danny O'Hare^{2,*} & Soo-Ik Chang^{3,*}

¹Department of Chemistry, University of Göteborg, SE-412 96 Göteborg, Sweden, ²Department of Bioengineering, Imperial College London, London, UK, ³Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Korea

Nitric oxide (NO) is a critical biological mediator involved in numerous diseases. However, the short lifetime of this molecule in biological conditions can make its study *in situ* complicated. Here, we review some recent results on the role of NO in angiogenesis, obtained using a biocompatible micro-electrode array. This simple system allowed for the quick and easy quantification of NO released from cells grown directly on the surface of the sensor. We have used this technology to demonstrate that angiogenin induces NO release, and to partially elucidate its intracellular transduction pathway. [BMB reports 2011; 44(11): 699-704]

INTRODUCTION

Biochemistry and measurement of nitric oxide

The radical nitric oxide (NO) is a ubiquitous biological mediator (1). This low molecular weight compound is involved in vascular physiology (2, 3), immune reactions (4, 5) and in the neural system (6, 7). Its role in several pathologies is also critical. For instance, NO is a key factor involved with angiogenesis (8), and is therefore a central factor in tumor development; tumors being dependent on neovascularization to sustain their growth (9). Similarly, angiogenesis, and consequently NO, have a direct effect on hypoxia (10), which is usually identified as a marker of malignancy. Understanding the role of NO in these phenomena is a major requirement for our fundamental understanding of physiology and to identify new therapeutic targets.

NO is biologically produced by nitric oxide synthase (NOS). The substrate for NOS is L-arginine, and NO is co-produced with L-citrulline. Several isoforms of this enzyme exist: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). Selective in-

hibitors for these enzymes are available, but all these isoforms can be inhibited by N^ω-nitro-L-arginine methyl ester (L-NAME), an analogue of L-arginine (11). In biological samples, NO has a very short lifetime as it rapidly converts to nitrite or nitrate in the presence of oxygen or iron containing molecules such as hemoglobin, respectively (12).

Because of its high reactivity and short lifetime, biological measurements of NOS activity have proven to be very challenging. Most of the widely used methods, such as the Griess test (13, 14) or quantification of radiolabeled by-products of NO synthesis from L-arginine, such as [³H] L-citrulline (14, 15), are labor intensive, require heavy equipment and long preparation, and do not allow for real time measurements within a single sample. Fluorescent markers, such as diaminofluoresceins (16), are also available.

Electrochemical sensors are an attractive alternative to the methods previously presented due to their ease of miniaturization and low cost. They are also amenable to mass production and can be quickly integrated into larger electronic architectures. For instance, several electrochemical investigations of NO have been carried out (17, 18) using platinum modified electrodes (19-21), or chemically modified electrodes (22-25). Additionally, several array designs have been proposed (24, 26, 27).

In this review, we summarize the results we have recently obtained with a multiple microelectrode array (MMA). The architecture of this device is simple and allows robust measurements under biological conditions. After having presented the MMA, we will review the fundamental results we have obtained on the biochemistry of angiogenic factors.

THE MULTIPLE MICROELECTRODE ARRAY

Fabrication

The MMA was prepared using standard stereolithography. This technology is routinely used in microfabrication and is based on the successive deposition and etching of layers of different materials. This etching is usually achieved by spin-coating of a polymeric photoresist on the material to be patterned (this material is usually sputtered by chemical vapor deposition) and by cross-linking this photoresist with UV light across a nickel-chromium mask. After developing, the material is covered with a protective layer having the desired shape and can there-

*Corresponding authors. Danny O'Hare, Tel: +44-20-7594-5173; Fax: +44-20-7584-9817; E-mail: d.ohare@imperial.ac.uk; Soo-Ik Chang, Tel: +82-43-261-2318; Fax: +82-43-267-2306; E-mail: sichang@chungbuk.ac.kr
<http://dx.doi.org/10.5483/BMBRep.2011.44.11.699>

Received 14 October 2011

Keywords: Angiogenin, Biosensing, Cells-on-a-chip, Functional assay, Nitric oxide

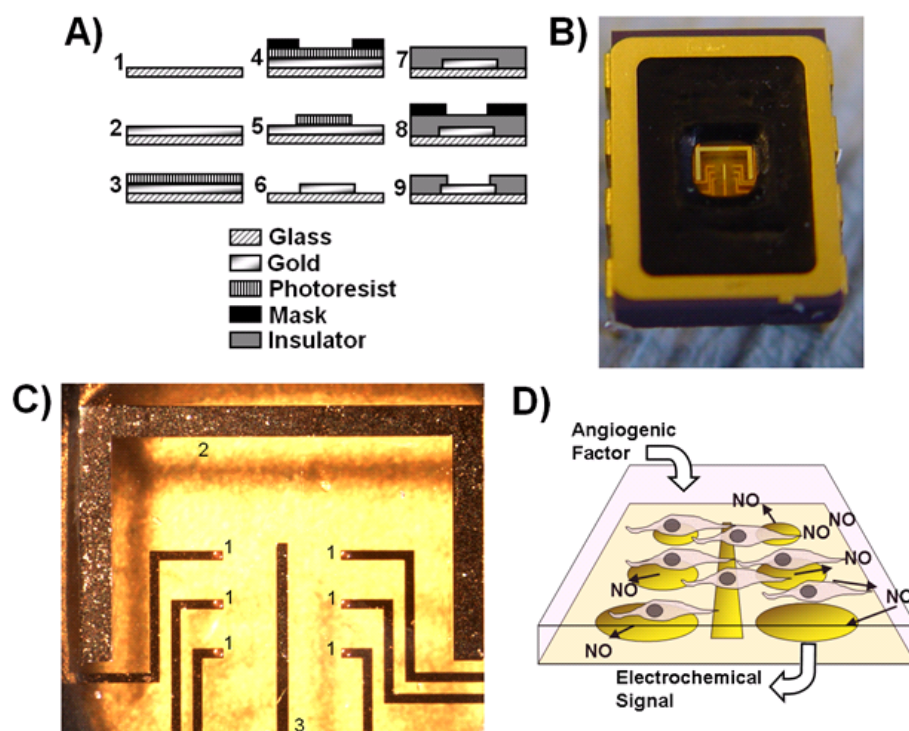


Fig. 1. Fabrication and description of the multiple microelectrode array (28). (A) Fabrication process: (1) The different layers are sputtered onto a clean glass slide. (2) Gold is deposited (onto an adhesion layer of titanium). (3) The photoresist is spin coated on the gold, (4) cross-linked with UV light through a chromium mask, (5) and developed. (6) The gold is etched and the photoresist is removed. (7) The insulating layer is deposited on the connections, (8) patterned and (9) etched to obtain the final device. (B) Picture of the final device packaged into an 8 pin DIL. (C) Focus on the active site showing the six 35 μm working electrodes (1), the counter electrode (2) and the pseudo reference electrode (3). (D) Principle of the electrochemical assay.

fore be etched, chemically or by plasma. The polymerized resist is then removed, revealing the patterned material.

In our case, a 1 μm thick layer of gold was sputtered over a titanium layer for improved adhesion (Fig. 1A). This layer was etched into several 50 μm wide connections for the working electrodes. A layer of insulator was then deposited and etched to form, recesses (2 μm deep, 35 μm in diameter) over the gold pads. Polyimide or silicon nitride were used as insulators, and silicon nitride was found to have a better stability than polyimide. The finished device shows 6 recessed working electrodes, a gold pseudo-reference, and a counter electrode. The chip is then packaged into an 8 pin DIL (Fig. 1B, C) and modified with a custom made PDMS gasket, allowing for the deposition and incubation of up to 500 μl of liquid (28). This device is designed to deposit cells on the top of the sensor in cell media, to add different drugs to the media, and to monitor the changes in NO levels with the electrodes, as shown on Fig. 1D.

Characterization and principles

The MMA was characterized electrochemically with ruthenium (II/III) hexaammine and oxygen. The differences in diffusion limited current, capacitance, and Tomeš potential from one electrode to another were quantified, and showed good reproducibility of measurements between the different channels (29). Identically, the diffusion limited current was found to be consistent with the theoretical value for steady state currents at a recessed electrode.

Furthermore, the device was dry-coated with fibronectin. This protein from the extracellular matrix was found to enhance the stability of the electrochemical device when inserted into a biological matrix (30, 31). Biomolecules, and in particular proteins, are very likely to adsorb onto the surface of the sensor, thus generating an insulating layer and partially inactivating the surface. Fibronectin has been found to limit this phenomenon, known as biofouling (32), even for complicated surface dependent reactions, such as oxygen reduction. Additionally, fibronectin offers improved biocompatibility and excellent adhesion of endothelial cells, as these cells have to be grown on the surface of the electrodes.

When a differential pulse voltammogram was run from 0.6 V vs. Ag|AgCl to 1.5 V vs. Ag|AgCl in the presence of cells, a peak current was identified at ~ 0.9 V vs. Ag|AgCl. A similar peak was found in the presence of NO or nitrite, thus indicating that this peak is probably generated by one of these species, but no interaction from other common biological interferents could be detected in that potential range (33). Therefore, tracking the changes of this peak was expected to allow measurements of NO release. In particular, the ratio

$$\text{Current ratio} = \frac{\text{peak current (after addition of secretagogue)}}{\text{peak current (before addition)}}$$

showing the variations in peak current before and after the addition of growth factors was the value of interest, as it allows

for a functional assay of cellular activation after stimulation by a growth factor.

The selectivity of this NO functional assay was established by i) investigating the origin of the peak and ii) using selective inhibitors. Addition of sulfamic acid, a nitrite scavenger, almost abolished that peak, thus showing that it was probably mostly due to the oxidation of nitrite. Nitrite being a by-product of NO actually accounts for past release of NO, and is therefore a good marker for NO release in biological systems. This is very similar in principle to chemical methods used for NO analysis, such as Griess' tests (13). More importantly, to establish unambiguously that the increases in the magnitude of the 0.9 V vs. Ag|AgCl peak were due to higher releases of NO, negative controls were systematically conducted using L-NAME. The selectivity of this technology therefore arises from i) the potential range where this peak is expected to be recorded and ii) negative controls where NO release is inhibited.

This measurement method, where 2 successive sets of DPV are performed to calculate peak current ratios, was designed to obtain robust and high throughput measurements and to minimize the effect of the electrochemical sensing on the cells. Three and 2 DPVs were respectively performed before and after addition of growth factor to stabilize the surface of the sensor before the measurements were taken, and were experimentally found to improve the reproducibility of the sensing. DPV was chosen because of its improved sensitivity and short run times (in our setup, ~40 seconds), thus limiting the effects of the electrochemical sensing on cell physiology.

The peak current ratio was preferred to the analyte concentration deduced from a calibration curve. The current ratio method was found to be very robust. This normalization process cancels the effect of changes in electrode surface because of cell adhesion or variation in cell density, and allows for massive parallel measurements. In our studies, we have used the current ratio as a robust and biologically significant marker, and compared this value for different pharmacological treatments to elucidate the physiological status of the cell.

USING THE MULTIPLE MICROELECTRODE ARRAY TO ELUCIDATE THE BIOCHEMISTRY OF ANGIOGENIC FACTORS

We now focus on the biochemical results obtained with the MMA to emphasize the biological significance and usefulness of our system. The device presented in the previous part of this paper was used to investigate the biochemistry of vascular endothelial growth factor (VEGF) and angiogenin (ANG). These *in situ* functional assays were focused on the intracellular cascade leading from the binding of the angiogenic factor to the evoked release of NO. In particular, special attention was given to the role of kinases into this mediation.

Vascular endothelial growth factor

VEGF is a well-studied angiogenic molecule involved in sev-

eral types of cancers and angiogenesis related diseases (34). It is known that VEGF induces NO release in endothelial cells, principally via mediation through phosphatidylinositol 3 (PI-3) and Akt kinases (35). To establish a proof of concept for our setup, pig aortic endothelial cells (PAEC) were grown on the surface of the fibronectin coated sensor and exposed to VEGF (33). It was found that the peak described in the previous paragraph increased by 40% 2 hours after addition of VEGF. As this increase was inhibited after addition of L-NAME, we could establish that this rise was an indicator of NO release. The same method was repeated in the presence of LY 294,002 and PD 98,059. These compounds respectively inhibit the PI-3 (36) and the mitogenic-activated protein extracellular signal-regulated (ERK 1/2) (37) kinases. In agreement with the published literature, we have found that VEGF induced NO release was mediated through PI-3, but not through ERK 1/2.

Similar experiments were carried out on sections of guinea pig aortas. By simply depositing this piece of tissue onto the MMA, in cell medium, we could demonstrate that VEGF also induced NO release from this aortic endothelium.

This initial study (33) demonstrated the viability of our device as direct way to elucidate intracellular pathways leading to NO release.

Angiogenin

ANG is a 14.4 kDA molecule initially identified by Bert L. Vallee's group in 1985 (38). Its role and activity are only partially understood. It appears that this molecule plays a fundamental role in angiogenesis and neovascularization, as it has been reported to be a critical requirement for vascular growth, even when evoked by other angiogenic factors such as acidic and basic fibroblast growth factors, epidermal growth factor, or VEGF (39). This phenomenon suggests that ANG may be an important factor in tumor growth and a good pharmacological target, as it may contribute to malignancy by inducing sustained angiogenesis. For instance, blocking the nuclear translocation of angiogenin inhibits the growth of human tumor xenografts in athymic mice (40). Identifying the cellular targets of ANG and understanding its mechanism of action offers intriguing perspectives for the treatment of angiogenesis related diseases.

Using the MMA, we have demonstrated that ANG induces NO release in human umbilical vein endothelial cells (HUVECs), as ANG induced a peak increase in our assays, and this peak was inhibited by L-NAME (41). This release was time dependent, and the signal measured in our assay reached a maximum 30 minutes after addition of ANG. Interestingly, this release was also dose dependent, as the response increased with the dose of ANG added to the cell media. A threshold for response has been identified between 250 ng ml⁻¹ and 1 µg ml⁻¹. This value is consistent with the basal blood level of angiogenin in humans (359 ± 59.9 ng.ml⁻¹) (42), and may therefore not be sufficient to induce a significant cell response. This result was then confirmed by confocal microscopy, as ANG was shown to increase the phosphorylation of eNOS.

Electrochemical measurements of ANG induced NO release were repeated using stem-cell derived endothelial cells, and similar responses were recorded (28). Differentiation of these stem cells into endothelial cells is usually assessed by immunostaining with anti-Von Willebrand factor (43), but use of NO release after addition of angiogenic factors could be used as a possible alternative criterion for differentiation.

ANG has been reported to have 4 different mechanisms of action (44):

- * ANG can induce cell migration by binding to actin receptors and cleaving the extracellular matrix (45).
- * ANG has a ribonucleolytic activity (46).
- * After binding to its cell membrane receptors, ANG triggers an intracellular biochemical cascade, mediated in particular via ERK 1/2 (47) and Akt (48), or via the phosphorylation of stress-associated kinase SAPK/JNK in smooth muscles (49).
- * ANG can induce nuclear translocation, *i.e.* enter the cell nucleus and probably interact with ribonucleotides (49, 50).

The last 3 points (RNase activity, kinase mediation, and nuclear translocation) are particularly relevant for our study of angiogenic factor induced NO release. The relationship between these events and ANG induced NO release have been investigated using the MMA in PAEC and HUVECs.

The same approach as the one described for VEGF has been used to study the kinase pathway leading to NO release. LY 294,002, but not PD 98,059, inhibited the increase in peak current after addition of ANG (41). This indicates that the ANG signal leading to eNOS phosphorylation is actually mediated through the PI-3/Akt kinase pathway and not by ERK 1/2, similarly to VEGF. However, activation of ERK 1/2 is known to be critical for angiogenesis (47). Even though it does not interfere with NO release, PD 98,059 inhibited cell proliferation in growth assays (41, 47), thus demonstrating that angiogenesis is a complex phenomenon involving contributions from several independent mechanisms, all of which are critical for cell growth.

An RNase inhibitor was used to investigate the role of the ribonucleolytic activity of ANG (28). This molecule binds with very high affinity to ANG ($K_i \sim 10^{-13}$ - 10^{-16} M) (51) and inhibits its activity in the chicken embryo chorioallantoic membrane (52). However, this compound had no significant inhibitory effect on NO release after ANG stimulation, thus showing that the ribonucleolytic property of ANG is not related to its ability to induce NO release in endothelial cells.

Neomycin, an aminoglycoside antibiotic, was used to inhibit ANG nuclear translocation (50, 53, 54). In particular, this drug has been used to inhibit tumor growth in athymic mice (40). In the MMA assays, presence of neomycin inhibited ANG induced NO release, which was thought to indicate that nuclear translocation is critical for NO generation. However, neomycin has been reported to show serious ototoxicity and nephrotoxicity (40). This tends to indicate that neomycin has several effects on cells, and that the inhibition of NO release could arise from an-

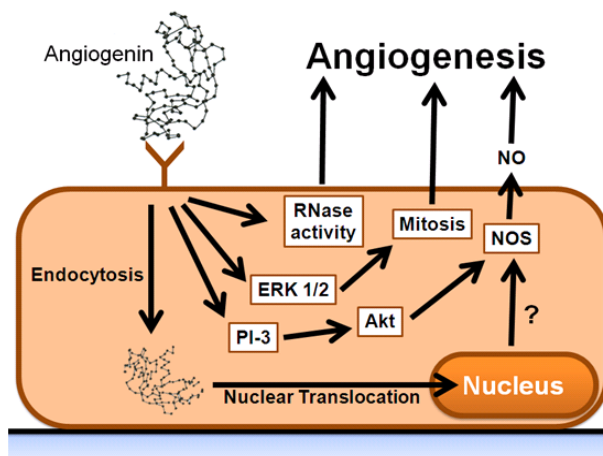


Fig. 2. Proposed pathway of angiogenin induced NO synthesis.

other factor other than blocking of nuclear translocation. In particular, inhibition of Akt phosphorylation in the presence of neomycin has been described (55), and the inhibitory effect of neomycin in the case of NO release is probably due to interactions with the kinase pathway rather than with nuclear translocation. Therefore, we cannot yet reach a conclusion on the role of nuclear translocation in ANG induced NO release. Neamine, a less toxic antibiotic produced by methanolysis of neomycin, shows the same antiangiogenic effects and also blocks ANG nuclear translocation (40), but does not block the phosphorylation of Akt (56). This molecule could therefore be a good candidate for selective inhibition of ANG nuclear translocation. Our current results on the relationships between ANG and NO are summarized in Fig. 2.

CONCLUSIONS

In this mini-review, we have summarized the results obtained with the MMA in a study of angiogenic factor induced NO release. The methodology was designed to obtain a robust device allowing measurements using different types of samples (cells or excised tissues), and was characterized with a well-known growth factor (VEGF).

More importantly, this device was used to demonstrate that ANG induces NO release and to examine and better understand the biochemical cascade leading to this event. This electrochemical chip has therefore already proven its versatility and usefulness as a tool for biochemical functional assays, and could find applications in drug testing, fundamental research, or screening of pathological bio-samples.

Acknowledgements

This work was supported by the National Research Foundation of Korea (Global Research Laboratory programme, K20904000 004-10A0500-00410) and the Chungbuk National University

(to S.-I.C.) in 2010.

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