

## Integrated Microdisk Gold Electrode Modified with Metal-porphyrin and Metal-phthalocyanines for Nitric Oxide Determination in Biological Media

Il Kwang Kim,\* Hyun Ok Bae,† Gi Soo Oh,† Hun Taeg Chung,† Young Jin Kim, and Hyun Ja Chun

*Institute of Basic Natural Science, Chemistry Department, Wonkwang University, Iksan City 570-749, Korea*

*†Department of Microbiology, College of Medical Science, Wonkwang University, Iksan City 570-749, Korea*

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An integrated gold microdisk electrode was constructed and modified with metal-porphyrin or metal-phthalocyanines for NO determination in biological media. Microanalysis of NO using square wave anodic stripping voltammetry in  $1 \times 10^{-2}$  M HClO<sub>4</sub> was optimal when the accumulation potential was 0.1 V, frequency 100 Hz, and the scan rate was 200 mV/s. When the electrode was modified with metal-porphyrin or metal-phthalocyanines, the anodic peak currents of NO increased due to the catalytic oxidation of NO. In case of Fe(II)-phthalocyanine modified electrode, the peak currents remarkably increased and the sensitivity was high. The calibration curve had good linearity in the range from  $3.6 \times 10^{-5}$  M to  $7.2 \times 10^{-7}$  M, and the detection limit was  $5.7 \times 10^{-7}$  M. For the structural stability and increased sensitivity, Fe(II)-phthalocyanine modified gold microdisk electrode coated with Nafion was applied to determination of NO released from cultured macrophase.

**Key Words :** Modified electrode, NO determination, Metal-phthalocyanine, Square wave anodic stripping voltammetry

### Introduction

Nitric oxide (NO), the unassuming molecule that suddenly grabbed the spotlight a little over a decade ago as a ubiquitous biological signaler in mammals, plays a mystic role in various phenomena from blood vessel dilation to smooth-muscle relaxation. It is also very important in neural transmittance related to cognition and control of sexual function, and other biochemical processes.<sup>1-4</sup>

NO was known to be capable of inactivating biologically important enzymes such as ribonucleotide reductase through its reaction with the metal center to form S-nitrosylation or sulfhydryl oxidation. NO was previously shown to have inhibition on the glucose oxidase (GOX) activity,<sup>5-7</sup> although no literature has yet been recorded about the mechanism of the inhibition of NO on GOX. The inhibition of NO on xanthine oxidase (XOD) activity was reported to be due to the NO reaction with an essential sulfur of the reduced molybdenum center of XOD to produce desulfo-type inactive enzyme.<sup>8</sup> Several physiologic phenomena in which NO is involved, such as the biologic mechanisms underlying symptoms of senility and antitumoral activities<sup>9,10</sup> would be better understood if NO could be directly detected in biological media.

For measurement and elucidation of NO effects in biological system,<sup>11-15</sup> various detection methods including ultraviolet spectrometry,<sup>16</sup> ESR spectroscopy,<sup>17</sup> laser absorption spectroscopy<sup>18</sup> chemiluminescence detection,<sup>19,20</sup> fiberoptic sensor,<sup>21</sup> and gas chromatography-mass spectrometer<sup>22</sup> have been reported. There are currently two amperometric methods available for measuring NO from intact tissues and single cells.

The first amperometric approach was based on the direct

electrooxidation of NO on a platinum electrode coated with different membranes such as chloroprene, nitrocellulose, silicone or Nafion, and cellulose acetate.<sup>23,34</sup> Shibuki<sup>35</sup> recently reported the measurement of NO release in the cerebellum using a microelectrode. He modified a miniature O<sub>2</sub> electrode by sealing its fire-polished 150-250 μm tip with a thin chloroprene rubber seal so that only low molecular weight gases could enter.

By introducing a platinum cathode within the pipette and holding it at positive voltage, he was able to detect NO, a readily oxidized gas, by its oxidation at the electrode surface. Pipettes were filled with 30 mM NaCl and 0.3 mM HCl (pH 3.5), and a Teflon-coated platinum wire was placed as close as possible to the membrane. A 200 μm silver wire was the anode. The cathode was kept 0.9 V from the anode and the anode was grounded. The principle of the assay is that if the anode is grounded there should be no current. There was a linear relationship between current and NO concentration *in vitro* over a small range (1-3 μM NO). This electrode was able to measure NO release in rat cerebellar tissue elicited by electrical stimulation.

The reproducibility of this electrode was affected by numerous parameters which are difficult to control, and especially by variations in the membrane thickness.<sup>36</sup>

A second, more sensitive technique is based on the observation that metalloporphyrins catalyze the oxidation of NO ( $\text{NO} \cdot - e^- \rightarrow \text{NO}^+$ ), and in so doing generate electrical current. Malinski and Taha<sup>37,38</sup> coated carbon fibers (chosen as a convenient, strong microprobe material) with a thin polymeric porphyrin layer and measured NO as the electrical current produced at 0.63 V. To minimize detection of NO<sub>2</sub><sup>-</sup> they coated the carbon fiber with Nafion, a negatively charged material that is highly impermeable to

anions. Nafion-coated fibers displayed no change in current when a 20-fold excess of  $\text{NO}_2^-$  was added to the sample.<sup>39-42</sup> This technique permitted detection of NO production from cultured endothelial and smooth muscle cells. There are several reports of porphyrinic sensor for in situ detection of NO production in various biological systems.<sup>43-48</sup> The techniques are recently explored a further extension of the electropolymerization and new electrocatalytic materials such as phthalocyanines, annulene derivatives, and cellulose acetate.<sup>49-52</sup> To date, a novel microchip NO sensor for real time<sup>53</sup> and updated review article for electrochemical NO sensors were reported.<sup>54</sup>

In this article, we report on a miniature integrated gold microdisk electrode system which was constructed and coated with each of Co(II)-porphyrin, Co(II)-phthalocyanine, and Fe(II)-phthalocyanine. We evaluated the optimum condition of Fe(II)-phthalocyanine electrode for direct measurements of NO by square wave anodic stripping voltammetry (SWASV) and used in analysis of NO generated from sodium nitroprusside (SNP) and macrophages (RAW 264.7).

### Experimental Section

**Reagents.** A NO saturated solution was obtained by bubbling NO gas (98.5%, Aldrich Co.) through deoxygenated distilled  $1 \times 10^{-2}$  M  $\text{HClO}_4$  solution for 40 min, using a value of 1.9 mM for its concentration at saturation.<sup>51,53</sup> A series of standard NO solutions were prepared by diluting aliquots of NO saturated solution. The prepared NO solutions were kept in a glass flask with a rubber septum, and stored in the dark to ensure stability for 3 h.

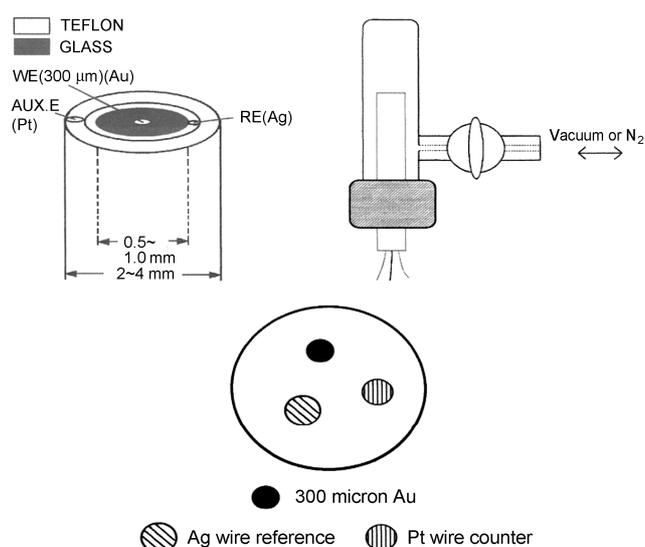
Co(II)-tetrakis (4-methoxy-phenyl)-porphyrin (Co(II)-TMPP), Co(II)-phthalocyanine (Co(II)-PC), Fe(II)-phthalocyanine (Fe(II)-PC), and sodium nitroprusside (SNP) were purchased from Aldrich Co. Deionized water was obtained by filtering through a Millipore Milli-Q filter. Nitrogen was bubbled through a vanadium chloride and a basic pyrogallol solution.

**RAW 264.7 macrophage cell line and culture.** The murine macrophage cell line (RAW 264.7) was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were maintained in complete Rosewell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C.

**Instruments.** Bundles of integrated gold microdisk electrodes were connected to a PARC model 303A electrode system and voltammograms were obtained with a PARC model RE0093 digital plotter and a PARC model 384B polarographic analyzer. SWASV of NO were obtained in  $\text{HClO}_4$  electrolyte solution under the nitrogen atmosphere.

Optimum conditions were evaluated by investigating the current intensity according to changes in the type of electrolytes, accumulation potential, accumulation time, frequency and scan rate. Aliquots of NO solution were subsequently added with a gas-tight syringe and the current response due to NO oxidation was recorded after each addition.

**Preparation of the integrated microdisk gold electrode.** In the miniature integrated three electrode system, gold wire



**Figure 1.** Top view of an IMGE system.

(300  $\mu\text{m}$  diameter) as working electrode and silver wire (500  $\mu\text{m}$  diameter) as the reference electrode, and Pt wire (500  $\mu\text{m}$  diameter) as the counter electrode were constructed as shown in Figure 1.

The gold working electrode was constructed by placing a 1 cm long gold wire into a cutted glass capillary tube. The hole of the capillary tube was sealed using a gas flame. Silver resin and copper wire were pushed into the opposite hole of the capillary tube to make an ohmic contact with the gold wire located inside and the capillary tube was dried in an oven at 80 °C for 2 h. The bundle of three electrodes was fixed with heat-shrinking tube, and the silver and platinum wires (1.5 cm long) were soldered to the copper wire. The bundle of electrodes was placed into a plastic tube (12 cm long) and filled with a homogeneous mixture of both Epon 825 resin (60 g) and methyl phenylene diamine (9 g). The plastic was peeled off after heating at 80 °C for 2 h and 130 °C for 6 h.

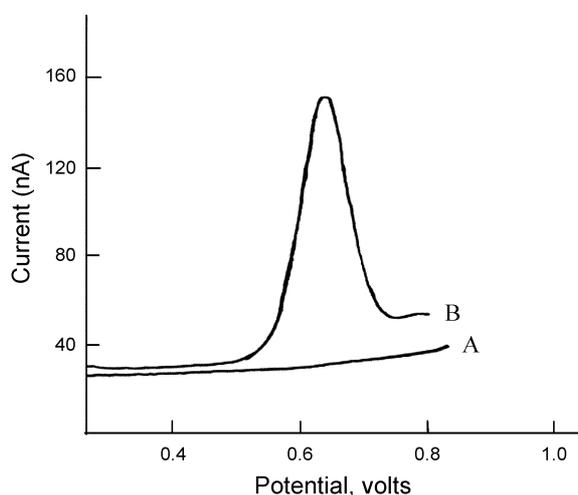
The electrode surface was rubbed with sandpaper, polished with alumina powder, and cleaned in a sonicator. A cyclic voltammogram (CV) was obtained by adding 0.1 M ferrocene used as an internal standard in 0.1 M tetra butyl ammonium perchlorate (TBAP) electrolyte solution, then the area (A) and diameter (r) of the working electrode were calculated. There was not a large difference in diameter of the electrode between before and after the construction. To modify the electrode, it was dipped into an N,N-dimethyl formamide (DMF) solution of the metal-porphyrin or metal-phthalocyanines. Prior to the determination of NO, the modified microelectrodes were further coated with Nafion by dipping in the 1.25% (w/v) Nafion solution and then placing the electrode under an infrared lamp to allow the ethanol to evaporation.

### Results and Discussion

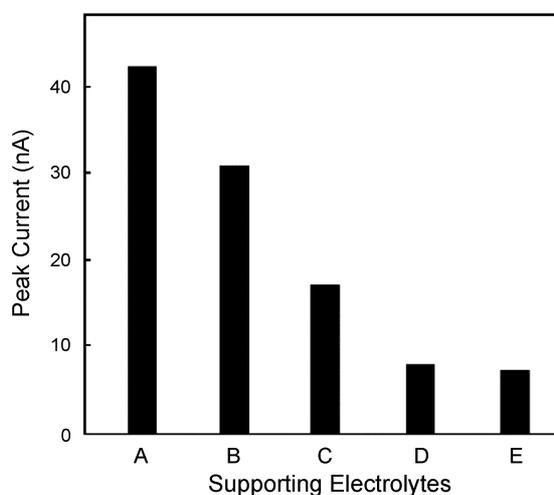
#### NO determination with the integrated microdisk gold

**electrode.** The SWASV method with the integrated micro-disk gold electrode (hereafter IMGE) was used to measure NO concentration at short time. On the  $1.8 \times 10^{-5}$  M NO in  $1 \times 10^{-2}$  M HClO<sub>4</sub>, the experiments with 200 mv/sec scan rate, 100 Hz frequency were performed to select suitable supporting electrolyte and the typical SWASV was showed in Figure 2. The peak currents of NO according to supporting electrolytes were showed in Figure 3. The value of the peak current was remarkably low when using a neutral supporting electrolyte (PBS or KCl), but was high when using an acid supporting electrolyte (HCl or HClO<sub>4</sub>) and was highest in the HClO<sub>4</sub>.

The measured peak currents according to concentrations of HClO<sub>4</sub> are shown in Table 1. The peak potential shifted toward positive potential as the concentration of HClO<sub>4</sub> increased because the hydrogen ion is contributing to the thermodynamic reversibility in electrochemical oxidation



**Figure 2.** Typical SWASV on the NO ( $1.8 \times 10^{-5}$  M) of IMGE in 0.1 M HClO<sub>4</sub>: scan rate; 200 mV/sec, accumulation potential; 0.1 volt, frequency; 100 Hz. A: Blank solution (0.1 M HClO<sub>4</sub>), B: added  $1.8 \times 10^{-5}$  M NO



**Figure 3.** Peak currents of  $1.8 \times 10^{-5}$  M NO in various supporting electrolytes using an IMGE. A: 0.1 M HClO<sub>4</sub>, B: 0.1 M HCl, C: Saline, D: Phosphate buffered solution, E: 0.1 M KCl

process of NO when the concentration of HClO<sub>4</sub> increases. The anodic peak observed at +0.62~0.75 V was consistent with previously reported oxidation potential of NO.<sup>35,43,44</sup> The peak currents were increased as the concentration of HClO<sub>4</sub> decreased, and was highest in  $1 \times 10^{-2}$  M HClO<sub>4</sub> solution. We used  $1 \times 10^{-2}$  M HClO<sub>4</sub> solution for the supporting electrolyte in the following experiments.

**Table 1.** Peak current of  $3.6 \times 10^{-6}$  M NO with various concentration of HClO<sub>4</sub> using an IMGE

Concentration (mol/L)	Peak potential (volts)	Peak current (nA)
0.01	0.656	50.5
0.05	0.676	47.7
0.10	0.694	42.4
0.50	0.726	29.9
1.00	0.756	16.2

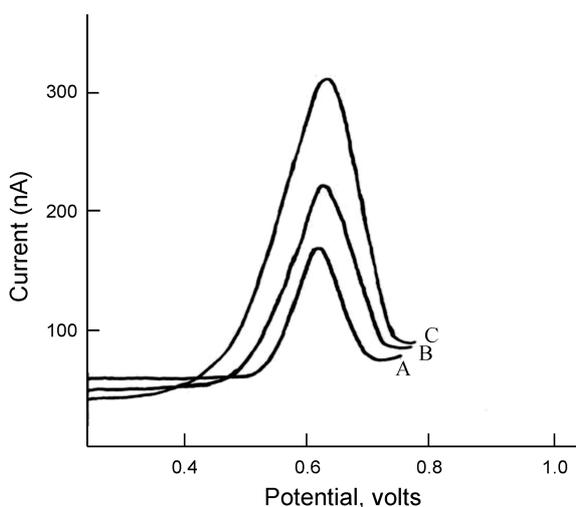
The peak currents were obtained by changing the accumulation potential from 0.4 to -0.3 V in  $1.8 \times 10^{-5}$  M NO solution, to determine the accumulation potential effect on the peak current of NO oxidation. The peak current gradually increased as the accumulation potential changed from 0.4 to 0.1 V, but it decreased as it changed from 0.1 to -0.3 V. Therefore, 0.1 V was chosen as the accumulation potential.

The peak currents were obtained changing the frequency in  $1.8 \times 10^{-5}$  M NO solution, to determine the effect of frequency on the anodic peak of NO. The diffusion current in SWASV was proportional to square root of frequency ( $\sqrt{f}$ ) and increased as frequency increased. In this experiment, a frequency of 100 Hz was chosen to obtain a stable peak current.

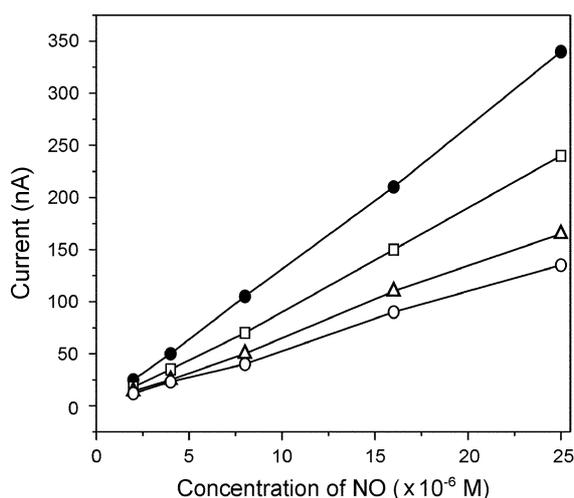
The peak currents were obtained by changing the accumulation time from 0 to 90s in  $1.8 \times 10^{-5}$  M NO standard solution, to determine the effect of the accumulation time on the anodic peak of NO. A maximum peak current obtained when the accumulation time was 30s, but the peak current decreased slightly when the accumulation time was over 30s. Under these conditions, the accumulation time does not effect a expected change influence to the peak current. In the following experiments, short accumulation time (10 sec) was applied because NO is unstable molecule.

The peak currents were obtained by changing scan rate after the accumulation potential (0.1 V), and the frequency (100 Hz) were fixed in  $1.8 \times 10^{-5}$  M NO, to determine the effect of the scan rate on the anodic peak of NO. An increase in the peak current was directly proportional to the square root of the scan rate ( $\sqrt{v}$ ) and the electron number ( $n^{3/2}$ ). We chose 200 mV/s as the scan rate to maintain a proper diffusion rate of the NO molecule and to shorten the analysis time.

**The effect of Co(II)-TMPP and Co(II)-PC on the NO oxidation.** The typical SWASV of the electrode modified with Co(II)-TMPP and Co(II)-PC was showed in Figure 4. The peak currents for the modified electrode were increased as follows Co(II)-PC > Co(II)-TMPP > bare IMGE. The peak currents were measured in various concentration and



**Figure 4.** Typical SWASV on the NO ( $1.8 \times 10^{-5}$  M) of IMGE modified with Co(II)-complex. A: bare IMGE, B: IMGE modified with Co(II)-TMPP, dipped in 0.1 mM Co(II)-TMPP for 45 min. C: IMGE modified with Co(II)-PC, dipped in 0.1 mM Co(II)-PC for 45 min.



**Figure 5.** The effect on the IMGE of dipping time and concentration of Co(II)-PC. -○- : bare gold, -△- : 0.1 mM·20 min, -□- : 0.5 mM·5 min, -●- : 0.1 mM·45 min.

dipping time to determine the effect of Co(II)-PC (Fig. 5). In the Figure 5, the peak current of longer dipping time in the lower concentration was higher than other's. The peak current of the electrode modified in 0.1 mM for 45min. increased three-fold compared with the bare IMGE.

**The effect of Fe(II)-TMPP and Fe(II)-PC on the electrochemical oxidation for NO.** The typical SWASV of the electrode modified with Fe(II)-TMPP and Fe(II)-PC were showed in Figure 6. In the Figure 6, the peak potentials of modified electrode compared with the bare IMGE were slightly shifted to the anodic potential. The peak currents of the modified electrode were increased as follows Fe(II)-PC > Fe(II)-TMPP > bare IMGE.

The peak currents were measured in various concentration and dipping time to determine the effect of Fe(II)-Pc, and showed in Figure 7. In the Figure 7, the peak current for the

modified electrode by dipping into 0.1 mM Fe(II)-PC for 45 min. was highest. The peak currents of the Fe(II)-PC modified electrode increased than the Co(II)-PC modified electrode in Figure 4. When the dipping time was long in diluted metal-TMPP or metal-PC, the redox-catalytic effect of the modified electrode was increased.

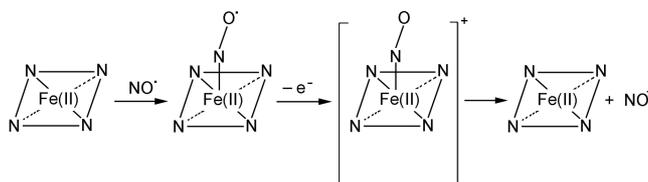
Malinski, *et al.*<sup>37-40</sup> have measured NO concentration on the carbon fiber electrode modified with Ni(II)-tetrakis (3-methoxy-4-hydroxy phenyl) porphyrin (hereafter Ni(II)-TMHP) by differential pulse voltammetry. The sensitivity of our IMGE modified with Fe(II)-PC was higher than Malinski's carbon fiber electrode modified with Ni(II)-TMHP because phthalocyanine has more stable structure than porphyrin. NO molecules are accumulated to the central metal of complexes by bonding formation as a bented axial ligand and oxidized at +0.6~0.7 V. The redox process of NO on the Fe(II)-PC complexes was showed in Schem 1.

**The effect of Nafion on the NO oxidation.** Malanski<sup>37,38</sup> and Friedemann<sup>27</sup> reported that a Nafion coating should provide selectivity to detect NO against  $\text{NO}_2^-$ . The cation exchanger Nafion film on the electrode prevented diffusion of  $\text{NO}_2^-$ , while the neutral NO radical diffuse easily through the coating layer. In this article, the effects of various Nafion concentration and the number of dipping times were investigated and showed in Figure 8. When the electrode coated with Nafion, the peak currents were decreased than non-Nafion Fe(II)-PC electrode, just as the current response was still higher than the bare IMGE.

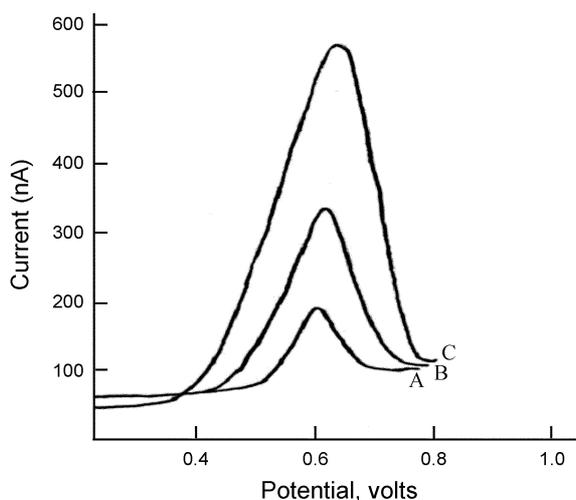
In addition, the peak currents of NO oxidation was decreased as a number of dipping times increased. If selectivity is not a concern, then Fe(II)-PC modified electrodes may be extremely sensitive to measure of low nanomolar NO. As a selected standard electrode, the linearity and detection limit of Fe(II)-PC electrodes coated three times with Nafion were determined.

The calibration curve was proportional to the concentration over a linear range from  $3.6 \times 10^{-5}$  M to  $7.2 \times 10^{-7}$  M and the detection limit was  $1.5 \times 10^{-7}$  M. The equation of standard calibration curve was  $Y = 27.43X - 5.86$ .

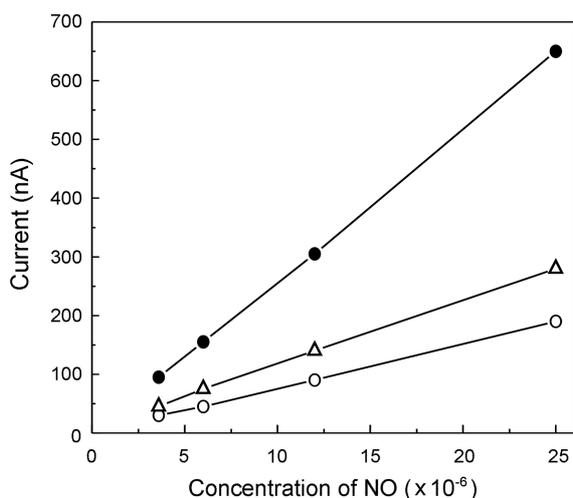
**Determination of released NO from SNP.** SNP used as a vasodilator in research on NO activity because NO is slowly formed through decomposition of SNP. SWASV were obtained under optimum condition of a sample solution made by SNP saturated solution diluted with  $1 \times 10^{-2}$  M  $\text{HClO}_4$  to investigate the NO concentration released from SNP. The peak potential of released NO was showed around 0.7 V which was as the same as that for NO solution. The result showed that the released NO from SNP was



**Scheme 1.** Suggested redox-catalytic process of NO molecule in Fe(II)-Pc.



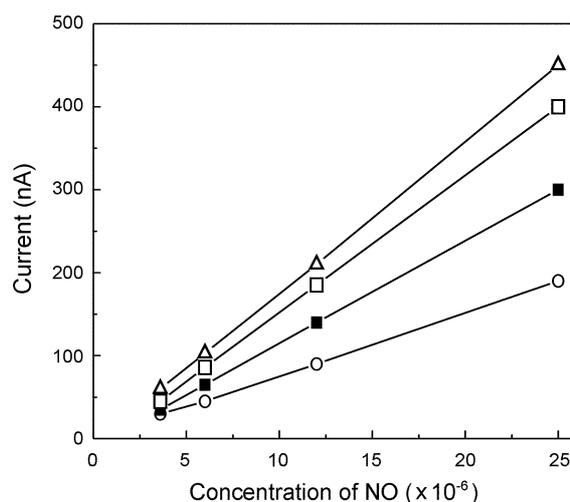
**Figure 6.** Typical SWASV on the NO ( $1.8 \times 10^{-5}$  M) of IMGE modified with Fe(II)-complex. A: bare IMGE. B: IMGE modified with Fe(II)-TMPP, dipped in 0.1 mM Fe(II)-TMPP for 45 min. C: IMGE modified with Fe(II)-PC, dipped in 0.1 mM Fe(II)-PC for 45 min.



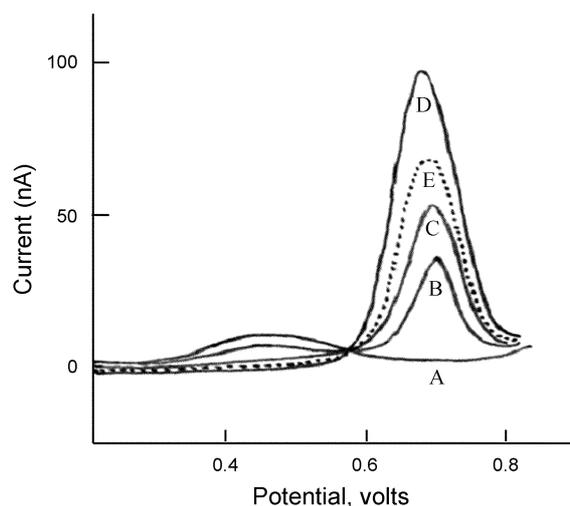
**Figure 7.** The effects on the IMGE of dipping time and concentration of Fe(II)-PC. -○-; bare IMGE -△-; 0.1 mM·30 min. -●-; 0.1 mM·45 min.

$0.21 \pm 0.03 \mu\text{M NO/mM}\cdot\text{min}$  when the value represents the mean  $\pm$  SD from 9 experiments. This means that 1 mM SNP dissolved in deoxygenated distilled water produces a peak current corresponding to  $0.21 \mu\text{M}/\text{min}$ . This value was consistent with those determined by the spectrophotometric measurements.<sup>55</sup>

**NO detection in RAW 264.7 macrophages.** NO was generated by culturing macrophage (RAW 264.7) cells in culture media, RPMI-1640, and incubating with liposaccharide and interferone- $\alpha$  for 12 h. The SWASV shown in Figure 6 was scanned under optimum conditions with an Fe(II)-PC modified electrode after pipetted 5 mL of the cultured media into 5 mL of electrolyte solution. In Figure 9, A was the SWASV of blank solution containing cultured media and electrolyte. The peaks of interfering substances from the



**Figure 8.** The effects on the Fe(II)-PC modified IMGE of dipping process in Nafion. -○-; bare IMGE -△-; 3 times for 5 min. in Nafion, -□-; 5 times for 5 min. in Nafion -■-; 8 times for 5 min. in Nafion



**Figure 9.** SWASV according to the NO concentration (2,3,6) on the Fe(II)-PC modified electrode into 10 mL of deoxygenated  $1 \times 10^{-2}$  M  $\text{HClO}_4$ . A: Blank solution (cultured media and electrolyte), B:  $2 \times 10^{-6}$  M, C:  $3 \times 10^{-6}$  M, D:  $6 \times 10^{-6}$  M NO in culturing media and electrolyte solution, E: Simple solution released from macrophage cell (converted value from standard calibration curve was  $4 \times 10^{-6}$  M NO)

cultured media appeared broadly between 0.4 and 0.6 V at Figure 9B, C and D were SWASV acquired in the process of adding a standard NO solution. The peaks increased with increased NO concentration. The oxidation peaks of the interfering substance of A decreased and the NO peak shifted to the cathodic potential as the NO concentration increased.

E in Figure 9 was a SWASV under the same condition but cell culture media containing macrophages. Value of the acquired peak current was approximately  $4.2 \pm 0.6 \mu\text{M}$ , compared with that of the standard calibration curve and converted to dilution ratio. This value was consistent with the result of the indirect determination by a specific chemiluminescent NO detector.<sup>56</sup>

### Conclusion

The IMGE was constructed and the SWASV method was applied to maximize ease of handling and quickly detect a micro-level concentration of NO. Microanalysis of NO using SWASV in  $1 \times 10^{-2}$  M HClO<sub>4</sub> as the supporting electrolyte was optimal when the accumulation potential 0.1 V, frequency 100 Hz, and the scan rate 200 mV/s.

The calibration curve had good linearity in the range from  $3.6 \times 10^{-5}$  M to  $7.2 \times 10^{-7}$  M, and the detection limit was  $1.5 \times 10^{-7}$  M. When the electrodes were modified with metal-porphyrin or metal-phthalocynine, the anodic peak currents of NO increased due to oxidation of NO by electrochemical catalytic effect. In case of Fe(II)-PC modified electrode, the peak currents remarkably increased and the sensitivity was highest among other modified electrodes. Fe(II)-PC modified gold microdisk electrode was applied to determination of NO released from cultured macrophage (RAW 264.7) in cell culture media (RPMI-1640). This Fe(II)-PC modified IMGE system could be used to NO monitor in real samples in future.

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