한국환경분석학회지 제 2권(제 2호) 149~157, 1999
J. of the Korea Society for Environmental Analysis

Modified Gold Microdisk Electrode for Nitric Oxide Determination in Biologic Media

Ho Sup Lee*, Hun Tag Jung**, Hyun Ja Chun and Il Kwang Kim

Department of Chemistry, College of Natural Science, Wonkwang University
**College of Oriental Medicine, WonKwang University*
***College of Medicine, WonKwang University*
Shinyong-dong, Iksan City, 570-749, South Korea

An integrated gold microdisk electrode was constructed and modified with metal-porphyrin or metal-phthalocyanines for NO determination in biologic media. Microanalysis of No using square wave anodic stripping voltammetry in 1×10^{-2}M $HClO_4$ 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 3.6×10^{-5} M to 7.2×10^{-7} M, and the detection limit was 5.7×10^{-7} M. For the structural stability and increased sensitivity, Fe(II)-phthalocyanine modified gold microdisk electrode coated with Nafion was applied to determine NO released from cultured macrophase (RAW 264.7) in cell culture media (RPMI-1640).

Key words : Modified electrode, NO determination, Metal-phthalocyanine, Square wave anodic stripping voltammetry.

1. Introduction

Nitric oxide (NO) is a regulating biomolecule involved in vasodilation, control of blood pressure, and the cellular immune response in human body. NO is also very important in neural transmission related to cognition (memory and forgetfulness), neural control of sexual function, and other processes.[1-4] Several physiologic phenomena in which NO is involved, such as the biologic mechanisms underlying symptons of senility, *etc.*, would be better understood if NO could be accurately detected in biologic media.

NO concentration can be measured indirectly by measuring the NO byproduct, nitrate,[6,7] or directly using spectroscopic and electrochemical methods.[8-11] The electrochemical method is most useful, because it is simple and quite sensitive. Shibuki *et al.*[12] reported the detection of NO from brain tissue at +0.9 V with a platinum micro-probe electrode. Ichimori *et al.*[13] used a NO selective electrode made from a Pt/Ir alloy coated with a three-layered membrane. Malinski et al.[14,15] used carbon fibers coated with tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin and Nafion® to minimize measuring of anionic interferents such as NO_2^-.

Devynec *et al.*[16,17] obtained linear measurements of NO concentrations ranging from 1.5 to 32 nM by coating a carbon wire with Ni-porphyrin and nafion, and measured NO within a range of μM by treating a gold microelectrode with the same compound. Friedemann *et al.*[18] used o-phenylene-diamine(O-PD) and Nafion® to modify the surface of 30 μm diameter carbon fiber electrode compared with glassy carbon electrode modified with various combinations of Nafion,® O-PD, or Ni(II)- tetrakis (4-hydroxy-3-methoxyphenyl) porphyrin in order

to decide which electrodes had the most sensitivity and selectivity for NO.

Ciszewski *et al.*[19,20] presented results of NO oxidation with a porphyrin-based sensor utilizing rotating disk experiments. Pallini *et al.*[21] studied different types of modified electrodes for biologic use. Jin et al.[22] described electrochemical microsensor for NO, which is based on an electropolymerized film of o-aminobenzaldehyde ethylenediamine Ni(II) and Nafion.® Electrochemical microelectrodes can be made extremely small and placed directly into biologic cells with minimal damage to surrounding tissues.[23]

In the present study, we determined NO concentration using voltammetry by constructing a miniature integrated gold disk microelectrode system modified by Co(II)-porphyrin, Co(II)-phthalocyanine, and Fe(II)-phthalocyanine. We detemined the optimum conditions for maximizing detection limits by surveying peak currents according to various analysis conditions, such as deposition potential, deposition time, frequency, etc., in an NO standard solution. We used the optimum conditions in an applied analysis of NO concentrations generated by sodium nitroprusside (SNP) and macrophages.

2. Experimental

2.1. Reagents

NO standards were prepared from saturated NO solutions. To produce a saturated NO solution (typically containing 1.8 mM NO), 1×10^{-2} M perchloric acid ($HClO_4$) solution was bubbled with purified nitrogen for 15 min to remove oxygen. The solution was then saturated with NO (98.5%, Aldrich Co.) for 40 min.[16,19] Standards were made fresh for each experiment and kept in a glass bottle with a rubber stopper. Dilutions of the saturated NO solution (2.0 mM) were prepared using deoxygenated $HClO_4$ solution.

Tetrakis(4-methoxy-phenyl)-porphin cobalt(III) (Co(III)-TMPP) and Co(II)-phthalocyanine (Co(II)- PC) and Fe(II)-phthalocyanine (Fe(II)-PC) and sodium nitroprusside, and Nafion® were all Aldrich Co. and used as purchased. Deionized water was obtained by filtering distilled water through a Millipore Milli-Q filter. Nitrogen was passed through a vanadium chloride and a basic pyrogallol solution before use. The cultured cells were produced by cultivating RAW 264.7 macrophages in the RPMI-1640 solution for 12 h.

2.2. Instruments

Bundles of three microdisk electrodes were connected to a PARC model 303A electrode system and voltammograms were obtained with a PARC model RE0093 digital plotter and an EG & PARC model 384B polarographic analyzer.

2.3. Consturction and Modification of the Microdisk Electrode

In the miniature integrated three-electrode system, gold wire (300 ㎛ diameter) as working electrode and silver wire (500 ㎛ diameter) as the reference electrode, and Pt wire (500 ㎛ diameter) as the counter electrode were constructed as shown in Fig. 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 blocked using a gas flame and the excess gold wire was removed by burning. 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℃ for approximately 2 h. The bundles

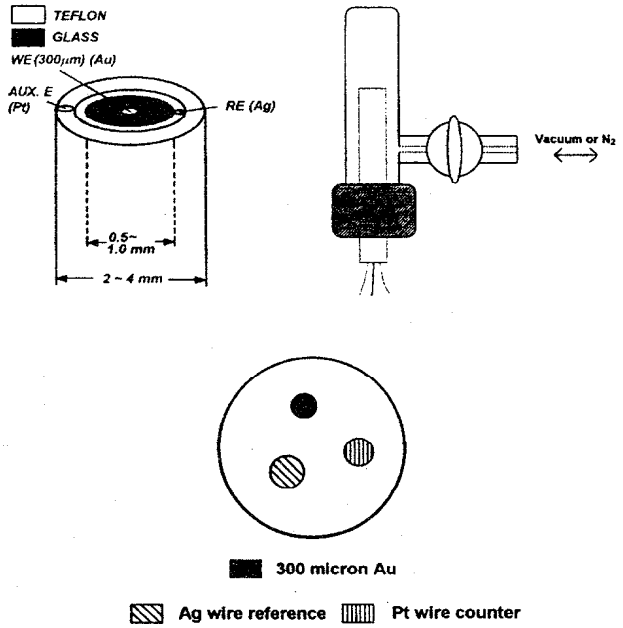


Fig. 1. Top view of the integrated gold microdisk electrode system.

of three electrodes were fixed with heat-shrink tubing, and the silver and platinum wires (1.5 cm long) were soldered to the copper wire. The bundles of electrodes were 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℃ for 2 h and 130℃ for 6 h.

The electrode surface was rubbed with sand-paper, polished with alumina powder, and cleaned using 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 between the electrodes before and after the construction and modification. To modify the electrode, it was dipped into an N,N-dimethyl formamide (DMF) solution in which the metal-porphyrin or metal-phthalocyanines were dissolved. Coverage of the modified electrode with Nafion® was obtained by sequential dipping in 1.25% aqueous-alcoholic solution of Nafion® as previously described.[14,16,18,24]

2.4. Experimental method

Square wave anodic stripping voltammetry (SWASV) was obtained by adding 10 mL of 0.1 M $HClO_4$ electrolyte solution to a cell, selecting the proper initial potential and pulse height under a nitrogen atmosphere, and scanning toward the anodic potential.

Optimum conditions were evaluated by investigating the current intensity according to changes in the type of electrolytes, accumulation potential, accumulation time, frequency, scan rate, *etc.* A standard calibration curve was obtained through the current values according to changes in the concentration of the saturated NO solution under optimum conditions.

3. Results and discussion

3.1. Preliminary Characterization of Gold Microdisk Electrode for SWASV of NO

As a preliminary experiment, an integrated gold microdisk electrode was constructed and the SWASV method was used to quickly detect a micro concentration of NO. The experiments with 200 mV/s scan rate and 100 Hz frequency were performed for the selection of a supporting electrolyte. The peak currents of NO were measured according to the type supporting electrolytes(Fig. 2). The value of the peak current was low when using a neutral supporting electrolyte (PBS or KCl), but was high when using an acid supporting electrolyte(HCl or $HClO_4$) and the peak current was highest in the $HClO_4$.

The measured peak currents using varying

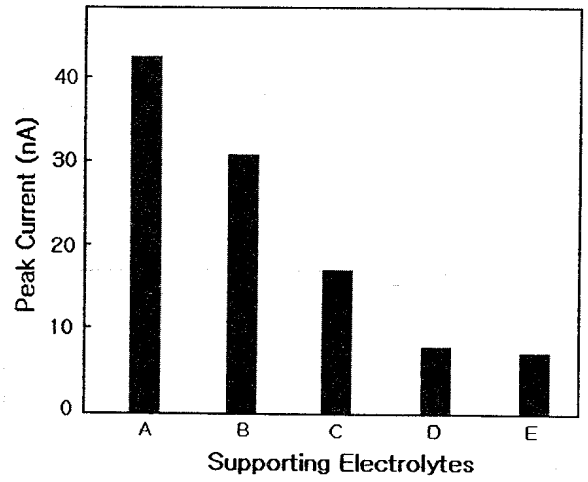


Fig. 2. Peak current of 3.6×10^{-6} M nitric oxide in various supporting electrolytes using an integrated gold microdisk electrode.
A : 0.1M $HClO_4$, B : 0.1M HCl, C : Saline, D : Phosphate buffered solution, E : 0.1M KCl

Table 1. Peak current of 3.6×10^{-6} M nitric oxide with various concentration of $HClO_4$ using an intergrated gold microdisk electrode system.

Concentration (mol/L)	Peak potential (volts)	Peak current (nA)
0.01	0.726	50.5
0.05	0.730	47.7
0.10	0.734	42.4
0.50	0.754	29.9
1.00	0.766	16.2

concentrations of $HClO_4$ are shown in Table 1.

The peak potential shifted toward the negative potential as the concentration of $HClO_4$ increased because the hydrogen ion diffuses over the electrode surface and acts competitively on the oxidation process of NO, when the concentration of $HClO_4$ increases. The anodic peak observed at +0.68 ~ 0.72 V is related to electrochemical oxidation of NO and this is consistent with previously reported oxidation potentials of NO.[12,16,23] There is an increase in the peak current as the concentration of $HClO_4$ decreases, and the peak current is highest in 1×10^{-2} M $HClO_4$ solution. We used 1×10^{-2} M $HClO_4$ solution for the supporting electrolyte in the following experiment.

The peak currents were obtained by changing the accumulation potential from 0.4 to - 0.3 V in 3.6×10^{-6} M NO solution, to determine the accumulation potential influences 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 into a cathodic potential of more than 0.1 V. Therefore, 0.1 V was chosen as the accumulation potential in this experiment. The peak currents were obtained by fixing accumulation potential at 0.1 V and changing the frequency in 3.6×10^{-6} M NO standard solution, to determine the influence of frequency on the anodic peak of NO. The diffusion current in square wave voltammetry is proportional to square root of frequency,[24] and the peak current increases as frequency increases. 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 90 s in 3.6×10^{-6} M NO standard solution, to determine the influence of the accumulation time on the anodic peak of NO. A maximum peak current obtained when the accumulation time was 30 s, but the peak current decreased slightly when the accumulation time was over 30 s. Under these conditions, the accumulation time does not largely influence to the peak current. The accumulation degree of NO on the electrode surface is dependent upon the diffusion coefficient, rate constant, and equilibrium constant. We performed this experiment in a state of zero accumulation time because NO is unstable.

The peak currents were obtained by changing the scan rate and by fixing the accumulation potential at 0.1 V, and the frequency at 100 Hz in 3.6×10^{-6} M NO standard solution, to determine

the influence of the scan rate on the anodic peak of NO. An increase in the peak current was almost constant with 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.

3.2. Effect of Co(II)-TMPP and Metal-PC Derivatives on Electrochemical Oxidation of NO

3.2.1. Effect of Co(II)-TMPP

The peak current was measured by constructing a modified electrode ; a bare gold microdisk electrode was washed with distilled water after dipping into DMF solution in which Co(II)-TMPP was dissolved. The peak currents of a modified electrode, and a bare gold microdisk electrode obtained through changes in Co(II)-TMPP concentration and dipping time were compared(Fig. 3). The peak currents for a modified electrode were increased than a bare gold microdisk electrode and the hugher peak current was obtained by dipping into 0.5 mM Co(II)-TMPP for 20 min.

3.2.2. Effect of Co(II)-PC

The peak currents were measured according to changes in Co(II)-PC concentration and dipping time to determine the effect of Co(II)-PC on the anodic peak current of NO. In the Fig. 4, the peak potential of a modified electrode was shifted in the direction of the anodic potential and all the peak currents increased. The longer the dipping time in a lower concentration (0.1 mM), the higher the peak current. The peak current of a modified electrode dipped in 0.1 mM for 45 min increased three-fold compared with a bare gold microdisk electrode.

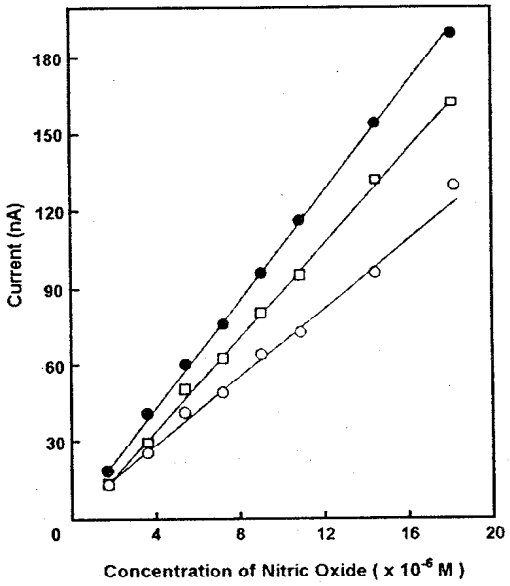


Fig. 3. Effect of dipping time and concentration of Co(II)-TMPP when using an integrated gold microdisk electrode modified with Co(II)-TMPP.
- ○ - : bare gold, - □ - : 0.1 mM • 20 min, - ● - : 0.5 mM • 5 min

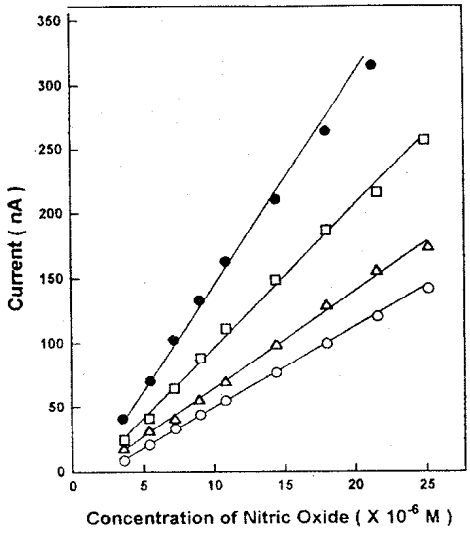


Fig. 4. Effect of dipping time and concentration of Co(II)-PC when using an integrated gold microdisk electrode modified with Co(II)-PC.
- ○ - : Bare gold, - △ - : 0.1 mM • 30 min, - □ - : 0.5 mM • 10 min, - ● - : 0.1 mM • 45 min.

3.2.3. Effect of Fe(II)-PC and Nafion® Coating

Fe(II)-PC modified electrode was constructed

in a same method of Co(II)-PC to determine the effect of Fe(II)-PC on the anodic peak of NO ; the peak currents were evaluated according to changes in concentration and dipping time, and numbers of Nafion treatment. Compared with a bare gold microdisk electrode in Fig. 5, the peak potential of a modified electrode shifted to the direction of anodic potential (+ 0.8 V) and all the peak currents increased. The peak current was highest for a modified electrode made by dipping into a 0.1 mM for 45 min, which increased five-fold. The peak currents of a modified electrode made with Fe(II)-PC increased more than that of a modified electrode made with Co(II)-PC. It means that Fe(II)-PC was more effective for an electrocatalytic oxidation of NO.

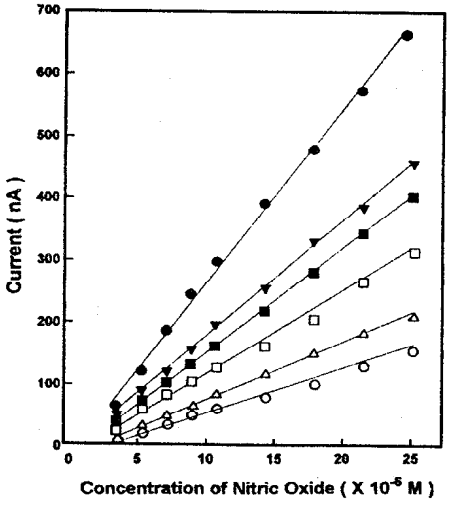
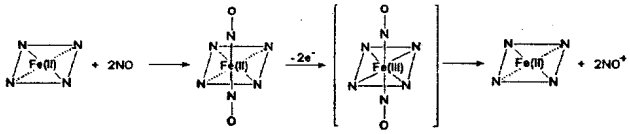


Fig. 5. Effect of dipping time and concentration of Fe(II)-PC when using an integrated gold microdisk electrode modified with Fe(II)-PC.
- ○ - : Bare gold, - △ - : 0.1 mM • 20 min, - ● - : 0.1 mM • 45 min, - ▲ - : 3 times for 5 min in Nafion,® - ■ - : 5 times for 5 min in Nafion,® - □ - : 8 times for 5 min in Nafion.®

Metal-porphyrin and PC derivatives worked as effective redox-catalysts, when the dipping was long time in diluted metal-porphyrin or metal-PC. This suggests that the well-ordered self-assembled layer of the complexes is formed on the surface and the accumulation of NO to the layer increased. NO molecules accumulate by bonding as an axial ligand to the central metal of complexes adsorbed on the electrode surface.[26,27] Co(II)-TMPP or Fe(II)-PC system, NO molecules are oxidized in the processes at which the central metal atom was first oxidized at +0.7 ~ +0.8 V, and the oxidized central metal is reduced (Scheme 1).



Scheme 1. Redox-catalytic process of NO molecule in porphyrin or phthalocyanine complex modified electrode system.

Malinski[14,15] modified carbon fiber with tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin and monitored NO with differential pulse voltammetry. The sensitivity of this gold microdisk electrode modified with Fe(II)-PC was higher than that of Malinski's carbon fiber electrode. Metal-PC which we could obtain an NO oxidation current with ease and sensitivity has more stable structure than metal-porphyrin.

As shown previously by Malinski, etc.[14] and Friedemann,[18] Nafion® coatings should provide selectivity against NO_2^-. The cationic exchanger Nafion® film coated on the gold electrode prevented the diffusion of NO_2^- from the bulk solution to the electrode surface, while the neutral NO radical diffused easily through the coating. We have investigated the usefulness of Fe(II)-PC modified electrodes coated with different concentration of Nafion® and the number of dipping times. With non-Nafion,® the peak current was highest in case of 0.1 mM Fe(II)-PC for 45 min and increased modifying with Fe(II)-PC. The Fe(II)-PC modified electrodes coated several times with Nafion® showed decrease in current

response, but still higher than a bare gold microdisk electrode or electrodes modified in 0.1 mM Fe(II)-PC for 20 min.

In addition, the peak currents of NO oxidation decreased as a number of dipping times. If selectivity is not a concern, then Fe(II)-PC modified electrodes may be extremely sensitive to low nanomolar NO. Due to their stability, we have determined the linearity or detection limit of Fe(II)-PC electrodes coated three times with Nafion.® The calibration curve was proportional to concentration over a broad range from 3.6×10^{-5} M to 7.2×10^{-7} M, and the detection limit was 1.5×10^{-7} M. The equation of standard calibration curve was Y = 25.51X - 15.63.

3.3. Determination of Released NO from SNP

SNP is a vasodilator used in research on NO activity because NO is slowly formed following decomposition of SNP. Voltammograms were obtained under optimum conditions after dissolving SNP in deoxygenated distilled water, making a sample solution by diluting it and adding 5 mL each into 5 mL of 1×10^{-2} M $HClO_4$ supporting electrolyte to investigate the NO concentration released from SNP. The peak currents are shown around 0.7 V, which is as the same as that for NO. The result showed the generating NO was 0.21 ± 0.03 μM NO/mM · min when the value represents the mean ± SD from 9 experiments. That means 1 mM SNP dissolved in deoxygenated distilled water produces a peak current corresponding to 0.21 μM/min. This value is consistent with those determined by the spectrophotometric measurements.[28]

3.4. NO Detection in RAW 264.7 Macrophages

NO was generated by culturing macrophage (RAW 264.7) in cell culture media, RPMI-1640, and incubating with liposaccaride and interferone-α for 12 h. The SWASV shown in Fig. 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 Fig. 6, A is the SWASV of a blank solution containing no cells, and the peaks of contamination or interfering substances from the cultured media appeared broadly between 0.4 and 0.7 V. B, C. and E are 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.

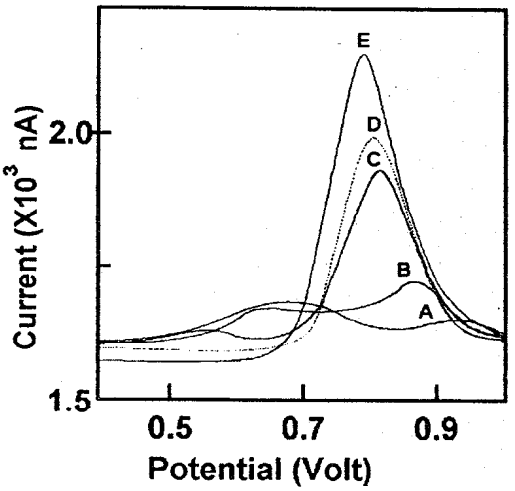


Fig. 6. Typical square wave anodic voltammograms according to the NO concentration on the Fe(II)-PC modified integrated gold microdisk electrode in the culturing media-supporting electrolytes (1:1) solution.
A : Blank solution ; 5 mL culturing media and 8 mL 1×10^{-2} M $HClO_4$
B : 1×10^{-6} M standard NO solution
C : 2×10^{-6} M standard NO solution
E : 3×10^{-6} M standard NO solution
D : SWAV of released NO from Macrophage cell ; estimated value in 4.2×10^{-6} M

D in Fig. 6 was a SWASV acquired under the same condition but cell culture media containing

macrophages. Value of the peak current acquired was approximately 4.2 μM, compared with that of the standard calibration curve and converted to dilution ratio. This value was consistent with the result (3 μM) of the indirect determination of NO using fluorophotometry.[29] Thus, the gold microdisk electrode modified with metal-PC can be use mostly for measuring NO in a biologic cells.

4. Conclusion

An integrated gold microdisk electrode was constructed and the SWASV method was used to maximize ease of handling and quickly detect a micro-level concentration of NO. Microanalysis of nitric oxide using SWASV in 1×10^{-2} M $HClO_4$ as the supporting electrolyte was optimal when the accumulation potential was 0.1 V, frequency 100 Hz, and the scan rate was 200 mV/s.

The calibration curve had good linearity in the range from 3.6×10^{-5} M to 7.2×10^{-7} M, and the detection limit was 1.5×10^{-7} M. When the electrodes were modified with metal-porphyrin or metal-phthalocynine, the anodic peak currents of NO increased due to the catalytic oxidation of NO. In case of Fe(II)-PC modified electrode, the peak currents largely increased and the sensitivity was high. For the structural stability and increased sensitivity, 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).

5. Acknowledgment

This work was supported by a grant of Wonkwang University (1998) and Korea Science & Engineering Foundation(KOSEF), and Chunbuk Provincial Government through Medicinal Resources Research Center (98-1603-01A3).

References

1. S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacol.* **1991**, 43, 109-114.
2. A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.* **1993**, 223-226.
3. J. E. Albina, S. J. Cui, R. B. Mateo and J. S. Reichner, J. Immumol. **1993**, 150, 5080-5083.
4. J. Collier and P. Vallance, *Br. Med. J.* **1991**, 302, 297-302.
5. R. Rauls, C & EN. **1998**, Oct. 19, 13.
6. T. Pai, W. Payne and J. LeGall, *Anal. Biochem.* **1987**, 166, 150-156.
7. H. Miyoshi, S. Nippa, H. Mori and H. Yoneyama, *Bull. Chem. Soc. Jpn.* **1990**, 63, 3380-3384.
8. K. Ohta, N. Araki, M. Shibata, J. Hamada, S. Komatsumoto, K. Shimazu and Y. Fukushi, *Neurosci. Lett.* **1994**, 176, 165-168.
9. Q. Zhou, G. R. Hellerman and L. P. Solomonson, *Thromb Res.* **1994**, 77, 87-96.
10. F. L. Kiechle and T. Malinski, *Am. J. Clin.. Pathol.* **1993**, 100, 567-569.
11. M. Kelm, M. Feelisch, R. Spahr, H. M. Piper, E. Noack and J. Schrader, *Biochem. Biophys. Res. Commun.* **1988**, 154, 236-244.
12. K. Shibuki, *Neurosci. Res.* **1990**, 9, 69-76.
13. K. Ichimori, H. Ishida, M. Fukahori, H. Nakazawo and E. Murakami, *Rev. Sci. Instrum.* **1994**, 65(8), 2714-2718.
14. T. Malinski and Z. Taha, *Nature* **1992**, 358(20), 676-678.
15. T. Malinski, Z. Taha, S. Grumnfeld, A. Barewicz, P. Tomboulin and F. Kiechle, *Anal. Chim. Acta* **1993**, 279, 135-140.
16. F. Bedioui, S. Trevin and J. Devynck, *J. Electroanal. Chem.* **1994**, 377, 295-298.
17. F. Lantoine, S. Trevin, F. Bedioui and J. Devynck, *J. Electroanal. Chem.* **1995**, 392,

85-91.
18. M. N. Friedemann, S. W. Robinson, and G. A. Gerhardt, *Anal. Chem.* **1996**, 68, 2621-2628.
19. A. Ciszewski, G. Milczarek, E. Kubaszewski, and M. Lozynski, *Electroanalysis*, **1998**, 9, 628-632.
20. A. Ciszewski and G. Milczarek, *Electroanalysis*, **1998**, 10, 791-796.
21. M. Pallini, A. Curulli, A. Amine, and G. Palleschi, *Electroanalysis*, **1998**, 10, 1010-1015.
22. H. Tu, L. Mao, X. CaO, and L. Jin, Electroanalysis, 1999, 11, 70-74.
23. D. A. Wink, D. Christodoulou, M. Ho, R. Murray and T. Meyer, *A companion to methods in enzymology*, **1995**, 7, 71-77.
24. F. Pariente, J. L. Alonso and H. D. Abruna, *J. Electroanal. Chem.* **1994**, 379, 191-197.
25. A. J. Bard *"Electroanalytical Chemistry : Square Wave Voltammetry,"* Marcell Dekker New York, **1986**, Vol. 14, pp 259-267.
26. B. B. Wayland, L. W. Olson, *J. Am. Chem. Soc.* **1974**, 96, 6037-6042.
27. R. Guilard, G. Lagrange, A. Tabard and K. M. Kadish, *Inorg. Chem.* **1985**, 24, 3649-3654.
28. I. Ioamnidis and H. de Groot, *Biochem. J.* **1993**, 296, 341-347.
29. C. D. Jun, B. M. Choi, H. M. Kim, and H. T. Chung, *J. Immunol.* **1995**, 154, 6541-6547.