A New Determination Method of Nitric Oxide (NO) with a NO-selective Electrode Demonstrated by the Kinetic Analysis of NO Generation and Decomposition

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A new determination method of nitric oxide (NO) was proposed by a NO-selective electrode on the basis of the kinetic analysis for the time-dependent amperometric currents due to NO generation and decomposition. From the linear relationship between the observed currents and the estimated NO concentrations in NONOate (NOC7) at physiological pH, the method was concluded to be useful for quantification of NO.

Recently, nitric oxide (NO) has been shown to play many physiological and pathophysiological roles in biological systems.[1,2] Therefore, in vivo and in vitro NO detection is needed to investigate such roles of NO. For this purpose, several analytical methods such as fluorometry[3] and EPR spectrometry[3,4] have been used. In real-time in vivo quantification of NO, an amperometric NO-selective electrode has been used because of its convenience and high sensitivity for continuous monitoring of NO in local sites.[5–7] *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) has been generally used as a standard of NO-donor, and 1 mM (M = mol dm^{-3}) of SNAP was assumed to generate a 1.3 μM of NO.[8] The generation rate of NO from SNAP in solution was, however, very slow and affected by the light, temperature, and divalent cation (Cu^{2+} and Fe^{2+}).[9] Therefore, it was required to assess carefully the relationship among the observed currents, concentrations of a NO-donor, and concentrations of NO in the system. Another NO-donor, NONOate,[10] has been found to be stable in alkaline solution, but spontaneously generate NO at a neutral pH with its intrinsic rate constant in a constant stoichiometric manner (2 molecules of NO from 1 molecule of NONOate). When we aim at understanding the physiological roles of NO in living systems, the use of a NO-selective electrode combined with the kinetic analysis of NO profile from NO donors is essentially important. For this purpose, we developed a new and precise analytical method of NO with a NO-selective electrode, in which the time-dependent current due to NO generated from a NONOate was analyzed on the basis of a mass-balance equation.

1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminoethyl)-3-methyl-1-triazene (NOC7) as a NONOate and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, sodium salt (carboxy-PTIO) as a NO-scavenger[4] were purchased from Dojindo Chemical Co. (Kumamoto, Japan). A standard solution of NOC7 was freshly prepared in 0.1 M NaOH. Carboxy-PTIO was dissolved in a phosphate buffered saline (PBS, pH 7.4). All other reagents were of the highest purity commercially available. NO was monitored using NO-selective electrodes and instrument (NO monitor NO-501, Inter Medical, Nagoya, Japan). The working electrode was kept at approximately +0.5 V from the reference, and both electrodes were placed for 60 min in 10 mL volume of a volumetric flask containing 10 mL of PBS which was maintained at 37 °C and continuously stirred. PBS was equilibrated with the air for overnight before the experiment. After the background currents were achieved to be stable and constant, a standard solution of NOC7 was added to PBS and the currents due to NO were monitored. All measurements were carried out for 5-10 min at least four runs, and data were presented as the means ± SDs.

Time-dependent changes of NO concentration in an aerobic solution (20 kPa of O_2) depended on both the generation rate of NO from NOC7 and the decomposition rate of NO by autoxidation. Although decomposition of NO was reported to depend on a second-order rate for NO concentration ([NO]),[11] we observed that it depends on an apparent first-order rate for [NO]. Therefore, the generation and decomposition processes of NO in this system are described as the following mass-balance equations for NOC7 and NO:

$$\mathrm{d[NOC7]}(t)/\mathrm{d}t = -k_\mathrm{g} \cdot \mathrm{[NOC7]}(t) \quad (1)$$

$$\mathrm{d[NO]}(t)/\mathrm{d}t = 2 \cdot k_\mathrm{g} \cdot \mathrm{[NOC7]}(t) - k_\mathrm{d} \cdot \mathrm{[NO]}(t) \quad (2)$$

where [NOC7](t) and [NO](t) represent the time-dependent concentrations of NOC7 and NO, respectively, k_g is the first-order rate constant for generation of NO from NOC7, and k_d is the apparent first-order rate constant for decomposition of NO. Then, [NO](t) is calculated by Equation (3) derived from Equations (1) and (2) as follows:

$$\mathrm{[NO]}(t) = \frac{2 \cdot k_\mathrm{g} \cdot \mathrm{[NOC7]}_0}{k_\mathrm{d} - k_\mathrm{g}} \cdot \left(\mathrm{e}^{-k_\mathrm{g} \cdot t} - \mathrm{e}^{-k_\mathrm{d} \cdot t}\right) \quad (k_\mathrm{d} > k_\mathrm{g}) \quad (3)$$

where $[\mathrm{NOC7}]_0$ is the initial concentrations of NOC7 and k_g of NOC7 (0.14 min^{-1} at 37 °C in PBS[12]) is considered to be much smaller than k_d of NO. From Equation (3), the maximum concentration of NO ($[\mathrm{NO}]_\mathrm{max}$) is represented at the time (t_max) when d[NO](t)/dt is zero as follows:

$$[\mathrm{NO}]_\mathrm{max} = 2 \cdot [\mathrm{NOC7}]_0 (k_\mathrm{g}/k_\mathrm{d})^{\frac{1}{1-k_\mathrm{g}/k_\mathrm{d}}} \quad (4)$$

Because the observed currents were linearly proportional to [NO] in the solution, the time-dependent profiles of currents (current(t)) were used for the kinetic analysis of [NO] profiles.[13]

Figure 1(a) shows the time-dependent profiles of currents due to NO generated from NOC7 in PBS at 37 °C and the theoretical curves, that were fitted to the mean value on the basis of Equation (5).[13] Each profile shows a rapid rise up to a maximum and then a slow decrease of the currents according to a first-order kinetic. Whether the increase of currents was selective for NO or not was confirmed by addition of carboxy-PTIO in the monitored solution. The simulated curves were excellently fitted to the observed data. To determine NO concentrations in PBS at 37 °C,

Table 1. Maximum currents monitored by NO-501, the estimated parameters, and the determined NO_x in PBS at each concentration of NOC7

$[NOC7]_0$ (μM)	$Current_{max}$ (nA)	k_g (min^{-1})	k_d (min^{-1})	$[NO]_{max}$ (μM)	$\frac{[NO]_{max}}{2[NOC7]_0}$ (%)	$[NO_x]$ (μM)
1	0.21 ± 0.01	0.03 ± 0.01	0.58 ± 0.03	0.09 ± 0.02	4.7 ± 1.1	n.d.
2	0.37 ± 0.01	0.05 ± 0.01	0.50 ± 0.04	0.32 ± 0.04	8.0 ± 1.0	n.d.
5	0.70 ± 0.02	0.06 ± 0.01	0.72 ± 0.02	0.68 ± 0.21	6.8 ± 0.4	n.d.
10	0.78 ± 0.07	0.07 ± 0.02	1.53 ± 0.09	0.77 ± 0.21	3.9 ± 1.1	21 ± 9
50	2.18 ± 0.05	0.12 ± 0.01	3.51 ± 0.67	3.22 ± 0.84	3.2 ± 0.8	108 ± 3
150	4.39 ± 0.35	0.15 ± 0.02	6.07 ± 0.14	6.87 ± 0.84	2.3 ± 0.3	325 ± 5
300	7.85 ± 0.40	0.20 ± 0.02	9.05 ± 0.31	11.93 ± 1.00	2.0 ± 0.2	587 ± 20

the calibration lines by using the monitored maximum currents ($current_{max}$) and the calculated $[NO]_{max}$ were prepared as shown in Figure 1(b). Then the linear relationship between $current_{max}$ and $[NO]_{max}$ was obtained at the concentration range of 0.09–12 μM of $[NO]_{max}$, where the correlation coefficient with linear regression was greater than 0.999 for 7 concentrations of 4 repeated measurements. The detection limit at an S/N ratio of 3 and the quantification limit were 10 and 100 nM of $[NO]_{max}$, respectively.

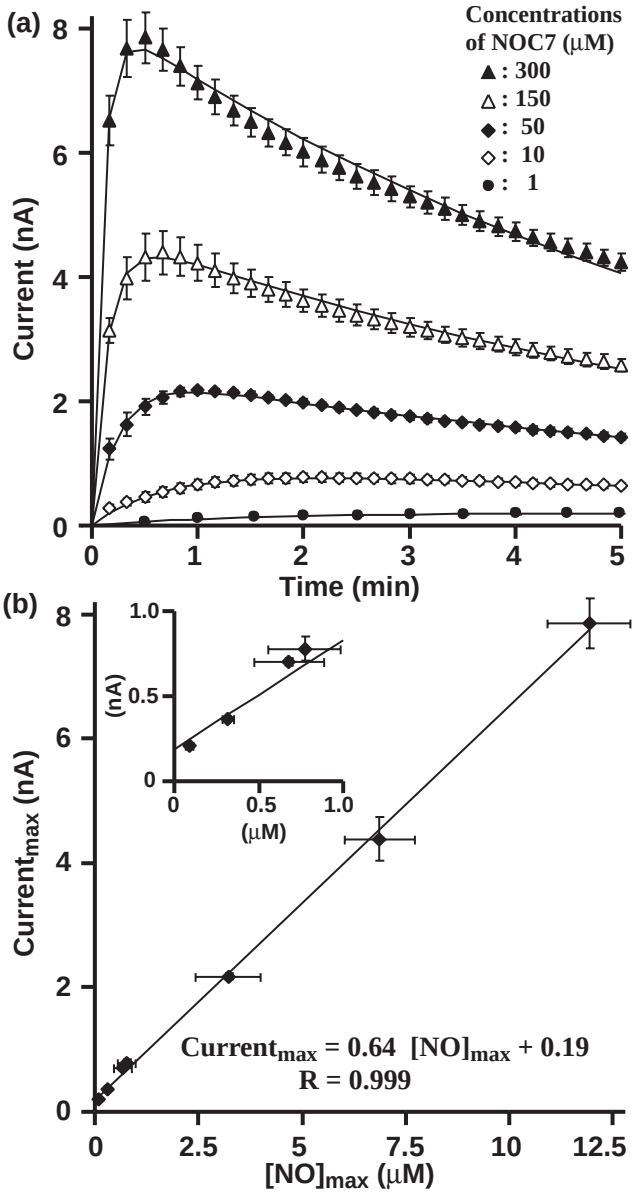

Figure 1. (a) Time-dependent changes of currents due to NO generated from NOC7 as monitored by NO-501, and the theoretical curves fitted to the mean values, and (b) calibration line of NO in PBS (pH 7.4) at 37 °C. Symbols represent the means ± SDs for 4 measurements.

To confirm the stoichiometric relationship between $[NOC7]_0$ and $[NO_x]$, the total concentrations of nitrite and nitrate, $[NO_x]$ in the medium after measurement of currents were determined by the Griess method with a TCI-NOX 1000 automated NO_x analyzer (Tokyo Kasei Co., Tokyo, Japan).[15] Approximately 2-fold of $[NO_x]$ to $[NOC7]_0$ were determined, as summarized in Table 1. The linear relationship between $[NOC7]_0$ and $[NO]_{max}$ ($r = 0.996$), and that between $[NO]_{max}$ and $[NO_x]$ ($r = 0.998$) were obtained. Although $[NO]_{max}$ values were proportional to both $[NOC7]_0$ and $[NO_x]$, those were found to be at most 2–8% of the cumulative concentration of NO generated from NOC7 which nearly equals to $[NO_x]$. The addition of 0–45 μM of bovine serum albumin, 0–2 mM of Ca^{2+} and Mg^{2+}, 0–2 μM of Fe^{3+}, Cu^{2+} and Zn^{2+}, and 0–2 mM of NO_2^- and NO_3^- did not affect the determination of NO at 50 μM of NOC7.

On the basis of these results, we propose here a new determination method of NO for the amperometric currents by a NO-selective electrode on the basis of the kinetic analysis. The method is now applied for the determination of NO in biochemical and biological systems.

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References and Notes

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