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Application of carbon fiber composite minielectrodes for measurement of kinetic constants of nitric oxide decay in solution

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Abstract

Carbon fiber microelectrodes and carbon fiber composite minielectrodes (CFM/CFCM) have been generally used for measurements of nitric oxide (NO) concentration in chemical and biological systems. The response time of a CFM/CFCM is usually from milliseconds to seconds depending on the electrode size, the thickness of coating layers on the electrode, and NO diffusion coefficients of the coating layers. As a result, the time course of recorded current changes (*I-t* curves) by the CFM/CFCM may be different from the actual time course of NO concentration changes (*c-t* curves) if the half-life of NO decay is close to or shorter than the response time of the electrode used. This adds complexity to the process for determining rate constants of NO decay kinetics from the recorded current curves (*I-t* curves). By computer simulations based on a mathematical model, an approximation method was developed for determining rate constants of NO decay from the recorded current curves. This method was first tested and valuated using a commercial CFCM in several simple reaction systems with known rate constants. The response time of the CFCM was measured as 4.7 ± 0.7 seconds ($n=5$). The determined rate constants of NO volatilization and NO autoxidation in our measurement system at 37 °C are $(1.9 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$ ($n=4$) and $(2.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ($n=7$), which are close to the reported rate constants. The method was then applied to determine the rate of NO decay in blood samples from control and smoking exposed mice. It was observed that the NO decay rate in the smoking group is >20% higher than that in control group, and the increased NO decay rate in the smoking group was reversed by 10 μM diphenyleneiodonium chloride (DPI), an inhibitor of flavin enzymes such as leukocyte NADPH oxidase.

Introduction

The physiological role of nitric oxide (NO) is closely related to its bioavailability, whereas NO bioavailability is dependent on the rate of NO generation, metabolism, and diffusion. Like studies of the NO generation process, studies of the NO metabolism process have provided important information for better understanding the physiological function of NO.

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There are multiple pathways for NO metabolism in the body. It is well known that NO can directly react with oxygen (autoxidation) and the reaction kinetics is second order with respect to NO. As a result, the rate of NO autoxidation will be reduced by 100 times as NO concentration decreases 10 times. Since NO concentration in the body is very low (at 1 μ M range or below), NO autoxidation by oxygen is too small to be the main NO metabolism pathway in the body [1–3]. Although NO autoxidation in water is slow at low NO level, experimental data and theoretical analysis show that this reaction may be appreciable in special locations such as cell membrane, due to the greater solubility of NO and O₂ in the cell membrane [4].

The reaction between NO and hemoglobin is very rapid. Since a large amount of hemoglobin is present in red blood cells in the lumen of blood vessels, this rapid reaction would significantly affect NO concentration in the vascular wall. This is because the vascular NO is mainly generated from endothelium, a layer of cell on the inner wall of blood vessels. To understand why the luminal hemoglobin can significantly change NO concentration in the vascular wall, computer simulation was used to illustrate how hemoglobin (Hb) on the one side of endothelium affects NO concentration on the other side of endothelium[5]. It was demonstrated that vascular NO concentration is proportional to the endothelial NO concentration which can be greatly reduced by the luminal Hb. The large amount of Hb in the lumen should almost abolish NO activity in the vascular wall; however, this does not occur in the body. Studies of NO catabolism rate by RBCs reveal that the rate of NO consumption by RBC-enclosed hemoglobin is greatly limited by the diffusion process through the cell-free zone, the unstirred plasma layer surrounding each RBC, and maybe the cell membrane and intracellular hemoglobin plasma[6–10]. These results provide explanations about why activity of the endothelium-derived NO is reserved even though there is a large amount of hemoglobin in the blood.

It has been well documented that NO can regulate oxygen consumption by inhibition of mitochondrial respiration. Now accumulating evidence shows that NO can also be consumed by isolated cells and inside tissues in an oxygen-dependent manner [11,12]. This inter-regulation between NO and oxygen may play an important physiological role in the body to adapt hypoxic conditions [12–15]. In these research areas, the study of NO metabolism kinetics is very critical and required for a comprehensive understanding of NO physiological functions.

Various techniques have been used in studying NO metabolism process. Among these methods, electrochemical NO sensors or electrodes have some special advantages because of their ability to directly measure NO concentration in solution. Two types of commercial NO electrodes have been generally used in laboratories. One is the Clark-type electrode, the other is the CFM/CFCM. The response time of these electrodes to a change of NO concentration is from milliseconds[16,17] to seconds[18]. In a previous paper, we have described how to use a Clark-type NO electrode to measure a fast reaction in which NO half-life is less than the response time of the NO electrode by solving diffusion-reaction equations [19]. In this study, we focus on the application of CFM/CFCM in measuring relatively slow NO reactions, and an approximate but accurate method is developed to determine kinetics and rate constants of NO decay without solving diffusion-reaction equations. Measurement errors are estimated based on theoretical analysis and computer simulations.

Experimental Materials and Methods

Preparation of NO Solutions

NO stock solution was prepared as described in previous papers[12,15]. Briefly, NO was scrubbed of higher nitrogen oxides by passage first through a U-tube containing NaOH pellets and then through a 1 M deaerated (bubbled with 100% argon) KOH solution in a custom-designed apparatus using only glass or stainless steel tubing and fittings. To prepare the saturated NO solution, the purified NO gas was bubbled for 15 min into the de-aerated buffer (0.2 M potassium phosphate, pH 7.4), which was contained in a glass sampling flask with a septum purchased from Kimble/Kontes (Vineland, NJ). The saturated NO concentration was nearly 2 mM.

Electrochemical Measurements of NO Concentration

The electrochemical system for measuring NO consisted of a CFCM (ISO-NOPF200 from World Precision Instruments (WPI), Sarasota, FL), a 4-port water-jacketed electrochemical chamber (NOCHM-4 from WPI, FL), a magnetic stirrer (WPI, FL), a Haake DC10-P5/U circulating bath, and an Apollo 4000 free radical analyzer (WPI, FL). The electrochemical chamber contained 2 mL of phosphate-buffered saline. The solution was stirred at a constant speed with the magnetic bar. The temperature of the chamber was held at 37 °C by the circulating bath. The CFCM was inserted (through the electrode port on the cap of the chamber) into the chamber. NO was added into the solution (through a hole on the cap of the chamber) with a Hamilton syringe (Hamilton Company, NV) by a bolus injection in the presence and absence of diluted blood samples to measure NO decay rate.

Animal model and preparation of Blood Cells

Male C57BL/6J mice, 11–12 weeks of age (Charles River Laboratories International, Inc. MA, USA), were housed at a 23 ± 2 °C, at 55% relative humidity, with 12h day-night cycle, and maintained on standard rodent chow and tap water *ad libitum*. After a week of acclimatization, mice were exposed to whole body mainstream and side stream cigarette smoke using the TE-10 cigarette smoking machine (Teague Enterprises, CA) and 3R4F reference research cigarettes (University of Kentucky) that deliver 9.4 mg tar/0.726 mg nicotine per cigarette under the standard Cambridge filter smoking condition. The smoking machine was programmed to give 3 sets of exposure. In each set, the machine puffs smoke over a period of ~24 min, followed by a break of fresh air for ~20 min. The total exposure time was ~72 min per day 5 days per week for 32 weeks. Age-matched, air exposed mice served as controls. Blood was drawn from control mice and smoking mice after 24 hours from the last exposure into a heparinized tube. Blood samples were centrifuged at $2300 \times g$ for 10 min. The supernatant containing the plasma was discarded, and the RBC/WBC pellet was washed three times with phosphate buffered saline (15 mM phosphate (potassium) plus 0.9% NaCl, pH 7.4). The packed RBCs/WBCs were then resuspended in the same buffer and stored on ice for use. All animal procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee at The Ohio State University, Columbus, Ohio, and conformed to the Guide for the Care and Use of Laboratory Animals.

Mathematical Model describing NO diffusion processes around a carbon fiber composite NO electrode

The sensing part of the CFCM is the micro-cylinder with a diameter of 200 μm and a length of 5 mm. The surface of the CFCM is coated with multi-layered NO-selective membrane. Since the solution is stirred, the NO concentration almost uniformly distributes in the whole solution except the space near the surface of cylinder CFCM, where a NO concentration

gradient exists. The NO gradient layer consists of the selective membrane and a thin solution layer near the electrodes. We use the concept of effective diffusion layer to simplify the diffusion process. This concept assumes that the diffusion through multiple layers is approximately considered diffusion problem through a single layer, and an apparent NO diffusion coefficient can be used to describe NO diffusion process in this single layer. To solve the diffusion problem, we assume: (1) The cylindrical electrode with a radius of r_0 (100 μm) is surrounded by a diffusion layer with a thickness of L . A bulk of buffer solution exists out of the diffusion layer. NO does not exist in the diffusion layer at $t=0$. (2) NO concentration outside of the effective diffusion layer ($r \geq r_0 + L = r_1$) is uniform, which is $[NO]_0$ at $t=0$ and a function of time ($[NO]_b$) when $t > 0$. NO starts diffusing through the diffusion layer toward the CFCM when $t > 0$. (3) Since NO is completely oxidized at the electrode surface, NO concentration at the electrode surface ($r=r_0=100 \mu\text{m}$) is zero. The time-dependent diffusion equation for the cylindrical electrode can be written as:

$$\frac{\partial[NO]}{\partial t} = D \frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial[NO]}{\partial r} \right) \quad (1)$$

Let $u=[NO]/[NO]_0$, $R=r/L$ and $R_0=r_0/L$, then Eqn. (1) can be converted as:

$$\frac{\partial u}{\partial t} = \frac{D}{L^2} \frac{1}{R} \frac{\partial}{\partial R} \left(R \frac{\partial u}{\partial R} \right) \quad (2)$$

According to assumptions (1)–(3), boundary conditions can be written as:

$$u=0 \quad \text{at } t=0 \quad (3)$$

$$u=0 \quad \text{at } t>0 \text{ and } R=R_0 \quad (4)$$

$$u=[NO]_b/[NO]_0 \quad \text{at } t>0 \text{ and } R=(r_0+L)/L=R_0+1 \quad (5)$$

The current $i(t)$ at the CFCM is proportional to NO concentration gradient at the surface of the CFCM, so we have:

$$i(t) = g \frac{\partial c(t)}{\partial r} \Big|_{r=r_0} = \frac{g[NO]_0}{L} \frac{\partial u(t)}{\partial R} \Big|_{R=R_0} \quad (6)$$

where g is a constant. If $[NO]_b$ is a constant, or $[NO]_b=[NO]_0$, the diffusion limited current (the maximal steady-state current) i_d can be obtained when t is large enough ($t \geq t_m$):

$$i_d = \frac{g[NO]_0}{L} \frac{\partial u(t_m)}{\partial R} \Big|_{R=R_0} \quad (7)$$

Here t_m is defined as the time to reach 99% of the maximal current. If NO concentration decreases with time after the NO is injected into solution, the current recorded by electrode will first increase immediately after NO is injected and then decrease with time. In this case, t_m is defined as the time at the current peak. The normalized current i_n is defined as:

$$i_n(t) = \frac{i}{i_d} = \frac{\left. \frac{\partial u(t)}{\partial R} \right|_{R=R_0}}{\left. \frac{\partial u(t_m)}{\partial R} \right|_{R=R_0}} = A \left. \frac{\partial u(t)}{\partial R} \right|_{R=R_0} \quad (8)$$

$$\text{where } A = \frac{1}{\left. \frac{\partial u(t_m)}{\partial R} \right|_{R=R_0}}.$$

If $[NO]_b$ is known or given, then the parameters D and L or the apparent parameters L^2/D and R_0 are the only two undetermined parameters in Eqns. (2)–(5) or in the function $i_n(t)$. In this case, L and D (or L^2/D and R_0) can be determined by finding the following Least-Square method:

$$f(L, D) = \sum_i [i_n - I_n]^2 \geq 0 \quad (9)$$

Where I_n is the normalized current that is measured experimentally and i_n is the normalized current that is calculated using Eqns. (2)–(8).

Data Analysis

Matlab R2009a was used to solve Eqns. (1)–(5) for finding the time-dependent $[NO]$ (NO decay curves) with different rate constants, to find current i , i_d and i_n from Eqns (6)–(8), and to determine values of L^2/D from experimental curves using Eqn. (9). Linear regression was performed using SigmaPlot 11.0 to find rate constants from experimental and computer simulated NO decay curves. The final rate constants were given as mean $\pm$ SE.

Results

Measurements of parameters L and D for the CFCM

To simplify data analysis in measurements of this parameter, $[NO]_b$ was kept constant in the related experiments as described in the previous study on Clark type electrode[19]. In the experiments, oxygen in solution was first removed by bubbling N_2 into the solution for at least 15 minutes, and then the cap of the chamber was closed and pushed down to the solution surface. Since there was no oxygen in the solution and no gas phase above the solution, the autoxidation of NO was eliminated and NO in the solution could not diffuse into the gas phase. Thus, NO in the solution is relatively stable, so we can consider NO concentration is a constant in the period of measurements after NO is added into the solution. It was observed in our experiments that the response time of CFCMs with a diameter of 200 μm is 4.7 ± 0.7 seconds ($n=5$) at $T_{95\%}$. A typical normalized current measured in an experiment (solid line) is demonstrated in Fig. 1. Eqn. (9) is used to determine L and D (or L^2/D and R_0). Computed results show that the apparent parameter L^2/D is the main parameter that affect the function $f(L, D)$, whereas R_0 has little effect on the function. The dotted line in Fig. 1 is the normalized current i_n that was simulated from Eqns. (2)–(8) based on the determined L^2/D . The value of L^2/D from 5 measurements is 11.5 ± 1.7 s ($n=5$), while R_0 is varied between 0.5 and 10.

Measurement errors caused by the response time of a CFCM

Although Eqns. (2)–(8) can be used to determine the rate constant of an NO reaction measured by a CFCM, numerically solving these equations is not convenient for most researchers. It is necessary to find a simple method with a high accuracy to determine the

rate constant directly from experimental curves without solving the diffusion-reaction equations. Using the experimentally determined L^2/D and assuming $R_0=1$ that is in the range of 0.5 to 10, we simulated currents by assuming that NO decay follows first order kinetics at 4 different rates (Fig. 2). In this case, we have:

$$[NO]_b = [NO]_0 \exp(-k_1 t) \quad (10)$$

The dotted line in Fig. 2 is calculated from Eqn. (10) which is the theoretical decay curve of normalized NO concentration in the solution, and the solid line is the normalized current at the CFCM calculated from Eqns. (2)–(8). These simulated results show that the normalized current is very close to the normalized NO concentration when NO decay is slow or k is small, but the normalized current deviates from the normalized NO concentration when NO decay is fast or k is large (Fig. 2). If we read data points from a normalized NO concentration ($[NO]_n$ or $[NO]_b/[NO]_0$) and plot $\ln([NO]_n)$ vs t , we will obtain a straight line with a slope of $-k_1$. In contrast, if we read data from the corresponding normalized current and plot $\ln(I_n)$ vs t , the plotted curve will not be a straight line and the average slope may deviate from $-k_1$. However, if we cut off the segment of the curve on the left side of the current peak and shift the remained part of the current curve to the left to compare with the normalized NO concentration curve, the two curves are much closer to each other (Fig. 3). Reading data from the remaining part of the normalized current curves (Fig. 3) and plotting $\ln(I_n)$ vs t shows that the average slope from the plot of $\ln(I_n)$ vs t is very close to the one from the plot of $\ln([NO]_n)$ vs t until k is greater than 0.2/s (Fig. 4). Thus, we may accurately measure the NO decay rate from the normalized current curve if k or the NO decay rate is not too large. To define what is “not too large” here, computer simulations were performed at different decay rate constants to examine the error of rate constants determined from the normalized currents comparing to those from the corresponding normalized NO concentrations. It was observed that the error is less than 5% if $t_{hp} \geq 2.5t_p$ on the current curve (refer Fig. 2C), where t_p is time t at the current peak and t_{hp} is time t at the half-peak. Considering different noise sources in actual measurements, we set $t_{hp} \geq 3t_p$ as the minimal requirement for using this approximate method to analyze experimental data.

Application of the CFCM to measure the rate of NO autoxidation and volatilization in the solution

To test our approximation method presented above, we measured the rate of NO autoxidation and volatilization in the aerated buffer solution. It is well known that the rate of NO autoxidation is second order with respect to $[NO]$ and first order with respect to $[O_2]$ [1–3,19,20]. In addition to the autoxidation-caused NO decay, the physical process through diffusion out of solution can also cause decay or drop in NO concentration. The rate of this decay through a physical process (diffusion) is first order with respect to $[NO]$ [2,19]. Since the rate of NO autoxidation is second order on $[NO]$, its half-life is inversely proportional to NO initial concentration. When NO concentration is at levels of low μM or below, the rate of NO diffusion out of solution may have a significant contribution to the total NO decay rate. In Figure 5A, we demonstrated three typical current curves measured by the CFCM after NO (2 μM) was injected into the chamber containing buffer solution. The curve a was obtained in the deaerated solution (oxygen in the solution was removed by N_2) with a head space above the solution surface [19]. The curve b was obtained in the aerated solution (equilibrated with air) when the cap of the chamber was pushed down to the solution until the solution filled out the three holes in the cap (without head space). The curve c was obtained in the aerated solution when the solution open to air (with head space). From the three curves we can see that the ratios of t_{hp} to t_p (t_{hp}/t_p) for each curve are much greater than 3, so we can directly use data on the right side of the peak in each curve to determine the related rate constants. The plot of $\ln(I_n)$ vs time t (using data from curve a) is a straight

line (Figure 5B). From the slope we can determine the first order rate constant k_1 . The mean and standard error of k_1 obtained from 4 curves is $(1.9 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$ ($n=4$). The plot of $1/I_n$ vs time t using data from curve b is a straight line. The second order rate constant k_2 was determined as $(2.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ($n=7$), which is close to the reported rate constant of NO autoxidation at 37 °C [19]. Using data from curve c, we can not get a straight line by plotting either $\ln(I_n)$ or $1/I_n$ vs time t , indicating that NO decay in the aerated solution open to air does not simply follow a simple first order or second order kinetics when NO concentration is at levels of μM or below. If we assume that the NO decay in the aerated solution open to air follows mixed first and second order kinetics defined below:

$$\frac{d[\text{NO}]}{dt} = -k_1[\text{NO}] - k_2[\text{NO}]^2 \quad (11)$$

and assume that $I_n \approx [\text{NO}]_n = [\text{NO}]/[\text{NO}]_0$, then it is not difficult to prove the following equation:

$$\frac{I_n}{1 + \frac{k_2[\text{NO}]_0}{k_1} I_n} = \frac{1}{1 + \frac{k_2[\text{NO}]_0}{k_1}} \exp(-k_1 t) \quad (12a)$$

or

$$\ln(I_n(1 + \frac{k_2[\text{NO}]_0}{k_1}) / (1 + \frac{k_2[\text{NO}]_0}{k_1} I_n)) = -k_1 t \quad (12b)$$

Where $[\text{NO}]_0$ is the initial NO concentration, and k_1 and k_2 are the first order and the second order rate constants, respectively. Assuming $k_2 = 2.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $k_1 = 1.9 \times 10^{-3} \text{ s}^{-1}$, we

can obtain a new k_1 from the slope of the plot of $\ln(I_n(1 + \frac{k_2[\text{NO}]_0}{k_1}) / (1 + \frac{k_2[\text{NO}]_0}{k_1} I_n))$ vs time

t . Using the new k_1 to replace the old k_1 in $\ln(I_n(1 + \frac{k_2[\text{NO}]_0}{k_1}) / (1 + \frac{k_2[\text{NO}]_0}{k_1} I_n))$, and re-plot

$\ln(I_n(1 + \frac{k_2[\text{NO}]_0}{k_1}) / (1 + \frac{k_2[\text{NO}]_0}{k_1} I_n))$ vs time t to determine a new k_1 from the slope of the plot again. By repeating this process, the old k_1 will finally approach the new generated k_1 .

The final plot of $\ln(I_n(1 + \frac{k_2[\text{NO}]_0}{k_1}) / (1 + \frac{k_2[\text{NO}]_0}{k_1} I_n))$ vs time t was demonstrated in Fig. 5D, and the final k_1 is determined as $(1.9 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$ ($n=6$).

Application of the CFCM to measure rate constants of NO decay by blood cells

To apply the above approximation method to analyze experimental data, we measured NO decay in blood samples that were from mice with or without prior smoking treatment for 32 weeks. In the experiments, each blood sample was diluted 3000 times in a chamber containing 2 mL buffer (0.9 % NaCl + 15 mM potassium phosphate, pH 7.4). NO (1 μM) was added into the chamber a few times to oxidize oxyhemoglobin (oxyHb) in RBCs (Fig. 6A). After the completed oxidation of oxyHb, which was easily observed from a significant change in NO decay rate, 1 μM NO was added into the chamber two more times. It was observed that NO has a faster decay rate in the presence of diluted blood samples from mice with smoking treatment comparing to those without smoking treatment (Fig. 6B). By measuring t_p and t_{hp} we know that the ratio of t_{hp}/t_p for the two peaks is much greater than 3, so we can directly use experimental data on the right side of the current peak to determine

rate constants. Data analysis on these experimental curves shows that NO decay rates follow mixed first and second order kinetics which can be described by Eqn. (11). The second order reaction is contributed by NO autoxidation, so $k_2=2\times 10^3 \text{ M}^{-1}\text{s}^{-1}$. The plot of

$\ln(I_n(1+\frac{k_2[NO]_0}{k_1}))/(\frac{k_2[NO]_0}{k_1}I_n))$ vs t is a straight line (Fig. 6C). The rate constants k_1 for the control group and smoking group are $(9.9\pm 0.7)\times 10^{-3} \text{ s}^{-1}$ ($n=8$) and $1.21\pm 0.09\times 10^{-2} \text{ s}^{-1}$ ($n=8$), respectively. This increased decay rate in the smoking group was reversed by $10 \mu\text{M}$ diphenyliodonium chloride (DPI, Sigma) with $k_1=(10.3\pm 0.4)\times 10^{-3} \text{ s}^{-1}$ ($n=5$), but $10 \mu\text{M}$ DPI had no appreciable effect on NO decay rate in blood samples from the control group (data not shown).

Discussion

NO electrodes have been generally used to measure NO concentration in solution. However, what an NO electrode directly records is not the NO concentration in solution but the current

(i) that is proportional to NO concentration gradient at the surface of the electrode ($\frac{\partial c}{\partial r}|_{r=r_0}$). In theory, the ratio of i to $[NO]_b$ is a constant if $[NO]_b$ is a constant and t is large enough so that i has reached a steady-state. Under this situation, NO concentration can be easily determined from dividing the electrode current by the constant ratio. In contrast, if $[NO]_b$ varies with time, the ratio of i to $[NO]_b$ will not be a constant, but a time-dependent function. In this case, there may not be a simple relationship between the current and the NO concentration in the solution. Although we can find this relationship by solving diffusion equations (2)–(5) as demonstrated in Fig. 1, it is not convenient for most researchers to apply this complicated computation in determining NO decay rate constants. In this study, we presented an approximation method for determining NO decay kinetic constants directly from current curves obtained by the CFCM.

For a CFCM with a response time of several seconds, computer simulations show that the relative difference between I_n and $[NO]_n$ is tiny when the rate constant k_1 is $2\times 10^{-3} \text{ s}^{-1}$ (Fig. 2A). As k_1 increases to $2\times 10^{-2} \text{ s}^{-1}$, this difference is clearly bigger, but I_n is still similar to $[NO]_n$ (Fig. 2B). At $k_1=0.2 \text{ s}^{-1}$, I_n is significantly different from the normalized concentration (Fig. 2C), and the difference is further enlarged as k_1 increases to 2 s^{-1} (Fig. 2D). However, if we shift the current peaks to the left as shown in Fig. 3, we can see that I_n is very close to $[NO]_n$ in Figs. 3A and 3B, slightly different in Fig. 3C, and the big difference appears only in Fig. 3D. These results imply that if we consider data on the right side of the current peak in an I_n curve to be an approximate $[NO]_n$ curve, we may be still able to determine kinetic constants of NO decay even if the whole I_n curve is significantly different from the $[NO]_n$ curve (Fig. 2). The feasibility of this approximate analysis is confirmed in Fig. 4, in which we see that the values of k_1 obtained by plots of $\ln(I_n)$ vs time t are very closed to the given NO decay rate constants (Figs. 4A–4C). However, if NO decay rate is too large, the value of k_1 obtained from this approximate analysis still has a large difference or a big “measurement error” (Fig. 4D), indicating that it is necessary to find a simple way to judge under what conditions the approximate analysis is accurate enough.

Using computer simulations, we observed that the “measurement error” is less than 5% when $t_{hp}>3t_p$ or $t_p<(t_{hp}-t_p)/2$ for the CFCMs. The characteristic time t_p is the time when the current reaches its peak, which is related to response time of the electrode, while the difference of $(t_{hp}-t_p)$ is the time needed for the current drops from its peak to its half peak, which is related to NO decay rate. A series of specific experiments with known NO decay kinetics and/or rate constants, were used to further testify this approximate method. Results show that this approximation method accurately determined the kinetic characteristic for the NO volatilization from solution to gas phase, the second order kinetic rate constants for NO

autoxidation in solution, and the rate constants for the mixed first order and second order kinetics (Fig. 5). It is interesting that the decay rate of NO autoxidation in the chamber without a cap (open to air) does not simply follow the second-order kinetics as usual, but a mixed first order and second order kinetics. This is because NO autoxidation is a second-order reaction, which will be much more stable in solution as NO concentration decreases. When NO concentration is 1 or 2 μM , its half-life in aerated solution ($[\text{O}_2]=200\text{ }\mu\text{M}$) is greater than 10 or 5 minutes, respectively. In this case, the rate of NO volatilization from the solution into the gas phase in our measurement system is comparable to the rate of NO autoxidation so that the physical volatilization process makes a significant contribution to the observed NO decay. Theoretically speaking, NO in the nM range should be very stable in aerated solution; however, it may not be as stable as thought because of the physical volatilization loss.

Finally we applied this approximate method to determine NO decay in the presence of blood cells from mice with/without smoking treatment. It was previously reported that alveolar macrophages from cigarette smokers release increased amounts of superoxide production [21], and thiol-reactive stable compounds in cigarette smoke significantly raised endothelial superoxide production by activating NADPH oxidase that could be inhibited by 10 μM DPI [22]. Our results show that after hemoglobin was pre-oxidized by extra amount of NO, the rate of NO decay in the presence of blood cells from mice in smoking group is appreciably increased compared to mice in the control group. The increased NO decay rate can be inhibited by 10 μM DPI, suggesting that superoxide production from leakage of NADPH oxidase and/or other flavin enzymes is increased in smoking mice. The increased superoxide production reduces NO bioavailability and contributes to endothelial dysfunction, resulting in cardiovascular diseases [23–27].

In summary, we present an approximation method for measuring NO decay constants by a carbon fiber composite NO microelectrode with a response time of several seconds. Our results show that it is possible to determine NO decay rate constants directly from the normalized current data recorded by the NO electrode within an acceptable measurement error when the condition $t_{\text{hp}} > 3t_{\text{p}}$ is met.

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Figure 1.

Response current of the CFCM with a radius of 100 μm to a bolus injection of 2 μM NO in the chamber containing 2 mL deaerated solution at 37 $^{\circ}\text{C}$ (solid line: recorded by the electrode, dotted line: the best fitting curve using Eqns. (2)–(9). The apparent parameter L^2/D determined from the best-fitting curves is 11.5 ± 1.7 s ($n=5$).

Figure 2.

Computer-simulated normalized NO concentrations (dotted lines) that decay in the solution with first-order kinetics at four different rates and the corresponding normalized currents (solid line) at the CFCMs. A: $k_1 = 2 \times 10^{-3} \text{ s}^{-1}$, B: $k_1 = 2 \times 10^{-2} \text{ s}^{-1}$, C: $k_1 = 0.2 \text{ s}^{-1}$, and D: $k_1 = 2 \text{ s}^{-1}$. A significant difference between the normalized NO concentration (dotted line) and the normalized current exists when $k_1 \geq 2 \times 10^{-2} \text{ s}^{-1}$.

Figure 3.

Shift of solid lines (normalized currents) in Fig. 2 to the left to overlap their peaks with peaks of the normalized NO concentrations (dotted lines). A: $k_1 = 2 \times 10^{-3} \text{ s}^{-1}$, B: $k_1 = 2 \times 10^{-2} \text{ s}^{-1}$, C: $k_1 = 0.2 \text{ s}^{-1}$, and D: $k_1 = 2 \text{ s}^{-1}$. Solid lines match the dotted lines pretty well as k_1 is up to 0.02 s^{-1} (A & B). When k_1 increases to $0.2/s$, the main part of the solid line is still parallel to the dotted line except the curve part near the peak (C). A big difference is seen between the solid line and the dotted line at $k_1 = 2/s$ (D).

Figure 4.

Plots of $\ln(I_n)$ (solid lines) and $\ln([NO]_n)$ (dotted lines) vs time t . The values of k_1 determined from the slope of each plot of $\ln([NO]_n)$ vs t are: A: $2 \times 10^{-3} \text{ s}^{-1}$, B: $2 \times 10^{-2} \text{ s}^{-1}$, C: 0.2 s^{-1} , and D: 2 s^{-1} . Correspondingly, The values of k_1 determined from the slope of each plot of $\ln(I_n)$ vs t are: A: $2 \times 10^{-3} \text{ s}^{-1}$, B: $2 \times 10^{-2} \text{ s}^{-1}$, C: 0.196 s^{-1} , and D: 0.815 s^{-1} , respectively.

Figure 5.

Application of the CFCM in determining kinetic orders or rate constants of NO decay in solution. A: Currents were recorded by the CFCM after 2 μM NO was injected into the deaerated solution with a head space above the solution surface (curve a), in the aerated solution (equilibrated with air) without headspace above the solution surface (curve b), and in the aerated solution when the solution opens to air (curve c). (B) Plot of $\ln(I_n)$ vs t using data from the right side of the peak of curve a, and the determined k_1 is $(1.9 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$ ($n=4$). (C) Plot of $(1/I_n)$ vs t using data from the right side of the peak of curve b, and the determined k_2 is $(2.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ($n=7$). (D) Plot of $\ln(I_n(1 + \frac{k_1}{k_2[NO]_0})/(I_n + \frac{k_1}{k_2[NO]_0}))$ vs t using data from the right side of the peak of curve c, the determined k_1 is $(1.9 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$ ($n=6$) assuming $k_2 = (2.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 6.

Application of the CFCM in determining NO decay rate constants in diluted blood samples from mice in smoking and control groups. Each blood sample was diluted 3000 times. A: NO (1 μM) was added into the chamber a few times to oxidize oxyhemoglobin (oxyHb) in RBCs of a control blood sample until oxyhemoglobin is completely oxidized into methemoglobin where NO decay rate significantly changed as designated by the upward arrow (solid line). The dashed line is NO concentration decay curve after 1 μM NO was added into the buffer solution in the absence of RBCs/WBCs. The downward arrow is used to indicate the point on the dashed line where the NO concentration is equal to the NO concentration of the solid line designated by the upward arrow). B: Normalized currents recorded from the CFCM in the presence of diluted blood sample from smoking group (---), smoking blood sample + 10 μM DPI (...), and control group (—). C: Plots of

$\ln(I_n(1 + \frac{k_1}{k_2[NO]_0}) / (I_n + \frac{k_1}{k_2[NO]_0}))$ vs t are straight lines, indicating that NO decay rates follow mixed first and second order kinetics. The values of k_1 determined from the slope of the plots are $(9.9 \pm 0.7) \times 10^{-3} \text{ s}^{-1}$ (control, —), $(1.21 \pm 0.09) \times 10^{-2} \text{ s}^{-1}$ (smoking, ---), and $(10.3 \pm 0.4) \times 10^{-3} \text{ s}^{-1}$ (smoking + DPI, ...), respectively.