

Accounts

Strategies and Development of Molecular Probes for Nitrogen Monoxide Monitoring

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Current methods for monitoring nitrogen monoxide (NO) and new strategies for designing NO probes are reviewed. The production of endogenous NO must be monitored if we are to understand the physiological and pathological roles of NO. Many methods have been reported for this purpose, including colorimetry, fluorometry, electrochemical methods, and electron paramagnetic resonance (EPR). Among these methods, one of the most promising for practical use is EPR using (dithiocarbamato)iron(II) complex as a spin trap. In the structure–sensitivity relationship, it was found that an electron-donating group on the dithiocarbamato ligand is favorable for enhancing the sensitivity of NO detection due to the stabilization of the Fe(II) complex form. A new concept, "radical-exchange", is also introduced to solve the issue of low sensitivity in EPR methods. This concept has been used to develop two types of NO probe: spin trap and fluorescent probes. These reagents detect NO to a detection limit of 10 nM–sub-μM. This strategy, using radical exchange in conjunction with EPR, is potentially useful for the design of sensitive probes that can detect NO directly.

Since NO was recognized as an endothelium-derived relaxing factor,[1] the molecule has received much attention due to its unique activity in biological systems. Although NO is a very simple inorganic radical, it plays various important roles in organisms, such as that of vasodilator to control vascular tone,[2] that of neuromodulator in the central and peripheral nervous systems,[3] and that of effector molecule in the immune system.[4] NO's versatility stems largely from its chemical properties. NO is an inorganic radical, so it interacts directly with many biological components through redox reactions to change its chemical forms to ones having different bioactivities. Firstly, NO reacts with molecular oxygen to form N_2O_3 or N_2O_4 via the nitrogen dioxide radical. These products finally form nitrite and nitrate anions.[5] N_2O_3 may act as a nitrosation agent. The reaction of NO with superoxide anion forms peroxonitrite,[6] which is a susceptible molecule causing various pathological states, such as atherosclerosis,[7] neuronal death[8] or ischemia/reperfusion tissue damage.[9] In contrast, *S*-nitrosothiol, which is formed by the reaction of thiols and nitrosation agents derived from NO, protects cells and tissues against oxidative stress in many cases.[10] This reaction cascade makes NO's bioactivity difficult to understand. Therefore, to develop an NO monitoring method, it is necessary to directly monitor NO in order to distinguish it from various other NO-related derivatives. Another important characteristic of NO is its high diffusibility. NO can be found quite far from its origin. Thus, it must be monitored in real-time near the sites where it is produced and active. Spatial imaging of NO distribution is also preferable for measurements that use biological samples. Many methods have been reported for NO assays, including colorimetric,[11–13] fluorometric,[14–17] electrochemical,[18–20] chemiluminescent[21] and spin-trapping methods.[22–26] However, these methods are not yet fully capable of such monitoring. In this paper, we reviewed various current techniques for NO monitoring and explain our attempt to make a sensitive NO probe using a new concept.[17,27,28]

Overview of Current Methods for NO Monitoring

The current NO assay methods can be classified into the following three categories: (i) indirect assay using NO derivative, (ii) direct assay through the redox reaction of NO, (iii) direct assay using metal ions. Category (i) involves the Griess assay[11] or a fluorometric assay using either diaminonaphthalene (DAN),[14] diaminofluorescein (DAF),[15] or diaminorhodamine (DAR).[16] These methods monitor NO derivatives that are produced from NO in biological environments. These derivatives include nitrite, N_2O_3, or other nitrogen oxides. Since NO is a redox-active species, it should be detectable electrochemically. Therefore, various electrochemical methods have been reported in category (ii). The advantages of an electrochemical method include high sensitivity and real-time monitoring, although it is difficult to get spatial imaging. NO also tends to bind to some transition metal ions rapidly and tightly. Category (iii) uses this property of NO. Hemoglobin[22] or many

(dithiocarbamato)iron(II) complexes[23,24] are used for such methods, coupled with either colorimetry or the EPR technique.

NO Monitoring in Colorimetry and Fluorometry

In aerobic conditions, NO reacts with oxygen to produce nitrite and nitrate anions as final products through nitrogen dioxide radical and N_2O_3. Consequently, NO production should be monitored indirectly by the determination of nitrite anion. The Griess reagent is the most classical reagent for the determination of nitrite.[11] In this method, first, sulfanilamide reacts with nitrite under acidic pH to make diazonium salt. Then, the diazonium compound is converted into an azo dye by its reaction with 1-naphthylethylenediamine under alkaline condition (Fig. 1 a)). The concentration of nitrite can be estimated by measuring absorbance at 540 nm. DAN also reacts with nitrite under acidic conditions to give fluorescent triazole (λ_{ex} = 375 nm, λ_{ex} = 415 nm), which is detectable fluorometrically (Fig. 1 b)).[14,29] However, some parts of NO change to nitrate through the reaction with peroxonitrite or oxyhemoglobin. In this case, nitrate is converted to nitrite using nitrate reductase or manganese oxide. These methods are convenient for NO assays using many samples, but nitrite is formed from other chemical sources, too. These methods are also unsuitable for the real-time monitoring or spatial profiling of NO production. At neutral pH, DAN reacts with NO in the presence of oxygen.[30] In this case, the real reactant is a NO^+ equivalent such as N_2O_3 or some other nitrogen oxide. Using this reaction, real-time monitoring of NO was reported in microorganisms and rat neutrophile. DAF is a more sophisticated reagent in the same category (Fig. 1 c)).[15] DAF is practically nonfluorescent due to the charge transfer from the amino group to the xanthene fluorophore. In the presence of NO and oxygen, the fluorescence intensity at 515 nm based on the fluoresceine unit is enhanced by more than 100 times. The reagent can be introduced into living cells after acetylation. There are a lot of reports about in vivo monitoring of NO using DAF.[31,32] Recently, a reagent called DAR, which is similar to DAF but has a longer (575 nm) fluorescence wavelength, was reported.[16] Neither DAF nor DAR can monitor NO directly, because the real reaction species is some undetermined NO^+ equivalent. Thus, when DAF or DAR is used, the presence of a large amount of thiol or a different oxygen concentration may affect the estimation of the NO concentration. The slow reaction rate between DAF and NO ($k = 6.3 \times 10^6\ M^{-2}\ s^{-1}$) also leads to the underestimation of the NO concentration.[15] Nevertheless, DAF and DAR are still among the most promising reagents for in situ NO monitoring in living cells and tissues.

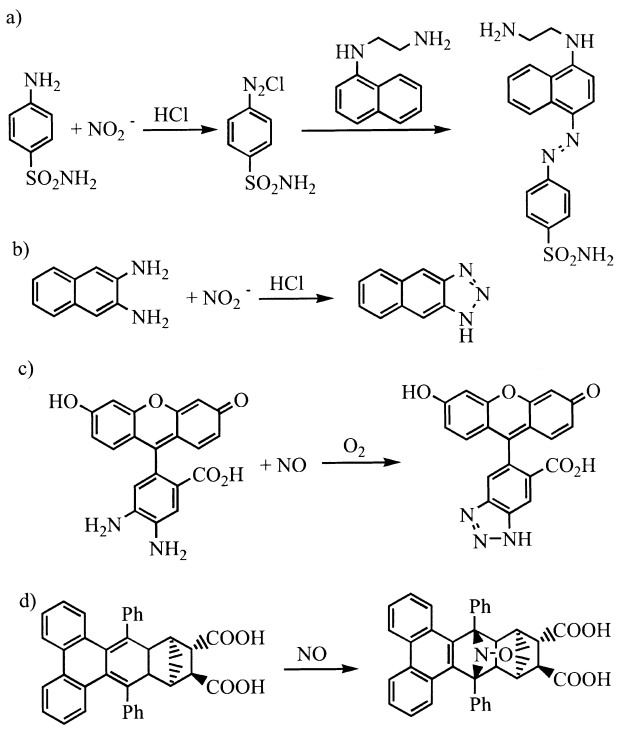


Fig. 1. Methods for NO-monitoring using colorimetry or fluorometry. a) Griess method, b) DAN (diaminonaphthalene) assay, c) DAF (diaminofluorescein) assay, d) FNOCT assay.

Another interesting fluorescent probe for NO is *o*-quinodimethane compounds (FNOCT) (Fig. 1 d)).[33] FNOCT reacts with NO directly to produce radical products that include nitroxide radical and phenanthrene moieties. Then the nitroxide radical, which tends to quench the fluorescence of phenanthrene, is extinguished by reducing agents in the biological sample. FNOCT can detect NO directly at the nM level from a NO releasing agent[34] or a living cell.[35] Unfortunately, the reaction rate between FNOCT and NO is small ($k = 60 \pm 8\ M^{-1}\ sec^{-1}$), and hydrogen peroxide may affect the measurement. A metal complex can be applied for the NO fluorescent probe. Lippard et al. reported a cobalt complex possessing two Dansyl (diaminonaphthalene) units as a pendant group.[36] This probe enhanced the fluorescent intensity at 505 nm in the presence of NO, but the probe can be used only in an organic solvent. Barker et al. reported the use of an optode for fluorescent NO monitoring.[37] This system used a gold colloidal particle, on which was adsorbed cytochrome *c* labeled with difluorofluorescein (Oregon Green). The fluorescence at 640 nm decreased with the binding of NO to the cytochrome. This technique was also applied to ratiometric measurement using extra-fluorescent polystyrene microspheres (λ_{em} = 685 nm). This optode was used to monitor NO generated from macrophages. However, the detection limit, at the μM level, was too low to detect physiological NO.

NO Monitoring Using Electrochemical Methods

NO can be oxidized to NO^+ or reduced to NO^-. If an electrode is used for either of these reactions, NO can be measured directly by monitoring the redox current. Three types of such electrodes have been reported for that purpose, as follows: (i) an electrode based on a Clerk-type oxygen electrode,[18,38] (ii) an electrode based on Pt/Ir alloy,[19] and (iii) a carbon fiber electrode coated with a porphyrin derivative.[20] Since biological samples contain many kinds of redox-active molecules (such as nitrite, ascorbate and catechol amines), which participate in electrochemical reactions at a potential similar to that of NO, these electrodes are usually coated with various membranes that disturb the permeation of ionic species.[39] Such an electrochemical method can detect NO directly, enabling highly sen-

sitive real-time monitoring. On the other hand, this type of method is not suitable for the spatial imaging of NO distribution. These electrodes also have to be calibrated before use every time, because the results are sometimes affected by the temperature at which they are used.

NO Monitoring Using EPR Spectroscopy

EPR is a promising method that satisfies the required conditions in endogenous NO monitoring, real-time monitoring and spatial imaging. Theoretically, NO should be detectable by EPR, because NO is a free radical. However, the technique has severe quantum mechanical limitations for diatomic molecules. Thus, NO has to be stabilized as a polyatomic adduct by using spin traps. Hemoglobin is the first spin trap for practical use with NO.[40,41] NO binds to the heme iron strongly, producing nitrosylhemoglobin. This species can be detected as a characteristic three-line hyperfine structure.[42–44] NO concentrations have been determined in various biological samples. Unfortunately, this method is affected by oxygen.[45,46] Thus, carbonylhemoglobin has been used instead of deoxyhemoglobin because the CO adduct does not react with oxygen.[47] The laborious preparation required for the spin trap, as well as the low temperature required (such as 77 K[42] or 110 K[43]) also make this method difficult to use. Nitronylnitroxides are another type of spin trap for NO (Fig. 2 a)).[25,48] They are stable organic radicals that react with NO, directly forming iminonitroxides with dramatic changes in EPR spectra. NO from releasing agents or biological samples have been measured using this type of compound. However, these compounds are readily reduced by various biological components, such as cystein, glutathione, and ascorbate, thereby extinguishing the radical. This problem can be overcome by encapsulating the trap in a lipid vesicle,[49] although this tends to broaden the spectrum.

Probably the most widely used and practical NO spin trap is the (dithiocarbamato)iron(II) complex (Fe^{II}(dtc)) (Fig. 2 b)).[23] Although the mechanism for detecting NO in this complex is similar to that for detecting NO in hemoglobin, the nitrosyliron(II) complex can be detected at room temperature. This method also possesses the following advantages: (i) many Fe^{II}(dtc) complexes are available for various uses, (ii) the reaction rate is large enough to detect NO in biological environments,[50] (iii) NO can be detected directly, (iv) the iron complex participates in fewer side reactions, such as the Fenton reaction or superoxide production.[51] The Fe^{II}(dtc) spin traps can be classified into two types: hydrophobic and hydrophilic. The former has been used mainly for in vivo NO monitoring. Diethyldithiocarbamate (DETC) is the most widely used dithiocarbamate in this category.[52–55] The Fe^{II}(detc) is usually dissolved in albumin[56] or yeast membrane[23] because of its poor solubility in aqueous media. In some cases, iron salt and DETC are applied separately in in vivo experiments.[57,58] In the hydrophilic Fe^{II}(dtc), (*N*-methylglucaminedithiocarbamato)iron(II) complex (Fe^{II}(mgd))[59–61] and (sarcosinedithiocarbamato)iron(II) complex (Fe^{II}(dtcs))[24,62] are the most popular NO spin traps. These complexes have already been used for NO monitoring in many biological systems both in vitro and in vivo.[60,63] Fe^{II}(mgd) is suspected to react with nitrite[64] and nitronyl[65] rather than NO, probably because of its redox potential value. Yoshimura et al. reported that Fe^{II}(dtcs) had better performance than Fe^{II}(mgd) or Fe^{II}(detc) with respect to the S/N ratio in EPR-CT imaging of NO in the rat abdominal region.[66]

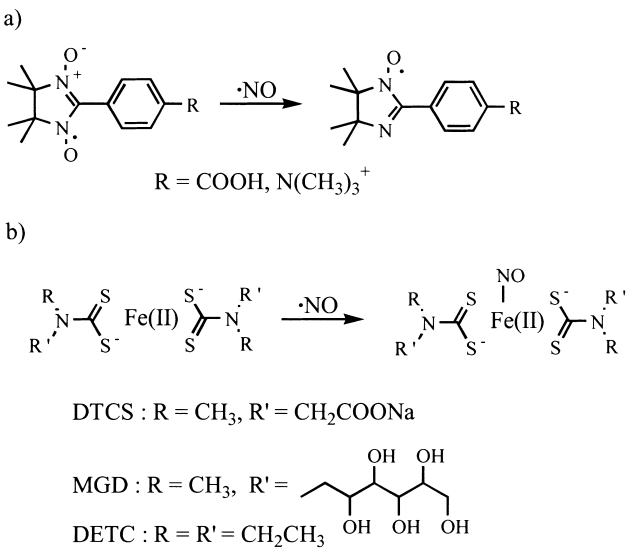


Fig. 2. Methods for NO-monitoring using EPR spectroscopy. a) PTIO (nitronylnitroxide) assay, b) Fe^{II}(dtc) ((dithiocarbamato)iron(II) complex) assay.

The Structure-Sensitivity Relationship in Fe^{II}(dtc) for NO Detection

Since Fe^{II}(dtc) is one of the most practical spin traps for NO monitoring, we synthesized the Fe^{II}(dtc) complexes listed in Fig. 3 to produce a more sensitive Fe^{II}(dtc), and we investigated the relationship between sensitivity in NO detection and the differences among substituent groups on DTC. All dithiocarbamates can be prepared by the reaction of corresponding secondary amines and carbon disulfide under an alkaline condition. Each dithiocarbamate and iron(II) sulfate heptahydrate was dissolved in 100 mM phosphate buffer solution (pH 7.4) at a concentration of 5 mM and 1 mM, respectively. Then 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7), which is a spontaneous NO-releaser, was added as 1 M sodium hydroxide solution to the above solution at a final concentration of 100 μM at 37 °C, pH 7.4. In the case of a hydrophobic Fe^{II}(dtc) complex, phosphate buffer containing 5 wt% SDS was used. Figure 4 shows a typical EPR spectrum of NO adduct of a Fe^{II}(dtc) complex. Just after NOC7 was added, the three-line spectrum started to increase at a rate corresponding to the rate at which NO was released. The obtained signal intensity was normalized using an EPR signal intensity of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxide (TEMPOL) and manganese dioxide.

Figure 5 indicates the time course of the signal increase by NOC7 in a hydrophilic Fe^{II}(dtc) complex possessing a carboxyl group. DTA-MA, whose structure is described in Fig. 3, has an electron-donating methyl group on the methylene carbon in DTCS. The sensitivity of NO detection was doubled in Fe^{II}(dtc-ma) compared with that of Fe^{II}(dtcs). On the other hand, the sensitivity of NO detection in Fe^{II}(dtc-ida), whose dithiocarbamate included an extra electron-withdrawing car-

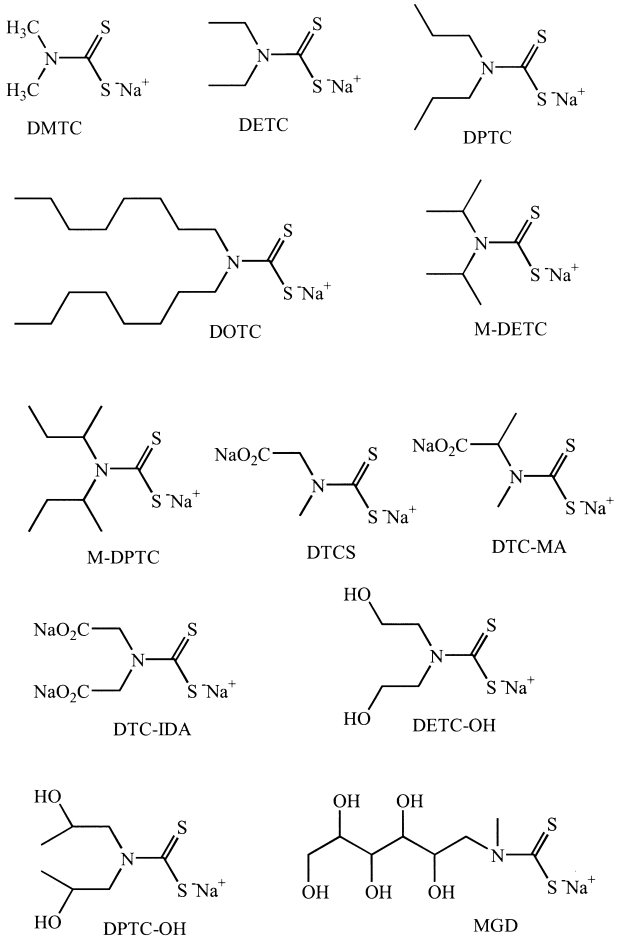


Fig. 3. Various dithiocarbamate we synthesized for investigating the relationship between the chemical structure and sensitivity of NO detection.

boxyl group in DTCS, decreased dramatically. These results suggested that an electron-donating group is preferable for the design of a Fe^{II}(dtc) complex sensitive to NO. In Fe^{II}(dtcs) and Fe^{II}(dtc-ida), the time course of signal production is slower than that of NO release from NOC7. This indicates that the unfavorable effect of the electron-withdrawing substituent for the NO detection may be related at least in part to the slower reaction rate of the Fe(II) complex with NO. In Fe^{II}(dtcs) and Fe^{II}(dtc-ida), the signal intensity was slightly decreased in the first several minutes compared to the initial intensity. Although the reason for this is unclear, it may be caused by a reductive nitrosation or re-generation of NO from nitrosodithiocarbamate, which may be formed by NO and present an excess of dithiocarbamate due to the slow reaction rate of the Fe^{II}(dtc) complex and NO. If the weaker electron-withdrawing group, the hydroxyl group, is attached to the dithiocarbamate instead of to the carboxyl group, the sensitivity is improved. Figure 6 shows the time course of the signal increase by NO production from NOC7 in the case of the Fe^{II}(dtc) complex made by dithiocarbamate having the hydroxyl group. When we used the Fe(II) complex of DETC-OH, in which the two carboxyl groups in DTC-IDA were substituted with hydroxyl groups, the EPR signal intensity of the NO adduct was enhanced by eight times, becoming larger than that in Fe^{II}(dtc-ida). MGD,

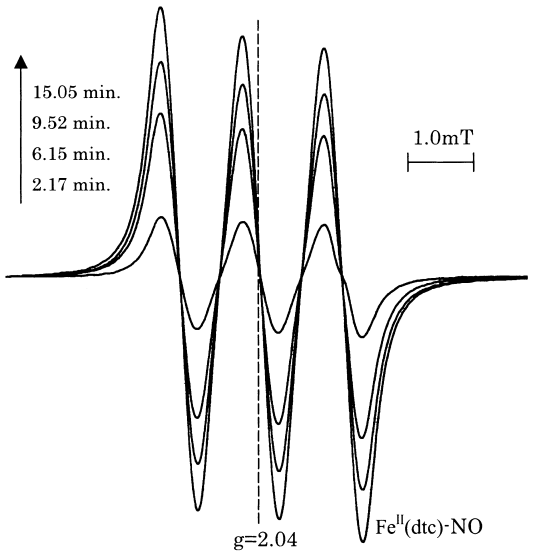


Fig. 4. Typical EPR spectrum of nitrosyliron(II) in the NO trapping experiment using (dithiocarbamato)iron(II) complex. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTCS and 1 mM iron(II)sulfate heptahydrate at 37 °C. The EPR spectrum was measured with following conditions; Resonance field 9.45 GHz, Field 332 ± 10 mT, Microwave power 1 mW.

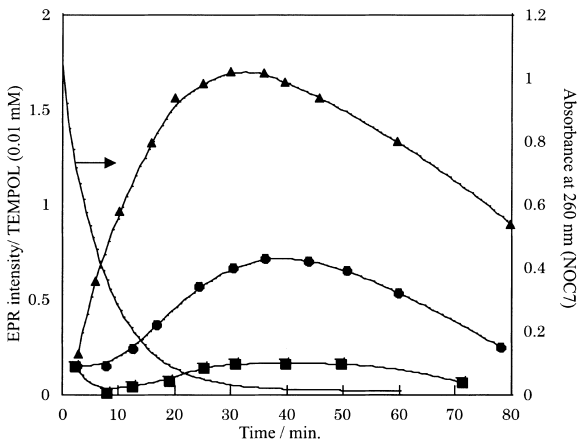


Fig. 5. Time-course of the change in EPR signal intensity of various Fe^{II}(dtc) complexes using carboxyl-type dithiocarbamates with the trapping of NO from NOC7. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTC and 1 mM $FeSO_4$ at 37 °C. Time-course of decomposition of NOC7 (NO-release) that was monitored using the absorbance at 260 nm, is superimposed. ▲: Fe^{II}(dtc-ma), ●: Fe^{II}(dtcs), ■: Fe^{II}(dtc-ida).

which has many hydroxyl groups, tended to decrease in sensitivity compared with the case of DETC-OH, and the profile of the signal increase resembled the profiles in the cases of Fe^{II}(dtcs) and Fe^{II}(dtc-ida), indicating the slow reaction rate with NO.

In the hydrophobic Fe^{II}(dtc) complex, the sensitivity–structure relationship was different from that in the hydrophilic complex. When the sensitivity was compared among the

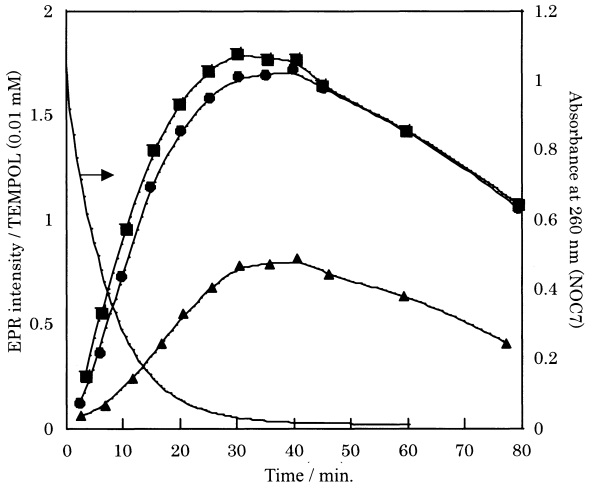


Fig. 6. Time-course of the change in EPR signal intensity of various Fe^{II}(dtc) complexes using hydroxy-type dithiocarbamates with the trapping of NO from NOC7. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTC and 1 mM iron(II)sulfate heptahydrate at 37 °C. Time-course of decomposition of NOC7 (NO-release) that was monitored using the absorbance at 260 nm, is superimposed. ▲: Fe^{II}(mgd), ●: Fe^{II}(detc-oh), ■: Fe^{II}(dptc-oh).

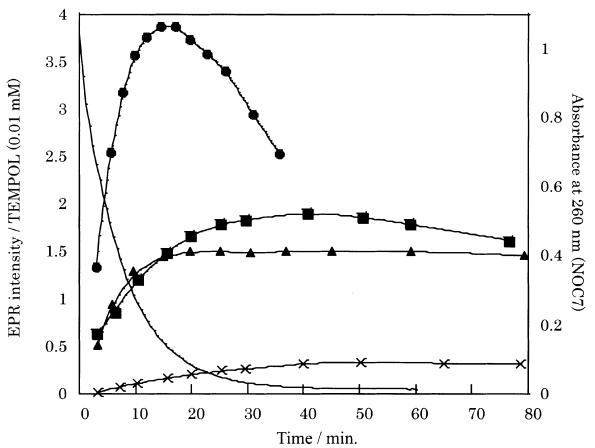


Fig. 7. An effect of alkyl chain in the dithiocarbamate to the sensitivity of NO-detection using hydrophobic Fe^{II}(dtc) complexes. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTC and 1 mM iron(II)sulfate heptahydrate and 5 wt% SDS at 37 °C. Time-course of decomposition of NOC7 (NO-release) that was monitored using the absorbance at 260 nm, is superimposed. ●: Fe^{II}(dmtc), ■: Fe^{II}(detc), ▲: Fe^{II}(dptc), ×: Fe^{II}(dotc).

Fe^{II}(dtc) complexes that had straight carbon chains, we found that sensitivity increased as chain length decreased (Fig. 7). This can be explained by the ease with which the complex in the sodium dodecylsulfate (SDS) micelle was encapsulated. Thus, the smallest Fe^{II}(dtc-ma) was the most sensitive among the four hydrophobic Fe^{II}(dtc) complexes investigated here. Fe^{II}(dotc), with its long alkyl chains, could not trap NO effectively because of the difficulty of dissolving into the SDS micelle. However, the stability of nitrosyl-Fe^{II}(dtc) was improved with the increase of the alkyl chain length in DTC. This may be related to the redox potential of the centered iron(II). When the sensitivity is compared using "compact" Fe^{II}(dtc) complexes, the effect of the electron-donating group on the ligand can be seen again; the effect is similar to that in the hydrophilic Fe^{II}(dtc) complex (Fig. 8). Introducing the methyl group into the dithiocarbamate enhanced the sensitivity, as was found when the signal intensities of NO-Fe^{II}(m-detc) and NO-Fe^{II}(detc) or NO-Fe^{II}(m-dptc) and NO-Fe^{II}(dptc) were compared.

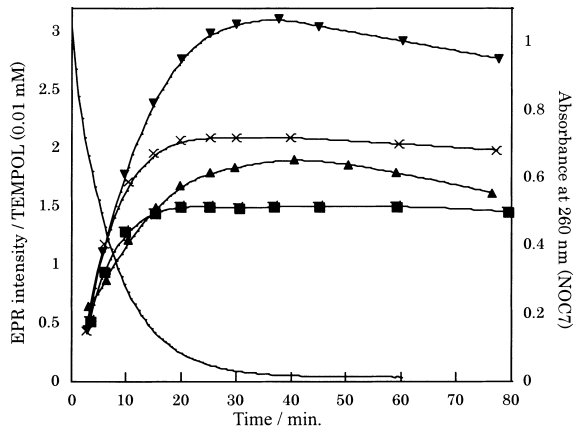
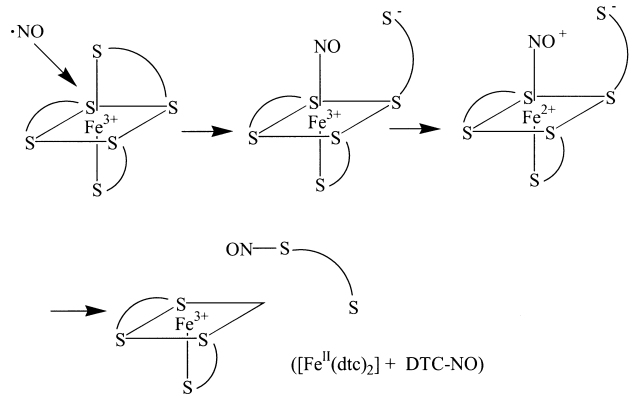


Fig. 8. Time-course of the change in EPR signal intensity of various "cmpact" hydrophobic Fe^{II}(dtc) complexes with the trapping of NO from NOC7. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTC and 1 mM iron(II)sulfate heptahydrate and 5 wt% SDS at 37 °C. Time-course of decomposition of NOC7 (NO-release) that was monitored using the absorbance at 260 nm, is superimposed. ▼: Fe^{II}(m-detc), ×: Fe^{II}(m-dptc), ▲: Fe^{II}(detc), ■: Fe^{II}(dptc).

To elucidate the electronic effect of the substituent groups on the dithiocarbamato ligand, a NO-trapping experiment was performed in the presence of 5 mM ascorbate using Fe(II) complexes of MGD, DTC-MA and DTC-IDA. The results showed enhanced sensitivities of all of the Fe^{II}(dtc) complexes examined. Also, the differences among the sensitivities using three Fe^{II}(dtc) complexes in the absence of ascorbate were totally cancelled (data not shown). The inconsistency between the time courses of EPR signal generation and NO release from NOC7 also disappeared. These results indicate that the electronic effect of the substituent group on the ligand is caused by the contamination of the Fe(III)-type complex that is caused by oxygen. If an electron-withdrawing substituent group is attached to the ligand, the Fe(II) complex would be labilized by enhancing the oxidation rate of the Fe(II) complex.

NO Trapping Using Fe(III) Complex

Yoshimura et al. reported that a Fe^{III}(dtc) complex can be used as a NO spin trap because Fe(II)-DTC is formed in situ by the reductive nitrosation (Scheme 1).[67] Thus, the NO detection was investigated using the Fe(II) and Fe(III) complexes of M-DETC and DTCS. Figure 9 indicates that the Fe(II) complex is more effective than the Fe(III) complex in trapping NO. In the Fe(III) complex, NO is also used for the reduction of Fe(III) to Fe(II). This decreases the actual concentration of NO that can



Scheme 1. Reductive nitrosiation.

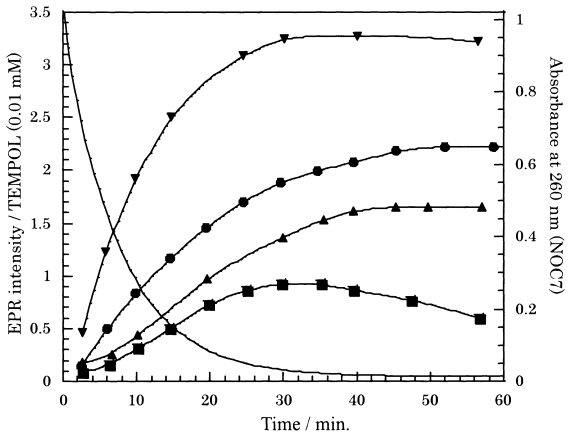


Fig. 9. Comparison of the change of the EPR signal intensity in the Fe(II)-type and Fe(III)-type complexes with the NO-trapping experiment. NOC7 (100 μM) was added to the 100 mM phosphate buffer solution (pH 7.4) containing 5 mM DTC and 1 mM iron(II)sulfate heptahydrate at 37 °C. In the case of hydrophobic Fe^{II}(dtc), 5 wt% of SDS was contained in the buffer solution. Time-course of decomposition of NOC7 (NO-release) that was monitored using the absorbance at 260 nm, is superimposed. ▼: Fe^{II}(m-detc), ●: Fe^{III}(m-detc), ▲: Fe^{II}(dtcs), ■: Fe^{III}(dtcs).

be used to form the EPR-detectable adduct, NO-Fe^{II}(dtc). However, the Fe(III) complex is stable for oxidation in an aerobic condition, in contrast to Fe^{II}(dtc). Thus, the Fe^{III}(dtc) complex still has some advantages for practical use, especially in in vivo experiments.

New NO Spin Trap Using "Radical-Exchange" Concept

In the previous section we investigated the relationship between the chemical structure of the dithiocarbamate and NO trapping ability. We then found some new Fe^{II}(dtc) complexes to be more sensitive NO spin traps than the widely used Fe^{II}(dtcs) and Fe^{II}(mgd). However, these new complexes enhanced NO detectability by two to three times at most, because detectable species belong to the same nitrosyliron regardless of which Fe^{II}(dtc) complex is used. A different type of NO spin trap based on a novel concept will be needed to obtain a still more sensitive spin trap for NO, because the detection limit of

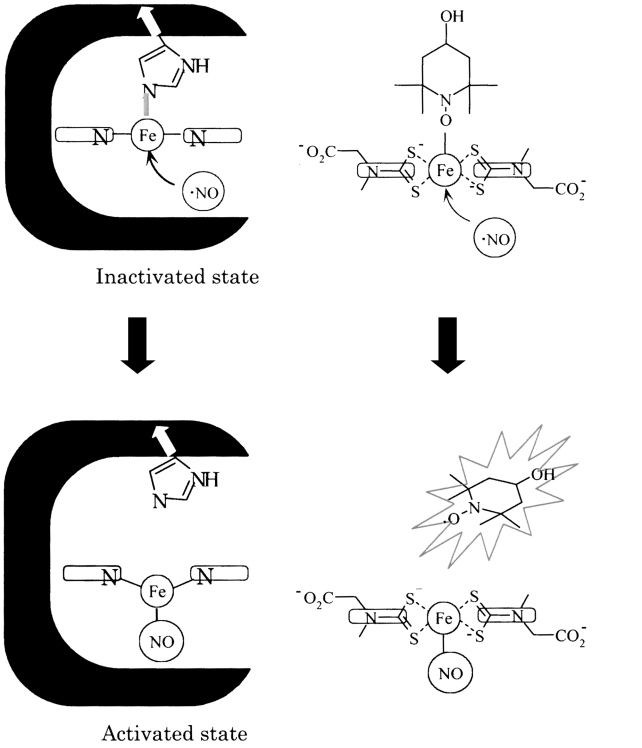


Fig. 10. A molecular mechanism of the activation in Guanylyl cyclase with NO and a new molecular switching system we designed.

NO using a Fe^{II}(dtc) complex was too low to allow the physiological NO to be monitored. To realize high sensitivity in NO monitoring, NO will have to be converted to some other radical species for detection with EPR. Stable organic radicals are a promising candidate for highly sensitive detection. Thus we designed a molecular switching system, in which NO is changed to a stable TEMPOL radical (Fig. 10).[27] NO is actually involved in a similar switching mechanism in a biological system. Guanylyl cyclase is one of the major targets of endogenous NO. The enzyme is activated with NO directly.[68–73] Figure 10 indicates the activation mechanism of the enzyme with NO. This enzyme includes heme, with which an imidazol in the histidine residue coordinates, in the regulatory domain. NO binds to the heme-iron(II) to dissociate the imidazol ligand through a strong electron-withdrawing effect. This event leads to a conformational change of the enzyme to the active state. We mimicked this system in designing our system. Thus, a planar Fe^{II}(dtc) complex and TEMPOL radical were used in place of the heme and the imidazol, respectively. When 200 μM of TEMPOL was added to the 100 mM phosphate buffer solution (pH 7.4) containing the Fe^{II}(dtcs) complex, which is formed with 1 mM iron(II)sulfate heptahydrate and 5 mM DTCS-Na salt at 37 °C, the EPR signal of the TEMPOL vanished immediately. This indicates that the TEMPOL interacts with the iron complex through electronic (redox) interaction to extinguish the radical. Probably an electron on the iron(II) was transferred to the TEMPOL to form Fe(III) and nitroxide anion. Then, 100 μM of NOC7 was added to the system. Figure 11 a)

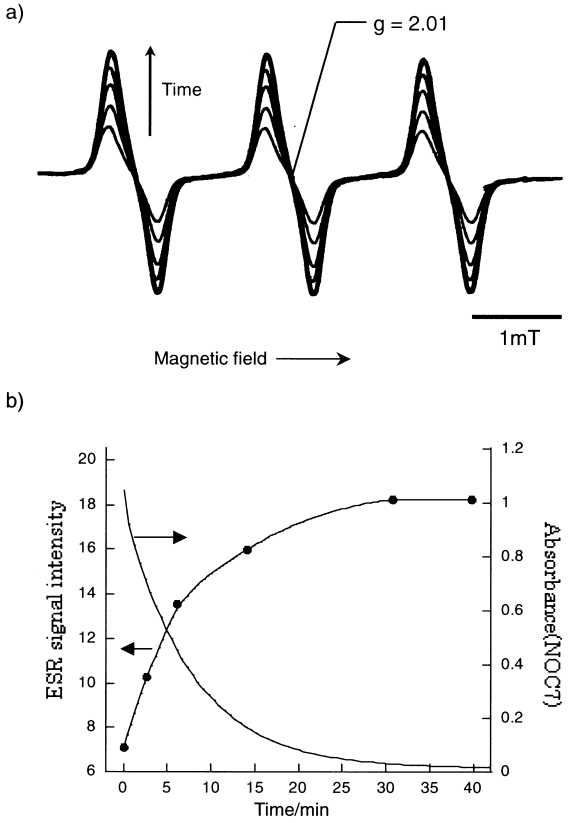


Fig. 11. Restoring of the EPR spectral of TEMPOL in the spin exchange system (200 μM) with nitric oxide released from NOC-7 (100 μM) (a) and a comparison of the time course of the EPR signal increase (●) and the NO release from NOC-7 (b). All experiments were performed at 37 °C in a 100 mM phosphate buffer (pH 7.4). The release of nitric oxide was monitored by a decrease of absorbance at 260 nm.

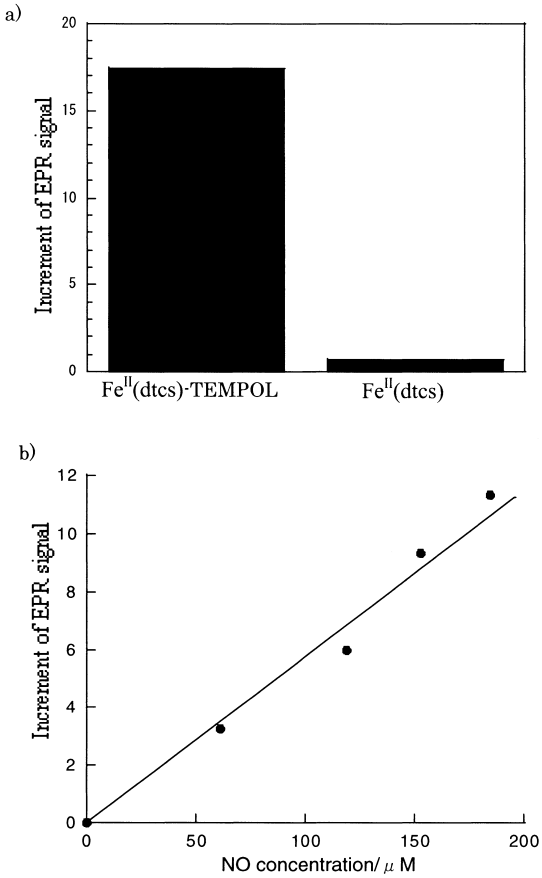


Fig. 12. A comparison of the increments of EPR signal intensities between the spin exchange system (200 μM) and the ordinary DTCS-Fe(II) complex (200 μM) after the trapping of NO released from NOC-7 (100 μM) (a) and the calibration line for the NO detection using the spin exchange system (b). All experiments were performed at 37 °C in a 100 mM phosphate buffer (pH 7.4).

shows the EPR spectral change of Fe^{II}(dtc)-TEMPOL after NOC7 was added. The EPR signal based on the TEMPOL gradually recovered, corresponding to the time course of NO release from NOC7 (Fig. 11 b)). On the other hand, if NOC7 was not added, the EPR signal did not change during a period of more than one hour. This clearly indicates that NO broke off the Fe(II)-TEMPOL interaction to restore the redox state of TEMPOL to its original radical form. The consistency between the time course of EPR signal restoration and that of NO-release from NOC7 means that NO binds to the Fe(II) complex much faster than NO is generated. Figure 12 a) compares the EPR signal intensity of the system and an ordinary NO spin trap, DTCS-Fe(II), after trapping of the NO from NOC7. The new "spin exchange" system is 25 times more sensitive than the system using Fe^{II}(dtcs). The spin exchange system can monitor NO to a detection limit of 10 nM (Fig. 12 b)). Recently, we found other Fe(II) complexes, such as Fe^{II}(cyclam) (cyclam = 1,4,8,11-tetraazacyclotetradecane) or Fe^{II}(salen) (salen = *N*,*N*′-ethylenebis(salicylideneaminato)) that participate in a similar charge-transfer interaction with TEMPOL and TEMPOL release with NO.[28] However, the rate at which the EPR signal vanished when TEMPOL was added to the Fe(II) complex, and the rate at which the EPR signal was restored when NOC7 was added, were much slower than in the case of Fe^{II}(dtcs), since the Fe^{II}(dtcs)-TEMPOL system should react only with NO directly, similar to other Fe(II) complexes. Actually, the system did not respond to other NO derivatives we examined: nitrite or peroxonitrite formed by 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1) (Fig. 13). *S*-nitrosothiol reacted with Fe^{II}(dtcs)-TEMPOL. The ordinary Fe^{II}(dtc) complex also reacts with *S*-nitrosothiol, but it is not clear whether this response is caused by the direct reaction with *S*-nitrosothiol or by the release of NO from the compound.

The new system introduced here detected NO with 25 times greater sensitivity than ordinary Fe^{II}(dtcs) complexes. The detection limit of NO, which was 10 nM in the NO-detection experiment using NOC7, is similar to that in FNOCT. However, the system still has some ambiguities regarding the accuracy of NO determination, because the iron(II) ion tends to be oxidized to Fe(III) which is ordinarily the case with Fe^{II}(dtcs) complexes. The signal intensity is affected by the concentration of dissolved oxygen. Thus, a calibration line for the determination of NO concentration should be used in every experiment.

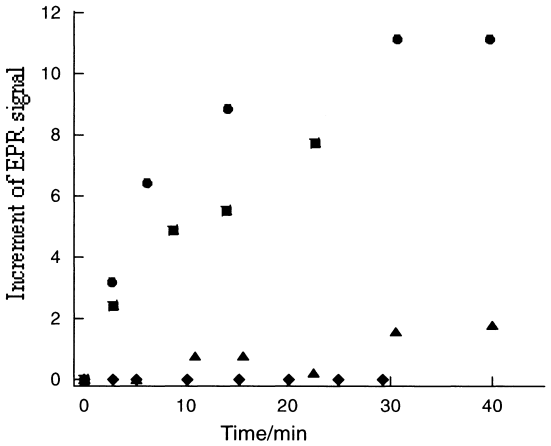


Fig. 13. The time-course of the EPR signal intensity change in the spin exchange system (200 μM) with various reagents. ●: NOC7 (100 μM), ■: *S*-nitrosoglutatione (GSNO) (100 μM), ▲: SIN-1 (100 μM), ◆: nitrite (100 μM). All experiments were performed at 37 °C in a 100 mM phosphate buffer (pH 7.4) in the presence of each NO-related reagent (1 mM).

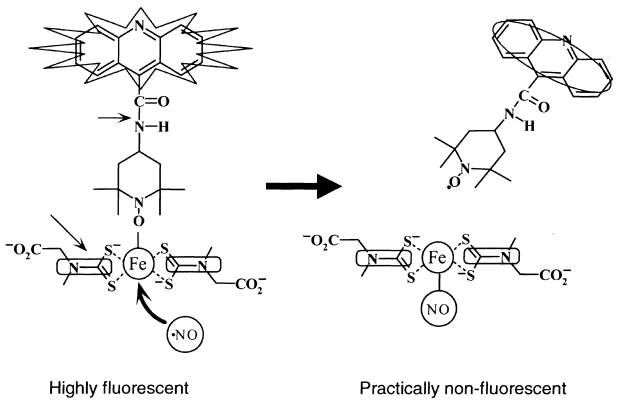


Fig. 14. A diagram of the NO-detection in the newly designed fluorescent probe using "radical-exchange" concept.

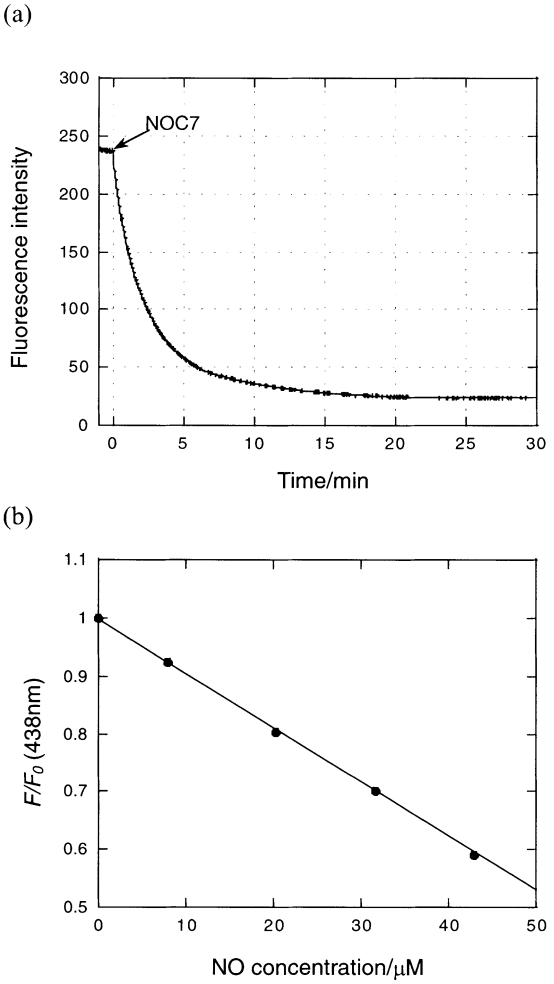


Fig. 15. The time course of the decrease in the fluorescence intensity at 438 nm with NO released from NOC7 (100 μM) (a) and the calibration line for the NO detection using the new fluorescent probe (b). The vertical axis indicates the ratio of the fluorescence intensity at 438 nm in the presence of various concentration of NO to that in the absence of NO.

Design of NO Fluorescent Probe Using the Radical Exchange Concept

Our molecular switching system has been applied to the design of a new fluorescent probe that reacts with NO directly. Figure 14 shows the first example of a fluorescent probe using the "radical-exchange" concept. Acridine-TEMPO was practically non-fluorescent because a free radical was present in the TEMPO unit. When the acridine-TEMPO (20 μM) was added to 100 mM phosphate buffer solution (pH 7.4) containing DTCS-Na (2.5 mM) and iron(II)sulfate heptahydrate (500 μM), the fluorescence at 438 nm based on the acridine moiety increased immediately, because the free radical in the TEMPO was extinguished through the charge-transfer interaction with Fe(II). Figure 15(a) shows the time course of the fluorescence change of this system after the addition of 100 μM NOC7 at 37 °C. The fluorescence intensity gradually decreased; the time course corresponded to that of the NO release from NOC7. A good linear correlation (r^2 = 0.9998) was found between the diminution of the fluorescence intensity and the NO concentration (Fig. 15(b)). This system would have been able to detect sub-μM levels of NO. Although the above probe simply decreases the fluorescence intensity with NO, a ratiometric probe also can be designed with the same strategy by using dual fluorescent labeling. Figure 16 shows the chemical structure and molecular mechanism of NO detection. In this system, PLOXYL-fluorescamine and methoxycoumalinomethyl-cyclam (Mmc-cyclam) were used in place of TEMPOL and DTCS-Fe(II) in the "radical-exchange" EPR probe. Mmc-cyclam was readily synthesized from cyclam and Br-methylmethoxycoumaline (Br-Mmc) by stirring in methylene chloride in the presence of triethylamine at room temperature. The methoxycoumaline (λ_{em} = 410 nm) and the fluorescamine derivative (λ_{em} = 385 nm) are a preferable pair for creating a fluorescence energy transfer (FRET), if the coumline unit is excited. When PLOXYL-fluorescamine (80 μM) was added to 100 mM

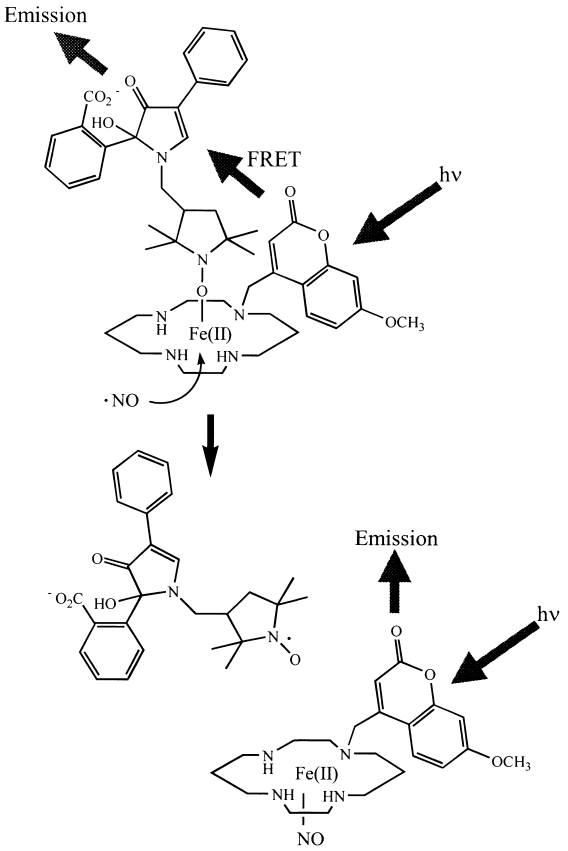


Fig. 16. A diagram of the ratiometric measurement of NO using the dual-fluorescent labeled probe.

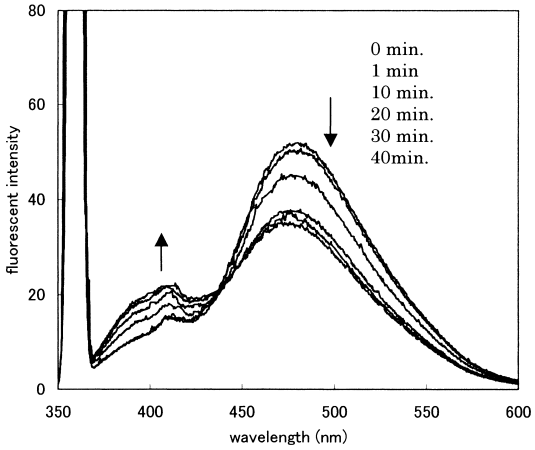


Fig. 17. A spectral change of the ratiometric fluorescent probe in the NO-trapping experiment using NOC7 as a NO-releaser. The experiment was performed at 37 °C in a 100 mM phosphate buffer (pH 7.4) in the presence of NOC7 (80 μM). The emission spectra were measured using 360 nm as an excitation wavelength.

phosphate buffer solution (pH 7.4) containing 20 μM Mmc-cyclam and 80 μM iron(II)sulfate heptahydrate, the fluorescence at 485 nm for the fluorescamine derivative increased immediately under an excitation wavelength of 310 nm for the coumaline unit. This increase occurred because of the effective FRET between the two fluorescent molecules. This indicates again that the PLOXYL bound to the Fe(II) through charge transfer interaction caused the radical quenching in PROXYL. NO from NOC7 shifted the maximum wavelength of the fluorescence from 485 nm to 410 nm (Fig. 17). This result means that NO effectively canceled the FRET.

Presumably, NO binding to the centered Fe(II) broke off the interaction between the PROXYL and the Fe(II) to release the PLOXYL-fluorescamin (Fig. 16). Thus, this probe may be useful for monitoring the NO concentration independent of the background fluorescence, by using the ratio of fluorescence intensity at 410 nm to that at 485 nm. However, detailed studies on the detectability of NO have not been performed. As mentioned in the previous section, the Fe^{II}(cyclam) system is not fully appropriate for practical application, because the reaction rate to NO is too slow to catch the small amount of NO in biological environments. To apply this strategy to practical ratiometric probes, a coumaline unit should be attached to a dithiocarbamate ligand.

Conclusion

NO monitoring is crucial to our understanding of the biological role of NO. NO will have to be monitored directly in real-time near the sites of production and action. Spatial imaging of NO distribution is also important. A lot of methods have been developed to monitor NO. Although some of these satisfy a portion of the above conditions for practical use, a perfect method has not yet been developed. The EPR method using the spin trap is one of the most promising because it can detect NO directly in real-time and also can be used for the spatial imaging of NO production. In such NO spin traps, (dithiocarbamato)iron(II) complexes are the most appropriate for practical use and are also widely used reagents. However, their sensitivity to NO is too low to detect the physiological NO that is supposed to be at the nM level. Thus, we firstly investigated the structure-sensitivity relationship of dithiocarbamato ligands to find more sensitive spin traps. As a result, substituent groups having an electron-donating property were useful for improving NO detectability, probably because of the stabilization of the Fe(II)-type complex. Fe(II) complexes of DTC-MA, DPTC-OH and M-DETC were all more sensitive probes than the widely used Fe^{II}(dtcs), Fe^{II}(mgd) and Fe^{II}(detc). However, the sensitivity improved only two- to three-fold at best, because all spin traps use the same EPR-detectable inorganic radical species, nitrosyliron(II). We then designed a new molecular switching system according to a new "radical-exchange" concept. In this system, the unstable NO radical is changed to the stable organic TEMPO radical. This system can detect NO with much greater sensitivity than can an ordinary Fe^{II}(dtc) complex using EPR. The concept was also applied to the design of fluorescent probes. Two types of NO fluorescent probes developed using the "radical-exchange" strategy were introduced here. Of course, these probes still have some issues that must be solved. Excess amounts of iron complex or ligand have to be used in order to make an effective system. These substances may affect the accurate determination of NO. However, the new concept introduced here is potentially useful for the design of more practical probes for NO monitoring. The connection of an organic radical species and a ligand with a linker arm may be needed to avoid the use of

excess ligand or other components while making the probing system.

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