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Fluorescent indicators for nitric oxide based on rhodamine chromophore

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Abstract

We have developed a novel fluorescent indicator for NO, DAR-M, which features long-wavelength excitation, high photostability and no pH dependency over a wide pH range.

Keywords: diamines; dyes; fluorescence; nitrogen oxides.

Direct detection of nitric oxide (NO) is important for the clarification of its many proposed biological functions.[1] We have reported diaminofluoresceins (DAFs) as fluorescent indicators for NO. By using the diacetyl derivative (DAF-2 DA), which permeates into cells and is quickly transformed into water-soluble DAF-2 by intracellular esterases, the temporal and spatial distribution of intracellular NO was visualized, and its usefulness for functional studies was confirmed.[2,3] However, some of the characteristics of DAF-2 are undesirable, such as low photostability, a wavelength range that overlaps autofluorescence in vivo, and pH dependency. In particular, the pH dependency is a critical problem. It is said that NO has something to do with the neurotoxicity after cerebral ischemia.[1] The application of DAF-2 DA to brain tissues under this condition is difficult, because intracellular pH falls below six during the ischemia.[4]

It was considered that DAF can be structurally divided into the diamine moiety as the reactive site and the xanthene moiety as the fluorophore. Aromatic vicinal diamines can trap NO under aerobic conditions to yield the corresponding triazole compounds. The change of the electron-donating ability of the functional groups causes the switch of fluorescence, owing to photo-induced electron transfer.[5] We supposed that this mechanism would also be applicable to the rhodamine fluorophore and that the introduction of a diamine moiety into rhodamine instead of fluorescein might overcome the problems.

We designed and synthesized diaminorhodamines (DAR-1 and DAR-2, Scheme 1) from *N*,*N*-diethyl-3-aminophenol and the corresponding acetoamido-nitrophthalic anhydride. The overall yields of DAR-1 and DAR-2 were 42% and 7.7%, respectively. DARs fluoresced after reaction with NO, as expected. The absorbance and fluorescence properties are summarized in Table 1. The wavelengths involved are

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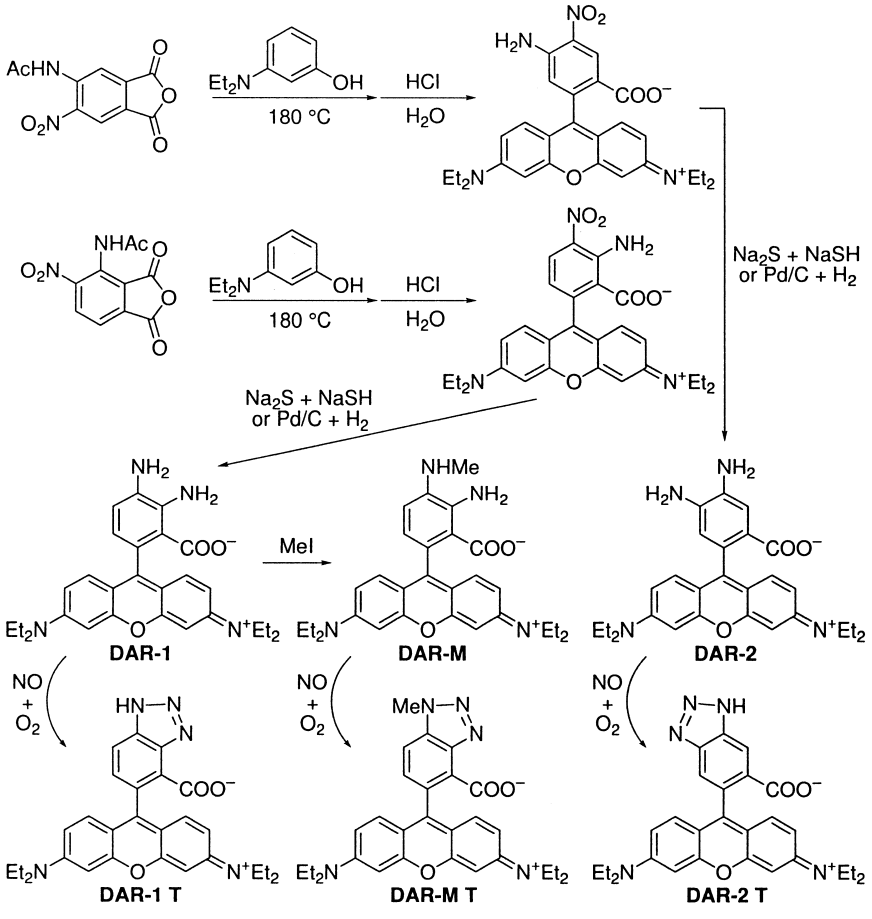
longer than those of DAFs. Although the quantum yields are relatively low, this does not represent a practical disadvantage; indeed, the signal to noise ratio is, if anything, sometimes higher because of the low background fluorescence at such a longer-wavelength, especially in the analysis of biological samples.



Scheme 1. Synthesis of diaminorhodamines (DARs) and their products of reaction with NO

Photostability was examined in sunlight.[7] The fluorescence intensity of DAR-1 T was not reduced at all after 3 h, while that of DAF-2 T was reduced to 1% of the initial intensity.

The effect of pH on the fluorescence intensity of DAR-1 T and DAR-2 T was also examined. The fluorescence intensity of rhodamine B, which is the chromophore of DARs, is stable above pH 4, since it has no phenolic OH. However, we observed instability of the fluorescence intensity around neutral pH as shown in Fig. 1. The instability may arise from a triazole proton, so, a methyl group was introduced into DAR-1 with methyl iodide (DAR-M, Scheme 1). It was selectively introduced into the amine with higher electron density.[8]

The absorbance and fluorescence properties of DAR-M (Table 1) were almost the same as those of DAR-1 or DAR-2. The augmentation of the fluorescence intensity of DAR-M upon addition of NO is shown in Fig. 2. Judging from NO standard curves calibrated with 10 μM DAR-M, the detection limit was 10 nM. The sensitivity of DAR-M was 1.4 times higher than that of DAR-1. It is considered that this

Table 1
Absorbance and fluorescence properties of dyes

Dye	Extinction coefficients and absorption maxima [$\times 10^4$ $M^{-1}cm^{-1}$, nm]		Fluorescence maximum of triazole form [nm]	Relative quantum efficiencies	
	Diamine	Triazole		Diamine	Triazole
DAR-1	12 , 550	8.7, 556	575	0.004	0.25
DAR-2	10 , 549	7.1, 552	571	0.006	0.34
DAR-M	9.8, 550	7.5, 558	574	0.007	0.29
DAF-2*	7.9, 486	7.3, 491	513	0.005	0.92

*See Kojima et al.[2]

All data were obtained at 20°C in 0.1 M sodium phosphate buffer, pH 7.4. Relative quantum efficiencies were obtained by comparing the area under the corrected emission spectrum of the test sample at 535 nm excitation with that of a solution of rhodamine B in ethanol, which has a quantum efficiency of 0.97 according to the literature[6]

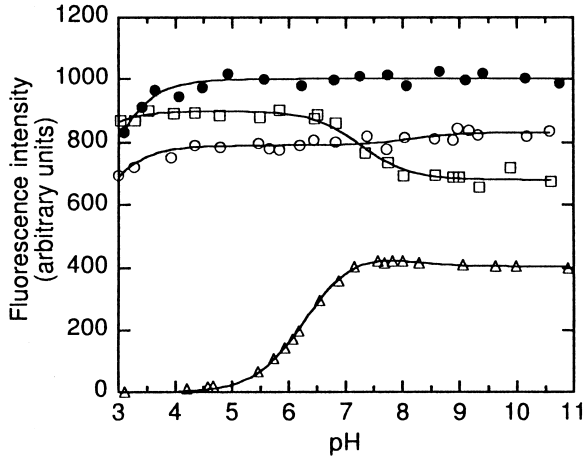


Figure 1. Effect of pH on the fluorescence intensity of DAR-Ts and DAF-2 T. Triazole forms of DAR-1, DAR-2 and DAR-M (final 1 μM) were added to sodium phosphate solution adjusted to various pH values. The pH was measured after mixing. The fluorescence intensities of DAR-1 T (open circle), DAR-2 T (open square) and DAR-M T (closed circle) were determined at 580, 575 and 575 nm with excitation at 565, 565 and 560 nm, respectively. The photomultiplier voltage was 950 V. The curves were fitted to the following equations: (DAR-1 T) intensity=$783/(1+10^{2.13-pH})$ +$42.7/(1+10^{6.69-pH})$, R=0.956; (DAR-2 T) intensity=$899/(1+10^{1.62-pH})$ −$218/(1+10^{7.27-pH})$, R=0.987; (DAR-M T) intensity=$1000/(1+10^{2.39-pH})$, R=0.951. DAF-2 T (open triangle): The data are from Kojima et al.[2]. The photomultiplier voltage was 400 V

increase of sensitivity results from a higher rate of trapping of NO due to the electron-donating effect of the methyl group. The fluorescence intensity of DAR-M T was stable above pH 4, as shown in Fig. 1.

In conclusion, we have developed an improved type of DAFs, DAR-M, which features longer-wavelength excitation, higher photostability and no pH dependency above pH 4. Thus, DAR-M should enable wider application of diamine compounds for detecting NO.

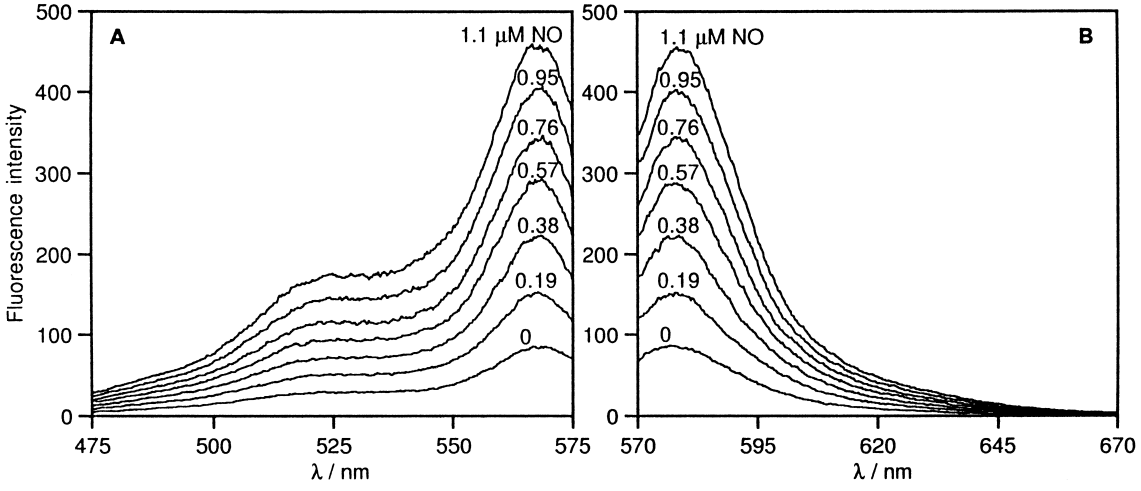


Figure 2. Excitation (A) and emission (B) spectra for DAR-M at 37°C in 0.1 M sodium phosphate buffer (pH 7.4) with NO concentrations ranging from 0 μM to 1.1 μM.NO solution was added to 10 μM DAR-M solution under aerobic conditions. The spectra were obtained from an average of five repeated measurements after the addition of NO solution. (A), determined with emission at 580 nm. (B), determined with excitation at 565 nm

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7. Bleaching of dyes (10 μM) was determined in 0.1 M sodium phosphate buffer (pH 7.4). The solutions in 30 ml vials were placed in full sunlight on a fine day in November in Tokyo.
8. It is supported by data of the amino protons in the ^{1}H NMR spectra. DAR-1 (300 MHz, DMSO-d_6): δ 1.09 (t, 12H, J=7.2 Hz), 5.01 (br, 2H), 5.87 (br, 2H), 6.07 (d, 1H, J=7.5 Hz), 6.38–6.43 (m, 4H), 6.57 (d, 2H, J=8.6 Hz), 6.78 (d, 1H, J=7.5 Hz). DAR-M (300 MHz, DMSO-d_6): δ 1.08 (t, 12H, J=6.8 Hz), 2.77 (s, 3H), 5.22 (br, 1H), 6.01 (br, 2H), 6.19 (d, 1H, J=7.7 Hz), 6.40–6.44 (m, 4H), 6.56–6.63 (m, 3H).