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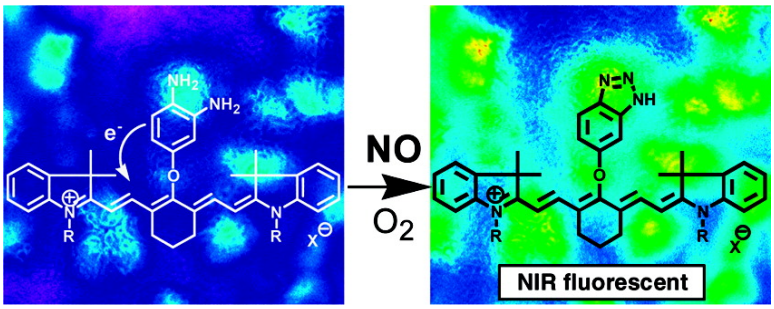

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Highly Sensitive Near-Infrared Fluorescent Probes for Nitric Oxide and Their Application to Isolated Organs

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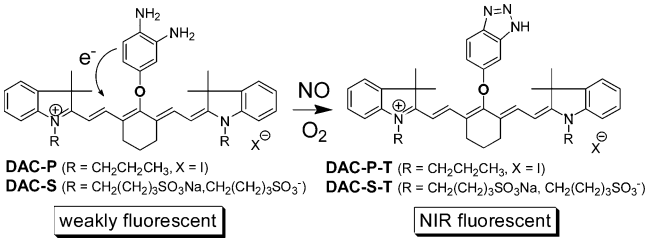
Nitric oxide (NO), which is synthesized through conversion of L-arginine to L-citrulline by NO synthase in vivo, is an important signaling molecule involved in the regulation of a wide range of physiological and pathophysiological mechanisms, and many disorders related to NO signaling impairment have been reported.[1,2] Therefore, methods for visualizing NO would be powerful tools to examine in detail the NO signaling mechanisms[3] and might eventually be useful for diagnosis as well.

Fluorescence imaging methods are generally superior in terms of sensitivity, selectivity, and ease of use. Recently, we have successfully developed several fluorescent probes for NO,[4,5] and these probes have been widely used in biological applications. However, they have severe limitations with regard to ex vivo applications to detect NO in isolated organs since their fluorescence lies in the visible region around 500−550 nm, which cannot penetrate deeply into human tissues. While visible light is highly absorbed by biological substances, such as hemoglobin, near-infrared (NIR) light at around 650−900 nm is less absorbed by such molecules and can penetrate more deeply into tissues. Moreover, it has the further advantage that autofluorescence is not observed upon NIR excitation. For these reasons, NIR fluorescence imaging is potentially very attractive for in vivo imaging.[6] In this report, we present novel NIR fluorescent probes which are highly sensitive to NO and establish their utility by imaging NO in isolated intact rat kidneys.

The probes are composed of two moieties: tricarbocyanine as the NIR fluorophore, which has a high extinction coefficient of about $1.5-2.0 \times 10^5$ M^{-1} cm^{-1}, and *o*-phenylenediamine as the NO-sensitive fluorescence modulator. Our strategy for detecting NO is based on the change of the electron-donating ability of *o*-phenylenediamine upon selective NO-mediated transformation of diamine into triazole under aerobic conditions. We predicted that *o*-phenylenediamine should quench the fluorescence of tricarbocyanine because electron transfer should occur from *o*-phenylenediamine to the excited fluorophore. On the other hand, the corresponding triazole formation should recover its NIR fluorescence since the triazole ring should not have sufficient electron-donating ability for such photoinduced electron transfer (PeT) to occur (Scheme 1). We named these new probes diaminocyanines, DACs, and the corresponding triazole compounds DAC-Ts. They can be classified into two types; DAC-P with two propyl groups was designed to penetrate cellular membranes without any modification, and DAC-S with sulfonate groups was expected to be highly soluble in water. Consequently, the appropriate type can be selected for particular purposes.

Scheme 1



Then, we examined the spectral properties of the probes. As we expected, the NIR fluorescence intensity of DACs greatly increased in an NO concentration-dependent manner (Figure 1). The fluorescence quantum yields of DAC-Ts were 14-fold higher than that of DACs. We next evaluated the effect of pH on the fluorescence. The fluorescence of DACs was quenched at pH above 6, and that of DAC-Ts were independent of pH from 2 to 12. That is to say, DACs work well under physiological pH conditions, although the fluorescence intensities of DACs were relatively high at pH below 6. We can explain this recovery of the fluorescence under acidic conditions in terms of protonation of the amino group reducing the electron-donating ability of *o*-phenylenediamine and abolishing the PeT; the pK_a value of protonated *o*-phenylenediamine is 4.47.[7] These spectral properties indicate that PeT is successfully regulated in these molecules. According to the Rehm−Weller equation,[8] the longer the wavelength of fluorophore is, the more difficult it is for PeT to occur. As far as we know, there is only one reported NIR fluorescent probe in which the fluorescence is controlled by the PeT mechanism.[9] Therefore, it is noteworthy that we could design these fluorescent probes and control PeT in the NIR region.

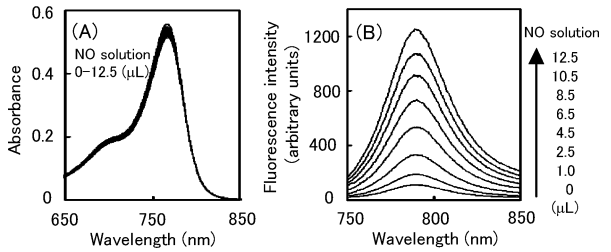


Figure 1. (A) Absorbance spectra and (B) emission spectra (excitation at 750 nm) of DAC-S (3 μM) in the presence of various amounts of NO at 37 °C in 0.1 M sodium phosphate buffer, pH 7.4. The spectra were obtained 15 min after the addition of a saturated NO aqueous solution (2 mM; 0−12.5 μL) to a solution of DAC-S (3 mL).

Furthermore, we compared the reactivity with NO of DAC-S with that of DAF-2,[4] which is a widely used NO fluorescent probe (Figure 2A). It was previously shown that the reaction rate of *o*-phenylenediamine with NO depends on electron density.[5] In the

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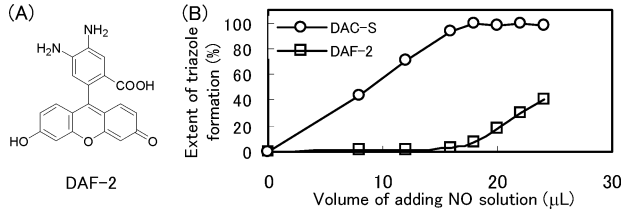

Figure 2. (A) Structure of DAF-2. (B) The extent of formation of the triazoles of DAC-S and DAF-2 (3 μM, 0.1% DMSO) after addition of various amounts of NO at 37 °C in 0.1 M sodium phosphate buffer, pH 7.4. The fluorescence intensity was measured 15 min after the addition of a saturated NO aqueous solution (2 mM; 0−24 μL) to a solution of DAC-S and DAF-2 (3 mL), and the extent of formation of the corresponding triazoles was calculated from the fluorescence quantum yields.

case of DAC-S, the electron density is increased by an electron-donating oxygen atom, while DAF-2 has an electron-withdrawing carboxylate group. Thus, DAC-S is expected to react faster than DAF-2. The result of competitive reaction of DAC-S and DAF-2 with NO is shown in Figure 2B. DAF-2 did not react with NO until almost all of the DAC-S was converted into DAC-S-T. When 8 μL of a saturated NO aqueous solution was added, 44% of DAC-S and 0.83% of DAF-2 were converted to the corresponding triazoles. Namely, the reaction efficiency of DAC-S with NO is at least 53 times higher than that of DAF-2 under an equimolar condition. This means that, although DAF-2 has functioned well in many kinds of cells so far, these new probes can potentially detect NO more effectively in biological tissues in the presence of endogenous competitors, such as thiols.

Finally, we applied DAC to isolated rat kidneys to examine whether it worked in ex vivo biological systems and whether we could observe the fluorescence change from outside the kidney without making sections. We selected DAC-P because it should be loaded more readily than DAC-S. Kidneys from male Wistar rats were isolated and perfused as described previously.[10] A diagram of the system is shown in the Supporting Information. As we expected, DAC-P was easily loaded into the kidneys simply by administering it into the right renal artery with the perfusate for several minutes and was hardly washed out throughout the observation. Then, NOC13,[11] which is an NO donor with a half-life of 4.7 min in aqueous buffer solution at pH 7.4 and 37 °C, was administered in the same manner. The NIR fluorescence images were captured every 20 s with a fluorescence stereomicroscope. We observed a fluorescence increase during the administration of NOC13 (Figure 3A). That is to say, DAC-P functions in ex vivo biological systems, and we could detect its fluorescence from outside the kidney. We noticed many circular patterns with a diameter of approximately 0.2 mm on the fluorescence images (Figure 3B−D). We confirmed that these patterns corresponded to the glomeruli inside the kidney by observing the fluorescence images of renal sections (Supporting Information). The observations are consistent with the structure of the glomeruli, which are covered with reticular blood vessels. DAC-P should be loaded into the endothelial cells during the perfusion. Thus, we succeeded in imaging NO in the isolated rat kidney using DAC-P.

In summary, we designed NIR fluorescent probes for NO, DACs, based on the PeT mechanism, synthesized them, investigated their spectral properties, compared the reaction rate with that of a widely used NO probe, DAF-2, and ascertained that they worked in isolated

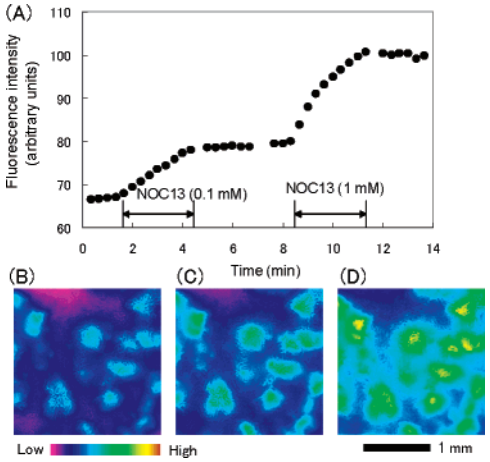

Figure 3. (A) Increase of the fluorescence intensity of DAC-P in rat kidney upon administration of NOC13. The fluorescence intensity is an averaged value calculated from an entire picture plane. The rat kidney was perfused with Krebs−Henseleit buffer at 5 mL/min. After the loading of DAC-P (5 μM) for 4 min, NOC13 (0.1 or 1 mM) was administered for 3 min (shown by arrows). (B) The captured NIR fluorescence image of a part of a rat kidney after the loading of DAC-P. (C) The image after the administration of NOC13 (0.1 mM). (D) The image after the administration of NOC13 (1 mM). All images are reproduced in pseudocolor.

rat kidneys. Because the reaction rate of DACs with NO is fast and the observation of their NIR fluorescence is less subject to interference by biological substances, our NO-detecting probes are expected to be applicable to not only cellular but also in vivo NO imaging, and work along this line is proceeding.

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Supporting Information Available: Full experimental procedures, synthesis, and characterization data for all compounds, spectral properties of DACs and DAC-Ts, pH profiles of DAC-S and DAC-S-T, a diagram of the perfused rat kidney system, and an NIR fluorescence image of a renal section. This material is available free of charge via the Internet at http://pubs.acs.org.

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