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Development of a Ratiometric Fluorescent Zinc Ion Probe in Near-Infrared Region, Based on Tricarbocyanine Chromophore

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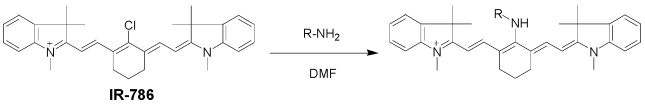
Cyanine dyes have been widely used in various fields and have been employed as fluorescent labels in fluorescence imaging studies of biological mechanisms. In particular, tricarbocyanine dyes have the advantage that light at their emission and absorption maxima in the near-infrared (NIR) region around 650−900 nm is relatively poorly absorbed by biomolecules, and so it can penetrate deeply into tissues. There is also less autofluorescence in this region, and so the characteristics of these dyes are favorable for in vivo imaging. In addition to the cyanine dyes for straightforward fluorescence labeling,[1−6] two types of tricarbocyanine dyes, whose fluorescence intensity changes upon specific reaction with biomolecules, have recently been developed. One is an NIR fluorescent probe for Ca^{2+} reported by Akkaya et al.[7] The other is a class of NIR fluorescent probes for nitric oxide (NO), DACs, developed by us, which can visualize NO in isolated rat kidneys.[8] Fluorescence modulation of DACs is controlled by photoinduced electron transfer (PeT), which causes a change of fluorescence intensity without affecting the emission and excitation wavelengths. Consequently, although DACs are useful as NO probes with off/on switching of fluorescence intensity in the NIR region, the fluorescence of these dyes tends to be influenced by the dye concentration, as well as by photobleaching and cellular environmental factors, such as pH and hydrophobicity. Moreover, the longer the wavelength of excitation, the more difficult it is to modulate fluorescence through the PeT mechanism because of the small value of ΔE_{00} in the Rehm−Weller equation.[9] To overcome these limitations, ratiometric fluorescent probes which exhibit a change of emission or excitation wavelength in the presence of biomolecules of interest are required. The utility of NIR fluorescence ratio imaging was demonstrated by Kircher et al.[10]

Peng et al. reported that amine-substituted tricarbocyanines have shorter wavelength of absorption, larger Stokes' shift, and stronger fluorescence intensity than nonsubstituted tricarbocyanines, owing to intramolecular charge transfer (ICT).[11] We synthesized a series of IR-786 derivatives in order to examine the relationship between the nature of the amine substituent and the photochemical properties (Scheme 1 and Table 1). We found that the lower the electron density of the amine, the longer the wavelength of the absorption maximum, with little change of the emission maximum. The results provide a rationale for the molecular design of novel ratiometric NIR probes, based on the difference in the electron-donating ability of the amine substituent before and after reaction with a biomolecule of interest.

Thus, we synthesized dipicolylcyanine (DIPCY, Scheme 2), having an amine-substituted tricarbocyanine as the NIR fluorophore. It has a high extinction coefficient of 7.0×10^4 M^{-1} cm^{-1} and a large Stokes' shift, and the dipicolylethylenediamine (DPEN) moiety

Scheme 1

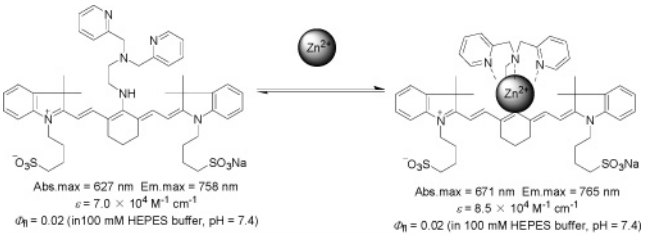


IR-786

Table 1. Correlation between the pK_a Values of Substituted Amines[12] and Photochemical Properties[13]

R	pK_a	abs$_{max}$ (nm)	em$_{max}$ (nm)	Φ_{fl}
hexyl	10.6	626	745	0.06
propyl	10.6	627	748	0.07
allyl	9.7	633	747	0.07
methoxyethyl	9.5	635	748	0.07
benzyl	9.4	647	744	0.07

Scheme 2



acts as a metal chelator. When the dipicolylamine coordinates to a metal ion, the electron density of the DPEN moiety will be decreased, lowering the electron-donating ability of the amine in the fluorophore. The IR-783 chromophore (Scheme S1), bearing highly water-soluble sulfonates, would be suitable as a mother structure for detection of metal ions in aqueous solution.

Zn^{2+} has a variety of essential physiological functions. Therefore, several groups have been making efforts to create rather long-wavelength fluorescent probes for Zn^{2+}.[14−18] However, no probes for Zn^{2+} in the NIR region have been developed. As shown in Figure 1, the reaction of DIPCY with Zn^{2+} produced a remarkable spectral change; the color of the solution changed from blue (Figure 1A) to teal (Figure 1B), and subsequent addition of an excess of *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a strong chelator of Zn^{2+}, resulted in recovery of the original color (Figure 1C). The formation of a 1:1 complex of DIPCY−Zn^{2+} was confirmed by ESI-MS spectrometry, and the absorption and excitation spectra of DIPCY in the presence of various concentrations of Zn^{2+} are shown in Figure 2A and 2B, respectively. At a sufficiently high concentration of Zn^{2+}, a 44 nm red shift of the absorption maximum was observed, which indicates that DIPCY could work as a ratiometric probe for Zn^{2+}.

The apparent dissociation constant (K_d = 98 ± 0.9 nM at pH 7.4 in HEPES buffer) for Zn^{2+} was determined by plotting the 760 nm fluorescence ratio for 627 and 671 nm excitation and was confirmed by plotting the absorption ratio at 627 and 671 nm

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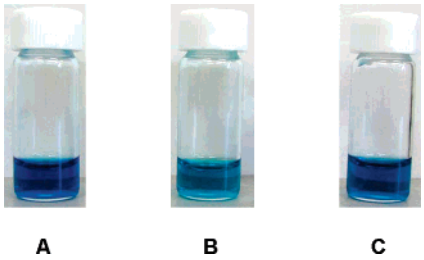

Figure 1. Color change of DIPCY in the visible region. (A) DIPCY solution in HEPES buffer: (B) and (A) +Zn^{2+}; (C) and (B) +excess of TPEN.

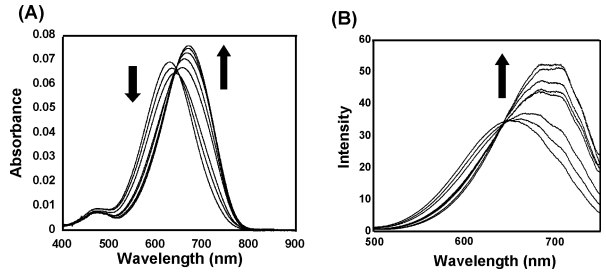

Figure 2. (A) Absorption spectra of 1 μM DIPCY at various Zn^{2+} concentrations (0, 0.01, 0.1, 0.8, 1.0, 2.0, 4.0, 10 μM) {in 100 mM HEPES buffer, pH = 7.4, I = 0.1 ($NaNO_3$)}. (B) Excitation spectra of 1 μM DIPCY at various Zn^{2+} concentrations (0, 0.01, 0.1, 0.8, 1.0, 2.0, 4.0, 10 μM) {in 100 mM HEPES buffer, pH = 7.4, I = 0.1 ($NaNO_3$)}. The emission was corrected at 760 nm.

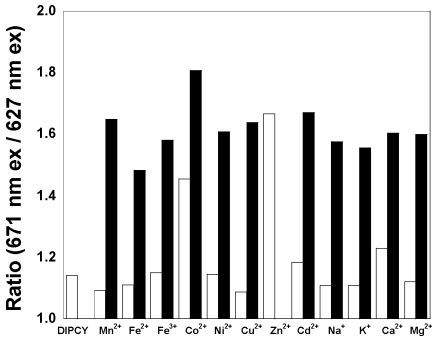

Figure 3. Metal ion selectivity of DIPCY. Bars indicate the fluorescence ratio (671/627 nm excitation, 760 nm emission). DIPCY (1 μM) was added to heavy metals (1 μM) and other agents (5 mM). All samples were measured in 100 mM HEPES buffer, pH = 7.4, I = 0.1 ($NaNO_3$). Colorless bars: each cation was added. Dark bars: each cation and zinc ion were added.

(Figure S1). The K_d values of ZnAFs with DPEN moieties, which were previously reported fluorescent Zn^{2+} probes,[19] are of sub-nanomolar order. The K_d value of DIPCY is rather high, in comparison to that of dipicolylamine (around 23 nM).[20] This is presumably due to the steric hindrance of the four methyl groups of the fluorophore. This K_d value suggests that DIPCY would be able to detect 10 nM to 1 μM Zn^{2+}, which is a suitable range for biological applications.[21]

Dipicolylamine is a chelator of Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Ag^{+}, Cd^{2+}, Hg^{2+}, and Pb^{2+}, in addition to Zn^{2+}. The metal selectivity of DIPCY is shown in Figure 3. We also found a fluorescence change of DIPCY with Co^{2+}. Upon addition of a 20-fold excess of Co^{2+}, a 39 nm red shift of the absorption maximum was observed. This property is interesting because Co^{2+} usually quenches fluorescence, like other heavy metals with partially filled d-shells. A 20-fold excess of Cu^{2+} produced a 62 nm red shift of the absorption maximum, while the fluorescence was completely quenched. The shifted absorption also gradually disappeared. Even if TPEN was added, the absorption did not recover, and the cyanine chromophore was decomposed. Namely, the Cu^{2+} complex with DIPCY is not stable. However, these free cations would have little influence in vivo since they exist at very low concentrations.[22]

In summary, we have successfully modified a tricarbocyanine to be a ratiometric fluorescent Zn^{2+} probe in the near-infrared region. This fluorescence modulation of amine-substituted tricarbocyanines should be applicable to dual-wavelength measurement of various biomolecules or enzyme activities. Studies along this line are in progress.

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Supporting Information Available: Full experimental procedures, characterization data for all compounds, and spectral properties of DIPCY. This material is available free of charge via the Internet at http://pubs.acs.org.

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