A Novel Fluorescent Probe for Zinc Ion Based on Boron Dipyrromethene (BODIPY) Chromophore

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ZnAB has the combined structure of *N*,*N*-bis(2-pyridylmethyl)ethylenediamine as a specific chelater for Zn^{2+} and 1,3,5,7-tetramethyl-8-phenyl-boron dipyrromethene as a fluorophore. Complexation of ZnAB with Zn^{2+} produces a remarkable enhancement of fluorescence intensity. ZnAB has the advantages of less sensitivity to solvent polarity and pH than fluorescein-based Zn^{2+} probes. Furthermore, it is not influenced by other cations, such as Na^+, K^+, Ca^{2+}, and Mg^{2+}, which exist at high concentrations under physiological conditions, even at 2.5 mM. The results show that ZnAB is a Zn^{2+} probe suitable for biological applications.

Key words zinc; fluorescence; boron dipyrromethene (BODIPY); photoinduced electron transfer; *N*,*N*-bis(2-pyridylmethyl)ethylenediamine

Zinc ion (Zn^{2+}) is found in every cell in the human body, and is the second most abundant heavy metal ion after iron. It is an essential component of many enzymes and transcription factors (*e.g.*, carbonic anhydrase, zinc finger proteins, *etc.*).[1] In addition to this protein-bound Zn^{2+}, chelatable Zn^{2+} is present, especially in the brain,[2] pancreas[3] and spermatozoa.[4] Certain neurons in the brain contain a relatively large pool of free Zn^{2+} sequestered in vesicles in their terminals. Such Zn^{2+} is released from nerve terminals by excitatory signals, and modulates the function of glutamate receptors. In the pancreas, Zn^{2+} is co-stored with insulin in secretary vesicles of pancreatic β-cells, and is released when insulin is secreted.[5] Zn^{2+} also suppresses apoptosis,[6] and induces the formation of β-amyloid,[7] which may be related to the etiology of Alzheimer's disease.

Although Zn^{2+} plays many physiologically important roles, the mechanisms involved are still poorly understood. Therefore, several chemical tools for measuring Zn^{2+} in living cells have been developed.[8—16] Fluorescent probes based on quinoline, fluorescein, other fluorophores or proteins, have been reported. Some of these probes can be used to monitor the change of Zn^{2+} concentration under physiological conditions, but they suffer from problems such as inadequate selectivity, insufficient sensitivity, and pH-sensitivity. We have already developed fluorescein-based probes, ZnAFs,[17,18] which have high selectivity and sensitivity, and ZnAF-Rs,[19] whose structure is based on benzofuran derivatives, and which enable ratiometric measurement. Here we report the design and synthesis of a new fluorescent probe for Zn^{2+} based on the BODIPY (boron dipyrromethene) chromophore. BODIPY has a high molar extinction coefficient and fluorescence quantum yield. Furthermore, it has the advantages of less sensitivity to solvent polarity and pH than fluorescein-based Zn^{2+} probes, and its structure can be modified to change its excitation and emission wavelengths.

Results and Discussion

Probe Design Based on Photoinduced Electron Transfer Our group has developed fluorescein-based probes for nitric oxide (DAFs),[20,21] for singlet oxygen (DPAXs, DMAXs),[22,23] and for Zn^{2+} (ZnAFs). As a basis for the design of these probes, we have utilized the photoinduced electron transfer (PeT) between the xanthene ring, which is an electron acceptor and fluorophore, and the benzoic acid moiety, which is an electron donor, and the probes exhibit fluorescence off/on switching that is dependent on the highest occupied molecular orbital (HOMO) level of the benzoic acid moiety. Amino- or oxygen-substituted BODIPYs have already been reported as pH or alkali metal and alkaline earth metal sensors.[24—27] However, BODIPY-based functional probes have not yet been developed for biological applications.

The fluorescence property of 1,3,5,7-tetramethyl-8-phenyl-BODIPY is thought to be controlled by electron transfer between the phenyl ring at the 8-position and 1,3,5,7-tetramethyl-8-phenyl-BODIPY (Chart 1a), because the dihedral angle between the benzene ring and BODIPY is almost 90° (Chart 1b), as in the case of fluorescein. The free energy change of the PeT process can be described by the Rehm–Weller equation,[28]

$$\Delta G_{PeT} = E_{1/2}(D^+/D) - E_{1/2}(A/A^-) - \Delta E_{00} - C$$

where $E_{1/2}(D^+/D)$ and $E_{1/2}(A/A^-)$ are the ground-state oxidation potential of the donor and the reduction potential of the acceptor, respectively, ΔE_{00} is the excitation energy, and C is the electrostatic interaction term. Since the reduction potential and the excitation energy of 1,3,5,7-tetramethyl-BODIPY (BODIPY 505/515; absorption maximum: 501 nm, reduction potential: -1.16 V *vs.* SCE) were nearly the same as those of fluorescein,[29] the threshold of fluorescence off/on in BODIPY was expected to be similar to that in fluorescein derivatives.

Thus, we designed ZnAB (Chart 2). *N*,*N*-Bis(2-pyridylmethyl)ethylenediamine, which is used as an acceptor for Zn^{2+} of ZnAFs, is directly attached to the benzene ring of 1,3,5,7-tetramethyl-8-phenyl-BODIPY. In the absence of metal cations, its fluorescence intensity should be weak because of fluorescence quenching by PeT, and the chelation of

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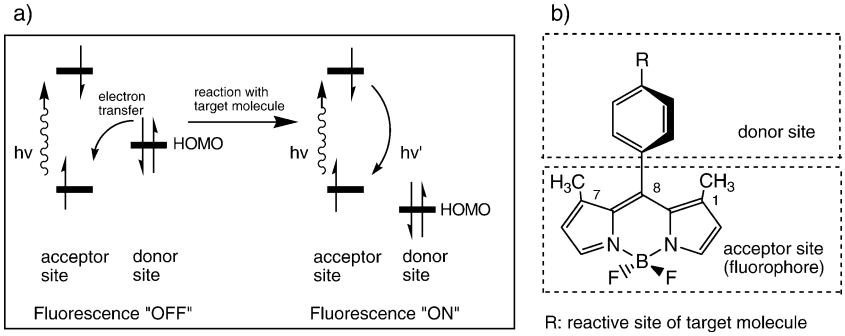

Chart 1. (a) Mechanism of Fluorescence OFF/ON Switching by Photoinduced Electron Transfer, (b) Design of Chemical Probe Based on Boron Dipyrromethene (BODIPY)

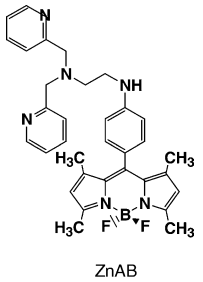

Chart 2. Chemical Structure of ZnAB

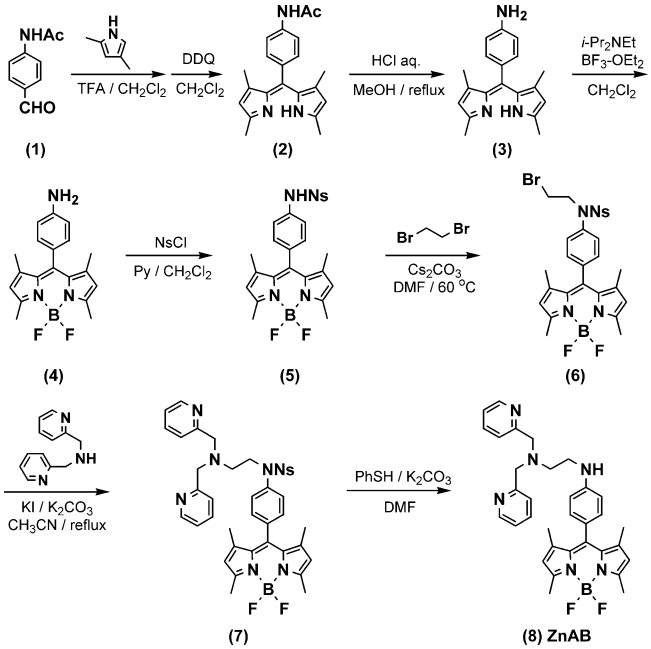

Chart 3. Synthetic Route to Compound (**8**): ZnAB

TFA: trifluoroacetic acid, Ns: 4-nitrobenzenesulfonyl, DDQ: 2,3-dichloro-5,6-dicyano-*p*-benzoquinone.

Zn^{2+} should induce a sufficient change of the HOMO level of the chelator-substituted benzene ring, so that the fluorescence intensity is increased because of the hindrance of PeT.

Synthesis of ZnAB The synthetic scheme for ZnAB is shown in Chart 3. Aminobodipy (**4**) was synthesized as previously reported,[30] and its amino group was protected with 4-nitrobenzensulfonyl group. Reaction with 1,2-dibromoethane, followed by 2,2′-dipycolylamine afforded compound

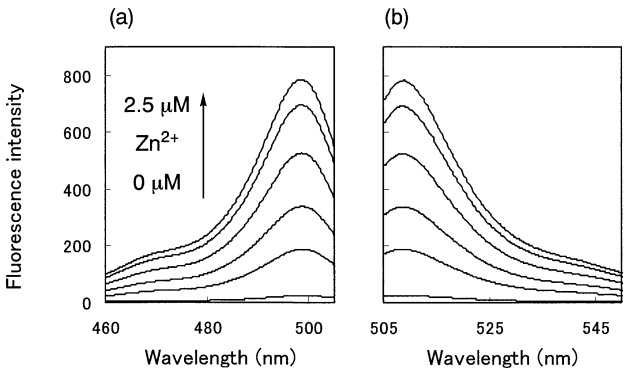

Fig. 1. (a) Excitation Spectra (Emission at 509 nm) and (b) Emission Spectra (Excitation at 499 nm) of ZnAB (2.5 μM) in the Presence of Various Concentrations of Zn^{2+} Ranging from 0 to 2.5 μM

These spectra were measured at pH 7.4 (CH_3CN : 100 mM HEPES buffer (I=0.1($NaNO_3$))=1 : 1).

(**7**). The 4-nitrobenzensulfonyl group of compound (**7**) was deprotected by treatment with PhSH and K_2CO_3 in DMF (*N*,*N*-dimethyformamide) to yield ZnAB, compound (**8**).

Fluorescence Properties of ZnAB with or without Zn^{2+} Under neutral conditions (pH 7.4), the final compound (**8**) (ZnAB) showed almost no fluorescence, and the fluorescence quantum yield was determined to be only 0.003. Upon addition of Zn^{2+}, the fluorescence intensity was increased by 30-fold (Fig. 1), and the fluorescence quantum yield was increased to 0.058. The wavelengths of the excitation and emission maxima were almost unchanged by Zn^{2+}: excitation at 499 nm and emission at 509 nm (Fig. 1).

Metal Ion Selectivity Metal ion selectivity was also examined. ZnAB was not influenced by other cations, such as Na^+, K^+, Ca^{2+}, and Mg^{2+}, which exist at high concentrations under physiological conditions, even at 2.5 mM, as shown in Fig. 2. These results are presumably due to the poor complexation of alkali metals or alkaline earth metals with the chelator of ZnAB. These cations also did not interfere with the Zn^{2+}-induced fluorescence enhancement. Among first-row transition metal cations, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} induced a slight enhancement of the fluorescence intensity. Although ZnAB probably forms complexes with these metal cations, the fluorescence is weakened because of electron or energy transfer between the metal cations and the fluorophore, a known fluorescence quenching mechanism.[31,32]

Kinetic Analysis of the Complex Formation of Zn^{2+} Upon addition of various concentrations of Zn^{2+}, the fluores-

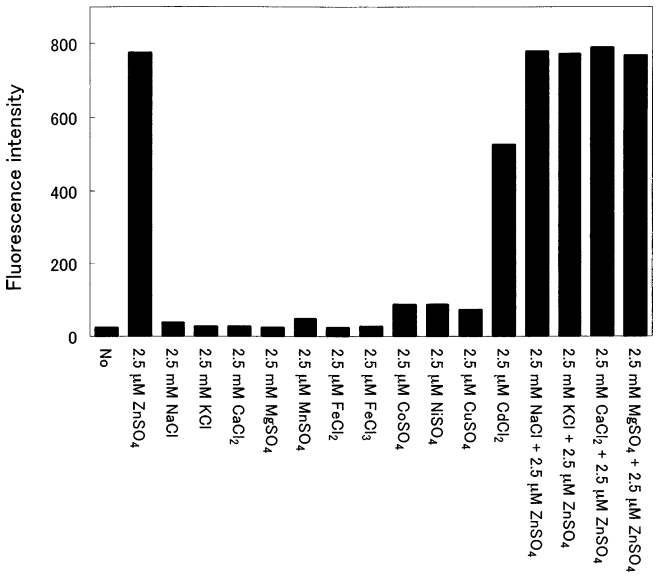

Fig. 2. The Relative Fluorescence Intensity of 2.5 μM ZnAB in the Presence of Various Cations

These data were measured at pH 7.4 (CH_3CN : 100 mM HEPES buffer (I= 0.1($NaNO_3$))=1 : 1).

Fig. 3. Job's Plot for Zn^{2+} and ZnAB which Forms 1 : 1 Complex

The total $[Zn^{2+}]+[ZnAB]=5.0\ \mu$M.

cence intensity of ZnAB (2.5 μM) linearly increased up to a 1 : 1 [ZnAB]/[Zn^{2+}] ratio, and the fluorescence and absorption spectra did not change between 2.5 and 50 μM Zn^{2+} addition. Furthermore, a Job's plot analysis revealed maximum fluorescence obtained at a 1 : 1 ratio (Fig. 3). Next, we measured the association (k_{on}) rate constant of ZnAB at 25 °C, using a stopped-flow spectrofluorimeter. The k_{on} value of ZnAB was $2.43\times10^5\ M^{-1}\ s^{-1}$, implying that the complexation is sufficiently fast to detect an increase of Zn^{2+} concentration within a few hundred milliseconds.

The Effect of pH on the Fluorescence Intensity The fluorescence intensities (arbitrary unit) of the Zn^{2+} complex with ZnAB were 509.8, 490.6 and 468.1 at pH 5.48, 7.14 and 8.49, respectively. The fluorescence intensity of the Zn^{2+} complex with ZnAF-2, which is a fluorescein-based Zn^{2+} probe, extremely decreases below pH 7.0, whereas that of ZnAB hardly changed above pH 5.0. This insensitivity to pH of the fluorescence is useful for applications to living cells, where pH changes are caused by certain biological stimuli.

In conclusion, we have developed a new fluorescent Zn^{2+} sensor molecule, ZnAB, which possesses high selectivity under physiological-like conditions. In addition, ZnAB should be useful as a prototype of other BODIPY-based fluorescent probes for Zn^{2+}, whose absorption and emission maxima are shifted to longer wavelength by chemical modification.[33—36] Such derivatization would enable multicolor imaging, which can distinguish many compounds simultaneously owing to the differences of excitation or emission wavelength, and should be applicable to complex biological systems.

Experimental

All reagents and solvents were of the highest commercial quality and were used without further purification. Silica gel column chromatography was performed using Silica gel 60 (Merck). Aluminum oxide column chromatography was performed using Aluminum oxide 90 active neutral (Merck). Aluminum oxide TLC was performed using Aluminium oxide 60 F_{254} (Merck). ^{1}H-NMR spectra were recorded on a JEOL JNM-GX400 instrument at 400 MHz; δ values are given in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with a JEOL MS700 mass spectrometer. Fluorescence spectroscopic studies were performed with a Shimadzu RF-5300. The slit width was 3.0 nm for both excitation and emission.

Synthesis of ZnAB (Chart 3). Preparation of Compound (2) To a solution of 4-acetamidebenzaldehyde (**1**) (10.0 g, 61.3 mmol) and 2,4-dimethylpyrrole (12.6 ml, 122.6 mmol) in 1000 ml of dichloromethane was added 100 μl of trifluoroacetic acid under an argon atmosphere, and the mixture was stirred for 4 h at room temperature. Then, a solution of DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) (13.6 g, 61.3 mmol) in 25 ml of THF and 25 ml of dichloromethane was added dropwise to the mixture over 30 min. Stirring was continued at room temperature for 1 h, then the mixture was washed with water. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was chromatographed on aluminium oxide and eluted with acetone to afford compound (**2**) (4.54 g, 13.6 mmol). (yield 22%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.34 (6H, s), 2.21 (3H, s), 2.35 (6H, s), 5.89 (2H, s), 7.24 (2H, d, J=8.8 Hz), 7.61 (2H, d, J=8.8 Hz). MS (FAB$^+$ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 334 ((M+H)$^+$).

Preparation of Compound (3) To a solution of compound (**2**) (3.77 g, 11.3 mmol) in 150 ml of methanol was added 150 ml of 1 N hydrochloric acid. The mixture was refluxed for 3 h, then cooled to room temperature, and extracted with dichloromethane. The organic phase was washed with water. The organic phase was dried over sodium sulfate and evaporated *in vacuo* to give compound (**3**) as a brown solid (3.30 g, 11.3 mmol). (yield 100%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.45 (6H, s), 2.36 (6H, s), 3.80 (2H, br), 5.91 (2H, s), 6.74 (2H, d, J=8.4 Hz), 7.05 (2H, d, J=8.4 Hz). MS (FAB$^+$ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 292 ((M+H)$^+$).

Preparation of Compound (4) To a solution of compound (**3**) (3.27 g, 11.2 mmol) in 500 ml of dichloromethane was added diisopropylethylamine (26.5 ml, 152 mmol) under an argon atmosphere, and the mixture was stirred at room temperature for 15 min. Furthermore, BF_3–OEt_2 (28 ml, 211 mmol) was added, and the mixture was stirred for 40 min. The mixture was washed with water and 2 N NaOH aq., and the aqueous phase was extracted with dichloromethane. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was chromatographed on silica gel and eluted with dichloromethane to afford compound (**4**) (3.01 g, 8.87 mmol). (yield 79%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.49 (6H, s), 2.54 (6H, s), 3.94 (2H, br), 5.96 (2H. s), 6.79 (2H, dd, J=6.4, 1.6 Hz), 7.02 (2H, dd, J=6.4, 1.6 Hz). MS (FAB$^+$ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 339 (M$^+$).

Preparation of Compound (5) To a solution of compound (**4**) (1.00 g, 2.95 mmol) and pyridine (0.48 ml, 5.90 mmol) in 20 ml of dichloromethane was added dropwise a solution of 4-nitrobenzenesulfonyl chloride (980.7 mg, 4.43 mmol) in 35 ml of dichloromethane over 10 min. The mixture was stirred overnight, then washed with water and brine, and dried over sodium sulfate. After evaporation of the dichloromethane, the residue was chromatographed on silica gel and eluted with dichloromethane to afford compound (**5**) (835 mg, 1.59 mmol). (yield 54%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.18 (6H, s), 2.42 (6H, s), 6.14 (2H, s), 7.25 (4H, m), 7.96 (2H, dd, J=8.8, 2.0 Hz), 8.34 (2H, dd, J=8.8, 2.0 Hz). MS (FAB$^+$ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 524 (M$^+$).

Preparation of Compound (6) A mixture of compound (**5**) (604 mg, 1.15 mmol), cesium carbonate (450 mg, 1.38 mmol), 1,2-dibromoethane (1.00 ml, 11.5 mmol), and 20 ml of *N*,*N*-dimethylformamide was stirred at 80 °C for 2 h. After cooling to room temperature, the mixture was diluted with dichloromethane and washed with water and brine. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was

chromatographed on silica gel and eluted with dichloromethane to afford compound (**6**) (520 mg, 0.824 mmol). (yield 72%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.40 (6H, s) , 2.56 (6H, s), 3.45 (2H, t, J=6.8 Hz), 4.07 (2H, t, J=6.8 Hz), 6.02 (2H, s), 7.26 (2H, d, J=8.4 Hz), 7.33 (2H, d, J=8.4 Hz), 7.78 (2H, d, J=8.8 Hz), 8.30 (2H, d, J=8.8 Hz). MS (FAB^+ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 630, 632 (M^+).

Preparation of Compound (7) A suspension of compound (**6**) (0.61 g, 0.966 mmol), 2,2′-dipicolylamine (0.52 ml, 2.90 mmol), potassium carbonate (0.33 g, 2.39 mmol), potassium iodide (0.41 g, 2.47 mmol) in 20 ml of acetonitrile was refluxed for 14 h. Acetonitrile was removed by evaporation, and the residue was diluted with 1 M potassium carbonate and extrated with dichloromethane. The organic phase was dried over sodium sulfate and evaporated to dryness. The crude product was chromatographed on silica gel (eluent: dichloromethane, dichloromethane–methanol 99.5 : 0.5 (v/v), 98 : 2 (v/v)) to afford compound (**7**) (0.19 g, 0.253 mmol). (yield 26%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.28 (6H, s), 2.55 (6H, s), 2.77 (2H, t, J=6.8 Hz), 3.88 (4H, s), 3.90 (2H, t, J=6.8 Hz), 5.99 (2H, s), 7.09 (2H, d, J=8.0 Hz), 7.18 (2H, d, J=8.0 Hz), 7.19 (2H, m), 7.54 (2H, d, J=8.0 Hz), 7.70 (2H, m), 7.71 (2H, d, J=8.8 Hz), 8.26 (2H, d, J=8.8 Hz), 8.52 (2H, d, J=4.8 Hz). MS (FAB^+ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 750 ($(M+H)^+$)

Preparation of ZnAB, Compound (8). 8-[4-*N*-[*N*′,*N*′-Bis(2-pyridinylmethyl)-2-aminoethyl]aminophenyl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene To a suspension of compound (**7**) (66.6 mg, 0.0888 mmol) and potassium carbonate (61.4 mg, 0.444 mmol) in 5 ml of *N*,*N*-dimethylformamide was added thiophenol (30 μl, 0.293 mmol), and the mixture was stirred for 3 h at room temperature. *N*,*N*-Dimethylformamide was removed by evaporation, and the residue was diluted with dichloromethane, washed with water, and dried over sodium sulfate. The residue was purified by aluminum oxide TLC with dichloromethane to give a brown solid. The brown solid was dissolved in a small amount of diethyl ether and reprecipitated with hexane to afford ZnAB, compound (**8**) (13.5 mg, 0.0239 mmol) (yield 27%) ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.49 (6H, s), 2.54 (6H, s), 2.93 (2H, t, J=5.9 Hz), 3.17 (2H, t, J=5.9 Hz), 3.94 (4H, s), 5.96 (2H, s), 6.65 (2H, d, J=8.8 Hz), 6.97 (2H, d, J=8.8 Hz), 7.17 (2H, ddd, J=7.8, 4.9, 2.0 Hz), 7.44 (2H, d, J=7.3 Hz), 7.64 (2H, td, J=7.8, 7.3, 2.0 Hz), 8.58 (2H, m). MS (FAB^+ in *m*-nitrobenzyl alcohol as a matrix): *m*/*z* 565 ($(M+H)^+$).

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