J|A|C|S
ARTICLES
Published on Web 04/11/2007

Design and Synthesis of a Library of BODIPY-Based Environmental Polarity Sensors Utilizing Photoinduced Electron-Transfer-Controlled Fluorescence ON/OFF Switching

Hisato Sunahara,[†,§] Yasuteru Urano,[†,‡] Hirotatsu Kojima,[†,§] and Tetsuo Nagano*,[†,§]

Contribution from the Graduate School of Pharmaceutical Sciences, the University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, CREST, JST, and PRESTO, JST, Honcho, Kawaguchi, Saitama 332-0013, Japan

Received November 28, 2006; E-mail: ff47015@mail.ecc.u-tokyo.ac.jp

Abstract: We systematically examined the mechanism of the solvent polarity dependence of the fluorescence ON/OFF threshold of the BODIPY (boron dipyrromethene) fluorophore and the role of photoinduced electron transfer (PeT). In a series of BODIPY derivatives with variously substituted benzene moieties at the 8-position, the oxidation potential of the benzene moiety became more positive and the reduction potential of the BODIPY fluorophore became more negative as the solvent polarity was decreased; consequently, the free energy change of PeT from the benzene moiety becomes larger in a more nonpolar environment. Utilizing this finding, we designed and synthesized a library of probes in which the threshold of fluorescence ON/OFF switching corresponds to different levels of solvent polarity. These environment-sensitive probes were used to examine bovine serum albumin (BSA) and living cells. The polarity at the surface of albumin was concluded to be similar to that of acetone, while the polarity of the internal membranes of HeLa cells was similar to that of dichloromethane.

Introduction

Environment-sensitive fluorescence probes are functional molecules whose fluorescence properties are dependent upon the hydrophobicity of the environments, and they are widely used in biochemical research.[1−6] These probes generally exhibit a low fluorescence quantum yield in aqueous solution but become highly fluorescent in nonpolar solution (e.g., 8-anilino-1-naphthalenesulfonic acid (ANS) and thioflavin T) or exhibit emission at longer wavelength in aqueous solution but at shorter wavelength in nonpolar solution (e.g., Prodan, Nile red).[7] However, the mechanism of the fluorescence changes is not well established, so it is difficult to develop new probes having different color and fluorescence ON/OFF threshold.

Photoinduced electron transfer (PeT) is a well-known mechanism through which the fluorescence of a fluorophore is quenched by electron transfer from the donor moiety to the acceptor fluorophore.[8] Recently, we have established rational design strategies for fluorescence probes based on the concept of PeT.[9−11] These findings allow us to design new fluorescence probes for various target analytes. The donor moiety of these fluorescence probes is structurally altered by the reaction or by binding with target molecules, resulting in a marked change of fluorescence properties.

Here we report a novel environment-sensitive BODIPY (boron dipyrromethene) probe library in which the fluorescence properties are well controlled by the PeT mechanism. Daub and co-workers[12−16] have described the solvent-dependent change of the optical properties of some BODIPY derivatives. They focused on the charge-transfer emission, which depends on solvent polarity, but the precise relationship between PeT-based quenching of fluorescence and the polarity of the solvent has not been established. We examined the mechanism of the solvent polarity-dependent changes in the fluorescence ON/OFF thresh-

† University of Tokyo.
‡ CREST, JST.
§ PRESTO, JST.

(1) Yeh, R. H.; Yan, X. W.; Cammer, M.; Bresnick, A. R.; Lawrence, D. S. *J. Biol. Chem.* **2002**, *277*, 11527−11532.
(2) Parasassi, T.; Krasnowska, E. K.; Bagatolli, L.; Gratton, E. *J. Fluoresc.* **1998**, *8*, 365−373.
(3) Saito, Y.; Miyauchi, Y.; Okamoto, A.; Saito, I. *Tetrahedron Lett.* **2004**, *45*, 7827−7831.
(4) Toutchkine, A.; Kraynov, V.; Hahn, K. *J. Am. Chem. Soc.* **2003**, *125*, 4132−4145.
(5) Ban, T.; Hamada, D.; Hasegawa, K.; Naiki, H.; Goto, Y. *J. Biol. Chem.* **2003**, *278*, 16462−16465.
(6) Ali, V.; Prakash, K.; Kulkarni, S.; Ahmad, A.; Madhusudan, K. P.; Bhakuni, V. *Biochemistry* **1999**, *38*, 13635−13642.
(7) Grabowski, Z. R.; Rotkiewicz, K.; Rettig, W. *Chem. Rev.* **2003**, *103*, 3899−4031.
(8) deSilva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566.
(9) Tanaka, K.; Miura, T.; Umezawa, N.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2001**, *123*, 2530−2536.
(10) Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, *125*, 8666−8671.
(11) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888−4894.
(12) Kollmannsberger, M.; Rurack, K.; Resch-Genger, U.; Daub, J. *J. Phys. Chem. A* **1998**, *102*, 10211−10220.
(13) Kollmannsberger, M.; Rurack, K.; Resch-Genger, U.; Rettig, W.; Daub, J. *Chem. Phys. Lett.* **2000**, *329*, 363−369.
(14) Rurack, K.; Kollmannsberger, M.; Resch-Genger, U.; Daub, J. *J. Am. Chem. Soc.* **2000**, *122*, 968−969.
(15) Rurack, K.; Kollmannsberger, M.; Daub, J. *Angew. Chem., Int. Ed.* **2001**, *40*, 385−387.
(16) Rurack, K.; Kollmannsberger, M.; Daub, J. *New J. Chem.* **2001**, *25*, 289−292.

10.1021/ja068551y CCC: $37.00 © 2007 American Chemical Society

old of the BODIPY fluorophore thermodynamically, and the results were applied to establish a library of environment-sensitive fluorescence probes, which we employed to estimate the polarity at the surface of a protein and at the membranes in living cells.

Experimental Section

Materials. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, or Aldrich Chemical Co., and were used without further purification. Special chemicals were tetrabutylammonium perchlorate (TBAP, electrochemical grade, Fluka). Bovine serum albumin (BSA, product no. A-7511, Sigma-Aldrich) was used without further purification. Organelle markers were from Molecular Probes. All the solvents employed were of spectrometric grade.

General Procedure for the Synthesis of TetramethylBODIPY Derivatives. An appropriate aldehyde (2 mmol) and 2,4-dimethylpyrrole (4 mmol) were dissolved in 250 mL of absolute CH_2Cl_2 under an Ar atmosphere. One drop of trifluoroacetic acid (TFA) was added, and the solution was stirred at room temperature overnight. When thin-layer chromatography (TLC) monitoring (silica; CH_2Cl_2) showed complete consumption of the aldehyde, a solution of 2 mmol of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in CH_2Cl_2 was added, and stirring was continued for 15 (or 20) min. The reaction mixture was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was roughly purified by column chromatography on aluminum oxide (CH_2Cl_2) to afford a brown-orange solid.

The solid and 4 mL of *N*,*N*-diisopropylethylamine (DIEA) were dissolved in 150 mL of absolute CH_2Cl_2 (or anhydrous toluene) under an Ar atmosphere, and the solution was stirred at room temperature for 5 min. Then 4 mL of boron trifluoride etherate (BF_3-OEt_2) was added, and stirring was continued for 30 min. The reaction mixture was washed with water and dried over anhydrous $MgSO_4$, filtered, and evaporated. The crude compound was purified by silica gel chromatography (CH_2Cl_2/MeOH or CH_2Cl_2/hexane) to afford an analytically pure sample.

Synthesis of 2-Methoxy-3-methylbenzaldehyde. A solution of 2-hydroxy-3-methylbenzaldehyde (1.10 g, 8.05 mmol) and cesium carbonate (8.00 g, 24.6 mmol) in dimethylformamide (DMF) (15 mL) was treated with iodomethane (3.47 g, 24.4 mmol). The mixture was stirred at room temperature for 10 h, water was added, and the reaction mixture was extracted with AcOEt (3 × 30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford a colorless oil (667 mg, yield 55%).

Synthesis of 10-Phenyl-9-anthracenecarboxyaldehyde. This was synthesized as previously reported.[17]

General Procedure for the Synthesis of AminoBODIPY Derivatives from NitroBODIPY Derivatives. Nitro derivatives were dissolved in MeOH. After the addition of 10% palladium−carbon (Pd−C), the mixture was stirred vigorously under a H_2 atmosphere. When TLC monitoring (silica) showed complete consumption of the starting material, the Pd−C was filtered off and washed with MeOH. The residue after evaporation of the filtrate was purified by column chromatography over silica gel (CH_2Cl_2/MeOH) to afford a violet powder.

Synthesis of 4,4-Difluoro-2,6-diacetyl-8-(2-methoxy-5-nitrophenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene. 2-Methoxy-5-nitrobenzaldehyde (312 mg, 1.72 mmol) and 3-acetyl-2,4-dimethylpyrrole (548 mg, 4.00 mmol) were dissolved in 250 mL of absolute CH_2Cl_2 under an Ar atmosphere. Two drops of TFA were added, and the solution was stirred at room temperature overnight. When TLC monitoring (silica; CH_2Cl_2) showed complete consumption of the aldehyde, a solution of 5.1 mmol of DDQ was added, and stirring was continued overnight. The reaction mixture was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was roughly purified by column chromatography on aluminum oxide (CH_2Cl_2) to afford a brown powder.

The brown powder (129 mg, 0.42 mmol) and DIEA (5.0 mL, 5.7 mmol) were dissolved in 20 mL of absolute CH_2Cl_2 (or anhydrous toluene) under an Ar atmosphere, and the solution was stirred at room temperature for 5 min. BF_3-OEt_2 (8.0 mL, 7.9 mmol) was added, and stirring was continued for 3 h. The reaction mixture was washed with water. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated. The crude compound was purified by silica gel chromatography (CH_2Cl_2/MeOH, 10:3) to afford an orange powder (90 mg, yield 9.3%).

Synthesis of 4,4-Difluoro-2,6-diacetyl-8-(5-amino-2-methoxyphenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene. Compound **13** (39 mg, 0.08 mmol) was dissolved in 10 mL of MeOH. After the addition of 10% Pd−C (8 mg), the mixture was stirred vigorously under a H_2 atmosphere. When TLC monitoring (silica; CH_2Cl_2/MeOH, 20:1) showed complete consumption of **13**, the Pd−C was filtered off and washed with MeOH. The residue after evaporation of the filtrate was purified by column chromatography over silica gel (CH_2Cl_2/MeOH, 20:1) to afford a violet powder (recrystallized from EtOH to afford orange needles) (14 mg, 0.03 mmol, yield 38%).

General Instruments. NMR spectra were recorded on a JNM-LA300 (JEOL) instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Mass spectra (MS) were measured with a JMS-DX300 (JEOL) for EI and a JMS-T100LC AccuTOF (JEOL) for ESI.

Cyclic Voltammetry. Cyclic voltammetry was performed on a 600A electrochemical analyzer (ALS). A three-electrode arrangement in a single cell was used for the measurement: a Pt wire as the auxiliary electrode, a Pt electrode as the working electrode, and a Ag/Ag^+ electrode as the reference electrode. The sample solution contained 0.1 M TBAP as a supporting electrolyte in the solvent, and argon was bubbled for 2 min before each measurement. Obtained potentials (Ag/Ag^+) were converted to those versus SCE by adding 0.25 V.

Fluorescence Properties and Quantum Yield of Fluorescence. Steady-state fluorescence spectroscopic studies were performed on an F 4500 (Hitachi). The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V. UV−vis spectra were obtained on a UV-1600 and a UV-1650PC (Shimazu). Each solution contained up to 1.0% (v/v) DMSO or $CHCl_3$ as a cosolvent (see the Supporting Information). The relative quantum efficiencies of fluorescence of BODIPY derivatives were obtained by comparing the area under the corrected emission spectrum of the test sample with that of a solution of fluorescein excited at 490 nm in 0.1 N NaOH, which has a quantum efficiency of 0.85 according to the literature.[18] The quantum efficiencies of fluorescence (Φ_{fl}) were obtained with the following equation (F denotes the area under the fluorescence band ($F = \Sigma I_{fl}(\lambda)$, where $I_{fl}(\lambda)$ is the fluorescence intensity at each emission wavelength), Abs denotes the absorbance at the excitation wavelength, and n denotes the refractive index of the solvent)

$$\Phi_{fl}^{sample} = \Phi_{fl}^{standard} \times (F^{sample}/F^{standard}) \times (n^{sample}/n^{standard})^2 \times (Abs^{standard}/Abs^{sample})$$

Computational Methods. All structures were computed using hybrid density functional theory (B3LYP) with the 6-31G* basis set as implemented in Gaussian 98W. Several starting geometries were used for the geometry optimization to ensure that the optimized structure corresponds to a global minimum.

Preparation of Cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA),

(17) Soutar, I.; Swanson, L.; Guillet, J. E.; Takahashi, Y. *Macromolecules* **1991**, *24*, 2815−2821.

(18) Parker, C. A.; Rees, W. T. *Analyst* **1960**, *85*, 587−600.

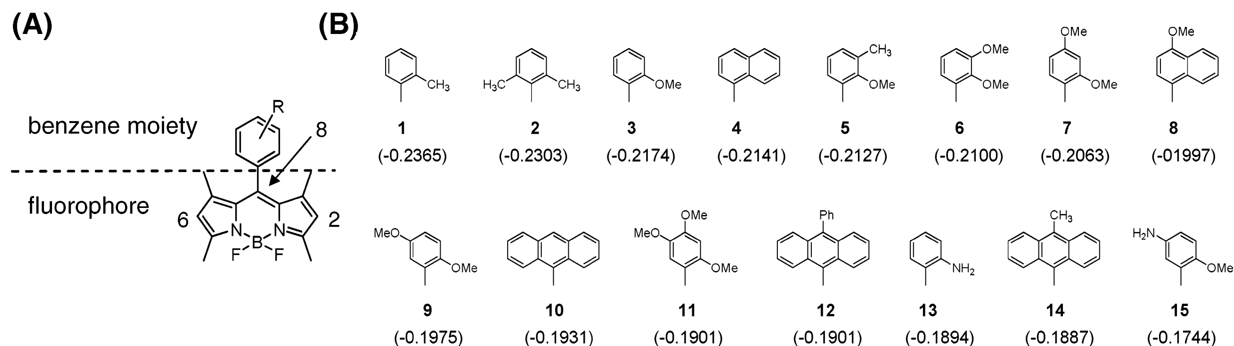


Figure 1. Structures of compounds used in this study: (A) BODIPY structure divided into two parts, the benzene moiety and the fluorophore; (B) structures of the benzene moiety at the 8-position of the BODIPY fluorophore. These are arranged according to HOMO energy level from 1 to 15.

supplemented with 10% fetal bovine serum (Invitrogen Corp.), 1% penicillin, and 1% streptomycin (Invitrogen Corp.) at 37 °C in a 5% CO_2/95% air incubator. The cells were grown on an uncoated 35 mm diameter glass-bottomed dish (MatTek, Ashland, MA), and washed twice with Hanks' balanced salt solution (HBSS) buffer (Invitrogen Corp.). The medium was replaced with HBSS buffer containing 50 nM BODIPY derivative dissolved in DMSO before imaging.

Confocal Laser Scanning Microscopy. Confocal imaging was performed on an Olympus Fluoview 500 confocal microscope. BODIPY derivatives were excited with the 488 nm line of an argon laser, and emission was detected over a range of 515−530 nm. For BODIPY derivative and organelle marker double detection, the samples were sequentially excited at 488 and 543 nm with argon lasers. The emission signals from BODIPY derivatives and organelle markers were detected over a range of 515−530 nm and over a range of 560−600 nm, respectively, to minimize overlap between the two signals.

Results and Discussion

1. Design and Synthesis of Pilot Compounds. The boron dipyrromethene (BODIPY) fluorophore (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) (Figure 1A) has a high extinction coefficient (>80 000 cm^{-1} M^{-1}), a high fluorescence quantum yield (often approaching 1.0, even in water), and is relatively insensitive to solvent polarity and pH.[19] Therefore, BODIPY derivatives are used as fluorescence tags and probes which function in water[20] or organic solvent.[21] Conceptually, the structure of BODIPY derivatives can be divided into two parts, i.e., the benzene moiety and BODIPY moiety, since they are twisted and conjugatively uncoupled.[22,23]

To systematically investigate the PeT-based fluorescence quenching of BODIPY derivatives in different environments, we synthesized various BODIPY derivatives in which the highest occupied molecular orbital (HOMO) energy levels of the benzene moiety at the 8-position were tuned over a wide range (Figure 1B). A previous report[24,25] suggested that the molecular rigidity arising from the introduction of methyl groups at the ortho positions of aryl-substituted BODIPYs minimizes the decrease of fluorescence intensity due to the nonradiative pathway. Thus, the BODIPY derivatives employed in this study commonly contain ortho substituents to block the nonradiative pathway and maintain a high fluorescence quantum yield.

2. Spectroscopic Properties of the BODIPY Derivatives. The absorbance and fluorescence emission spectra of the BODIPY derivatives were measured in various solvents. The HOMO energy levels of the benzene moieties, the absorbance and fluorescence properties, and the relative quantum yields of fluorescence (Φ_{fl}) in various solvents are summarized in Table 1. Figure 2, parts A and B, shows that the absorption and fluorescence spectra remained almost identical when the benzene moiety at the 8-position was changed even from benzene to naphthalene or anthracene, which suggests that there is no significant electronic coupling between the donor and fluorophore in the ground-state configuration. Figure 2, parts C and D, shows absorption and fluorescence spectra of 8-phenyl-BODIPY measured in various solvents. Other derivatives also showed almost identical absorption and fluorescence spectra in the various solvents tested here.

Figure 3 shows the relationship between the fluorescence quantum yield and the HOMO energy level of the benzene moiety calculated at the B3LYP/6-31G level. In DMSO, derivatives in which the HOMO energy level of the benzene moiety is more negative than −0.21 hartrees showed very high Φ_{fl} values. At HOMO energy levels more positive than −0.20 hartrees, the Φ_{fl} values dropped sharply, finally reaching $\Phi_{fl} \sim 0$. Thus, the threshold level between fluorescence OFF and ON (green column in the figure) was determined to be around −0.21 to −0.20 hartrees in the case of DMSO.

Similar behavior was observed in other solvents, but the position of the fluorescence ON/OFF threshold moved to higher HOMO energy regions with decreasing dielectric constant of the solvents. Finally, in the case of hexane, all the derivatives were highly fluorescent and the threshold could not be determined. These results suggested that PeT hardly occurred in nonpolar solvents, and an even higher HOMO energy would be necessary to quench fluorescence by PeT in hexane.

3. Quantitative Study of the PeT Process. It was plausible that the fluorescence of BODIPY was quenched by PeT in all

(19) Haugland, R. P. In *Handbook of Fluorescent Probes and Research Chemicals*, 9th ed.; Molecular Probes, Inc.: Eugene, OR, 2002.
(20) Gabe, Y.; Urano, Y.; Kikuchi, K.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 3357−3367.
(21) Pap, E. H. W.; Drummen, G. P. C.; Winter, V. J.; Kooij, T. W. A.; Rijken, P.; Wirtz, K. W. A.; Op den Kamp, J. A. F.; Hage, W. J.; Post, J. A. *FEBS Lett.* **1999**, *453*, 278−282.
(22) Kollmannsberger, M.; Gareis, T.; Heinl, S.; Breu, J.; Daub, J. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1333−133.
(23) Montalban, A. G.; Herrera, A. J.; Johannsen, J.; Beck, J.; Godet, T.; Vrettou, M.; White, A. J. P.; Williams, D. J. *Tetrahedron Lett.* **2002**, *43*, 1751−175.
(24) Yamada, K.; Toyota, T.; Takakura, K.; Ishimaru, M.; Sugawara, T. *New J. Chem.* **2001**, *25*, 667−66.
(25) Prieto, J. B.; Arbeloa, F. L.; Martinez, V. M.; Lopez, T. A.; Amat-Guerri, F.; Liras, M.; Arbeloa, I. L. *Chem. Phys. Lett.* **2004**, *385*, 29−35.
(26) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(27) Marcus, R. A. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1111−1121.
(28) Dean, J. A. *Langre's Handbook of Chemistry*; McGraw-Hill, Inc.: New York, 1992.

Table 1. HOMO Energy Levels and Spectroscopic Data of BODIPY Derivatives in Various Solvents

compd	HOMO energy (hartrees)	solvent	λ_{abs} (nm)	λ_{em} (nm)	Φ_{fl}
1	−0.2365	DMSO	503	514	0.875
		CH_3CN	498	509	0.923
		MeOH	499	509	0.915
		acetone	502	512	0.849
		CH_2Cl_2	502	512	0.874
		$CHCl_3$	503	514	0.862
		benzene	505	515	0.950
		hexane	502	511	0.925
2	−0.2303	DMSO	504	514	0.903
		CH_3CN	499	509	0.910
		MeOH	500	510	0.959
		acetone	500	510	0.891
		CH_2Cl_2	503	513	0.858
		$CHCl_3$	504	514	0.903
		benzene	505	515	0.921
		hexane	502	511	0.986
3	−0.2174	DMSO	504	515	0.889
		CH_3CN	499	509	0.860
		MeOH	500	510	0.950
		acetone	500	510	0.880
		CH_2Cl_2	503	513	0.961
		$CHCl_3$	505	515	0.889
		benzene	506	516	0.970
		hexane	504	512	0.855
4	−0.2141	DMSO	504	515	0.728
		CH_3CN	500	510	0.892
		MeOH	500	511	0.964
		acetone	500	510	0.966
		CH_2Cl_2	503	513	0.965
		$CHCl_3$	505	515	0.961
		benzene	505	516	0.922
		hexane	503	512	0.943
5	−0.2127	DMSO	505	517	0.857
		CH_3CN	500	511	0.873
		MeOH	500	512	0.893
		acetone	501	512	0.814
		CH_2Cl_2	504	516	0.885
		$CHCl_3$	505	518	0.904
		benzene	507	518	0.844
		hexane	504	514	0.916
6	−0.2100	DMSO	503	514	0.717
		CH_3CN	498	510	0.891
		MeOH	499	511	0.949
		acetone	499	511	0.854
		CH_2Cl_2	503	514	0.899
		$CHCl_3$	505	515	0.971
		benzene	505	518	0.925
		hexane	502	513	0.852
7	−0.2063	DMSO	504	514	0.785
		CH_3CN	499	509	0.894
		MeOH	500	510	0.942
		acetone	501	510	0.965
		CH_2Cl_2	503	514	0.923
		$CHCl_3$	505	514	0.929
		benzene	506	516	0.859
		hexane	504	513	0.948
8	−0.1997	DMSO	506	517	0.142
		CH_3CN	501	507	0.240
		MeOH	502	507	0.573
		acetone	502	513	0.715
		CH_2Cl_2	505	515	0.886
		$CHCl_3$	506	516	0.857
		benzene	507	514	0.763
		hexane	504	509	0.882
9	−0.1975	DMSO	504	517	0.011
		CH_3CN	499	508	0.011
		MeOH	500	511	0.053
		acetone	501	511	0.127
		CH_2Cl_2	503	514	0.818
		$CHCl_3$	505	516	0.979
		benzene	506	517	0.815
		hexane	504	513	0.975
10	−0.1931	DMSO	507	518	0.003
		CH_3CN	502	513	0.003
		MeOH	502	513	0.007
		acetone	503	513	0.022
		CH_2Cl_2	505	515	0.094
		$CHCl_3$	507	518	0.595
		benzene	508	516	0.924
		hexane	505	511	0.974
11	−0.1901	DMSO	505	n.d.	<0.001
		CH_3CN	500	n.d.	<0.001
		MeOH	502	n.d.	<0.001
		acetone	504	n.d.	<0.001
		CH_2Cl_2	504	515	0.002
		$CHCl_3$	507	517	0.101
		benzene	507	514	0.500
		hexane	505	511	0.880
12	−0.1901	DMSO	507	n.d..	<0.001
		CH_3CN	502	n.d.	<0.001
		MeOH	503	515	0.004
		acetone	503	513	0.011
		CH_2Cl_2	506	519	0.019
		$CHCl_3$	508	519	0.108
		benzene	509	516	0.685
		hexane	505	511	0.834
13	−0.1894	DMSO	505	n.d.	<0.001
		CH_3CN	501	n.d.	<0.001
		MeOH	501	n.d.	<0.001
		acetone	502	n.d.	<0.001
		CH_2Cl_2	504	517	0.004
		$CHCl_3$	505	517	0.024
		benzene	506	515	0.149
		hexane	502	512	0.875
14	−0.1887	DMSO	506	n.d.	<0.001
		CH_3CN	502	n.d.	<0.001
		MeOH	502	n.d..	<0.001
		acetone	503	n.d.	<0.001
		CH_2Cl_2	505	516	0.012
		$CHCl_3$	507	517	0.049
		benzene	508	516	0.539
		hexane	505	511	0.926
15	−0.1744	DMSO	502	n.d.	<0.001
		CH_3CN	498	n.d.	<0.001
		MeOH	499	n.d.	<0.001
		acetone	499	n.d.	<0.001
		CH_2Cl_2	503	n.d.	<0.001
		$CHCl_3$	505	515	0.003
		benzene	505	514	0.022
		hexane	503	513	0.861

[a] All data were measured in the presence of up to 1% $CHCl_3$ as a cosolvent. The fluorescence quantum yield was determined using fluorescein as a standard (Φ_{fl} = 0.85 in 0.1 M NaOH) (ref 18). HOMO energy levels of the corresponding benzene moieties were calculated with B3LYP/6-31G(d)//B3LYP/6-31G(d) by Gaussian 98W. n.d. = not detectable.

the solvents except hexane because only Φ_{fl} changed and no change in absorbance or emission wavelength was observed. In general, the feasibility of electron transfer between a fluorophore and a quencher can be judged from the change in

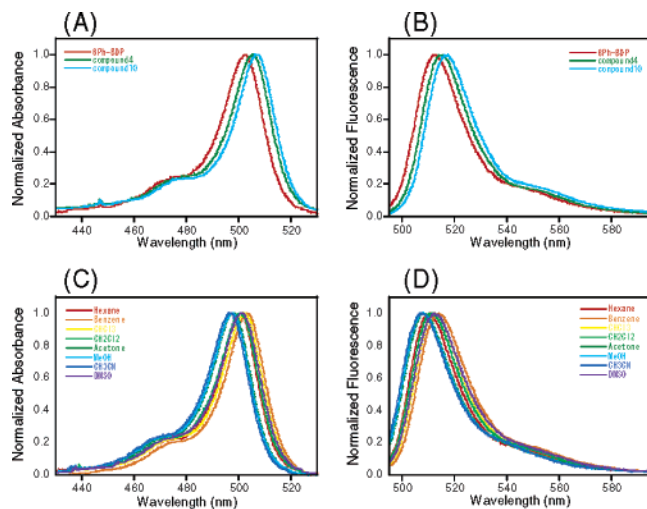


Figure 2. Absorption and fluorescence spectra of BODIPY derivatives: absorption (A) and fluorescence (B) spectra of 8-position-substituted BODIPY (red, benzene; green, naphthalene; light blue, anthracene) measured in $CHCl_3$; absorption (C) and fluorescence (D) spectra of 8-phenyl BODIPY in various solvents (red, hexane; orange, benzene; yellow, $CHCl_3$; yellow-green, CH_2Cl_2; green, acetone; light blue, MeOH; blue, CH_3CN; purple, DMSO).

Table 2. Oxidation Potential of the Benzene Moiety and Reduction Potential of BODIPY in Various Solvents[a]

	DMSO	CH_3CN	MeOH	CH_2Cl_2
	$E_{ox\ 1/2}$ (V vs SCE)			
ferrocene	0.31	0.37	0.40	0.51
9,10-dimethylanthracene	1.00[b]	1.04	1.06[b]	1.15
1,4-dimethoxybenzene	n.d.[c]	1.25	1.29[b]	1.40[b]
	$E_{red\ 1/2}$ (V vs SCE)			
compd **2**	−1.21	−1.19	n.d.[c]	−1.25

[a] The redox potentials were measured by cyclic voltammetry in DMSO, CH_3CN, MeOH, and CH_2Cl_2, containing 0.1 M TBAP with a scan rate of 0.1 V/s^{-1}. [b] Scan rate of 500 V/s^{-1}. [c] n.d. = not detectable.

free energy (ΔG_{eT}). The ΔG_{eT} value can be calculated from the Rehm−Weller equation,[29]

$$\Delta G_{eT} = E_{ox} - E_{red} - E_{00} - C$$

where E_{ox} and E_{red} are the oxidation potential of the electron donor and the reduction potential of the acceptor, respectively, E_{00} is the singlet excited energy of the fluorophore, and C is the work term for the charge separation state.

We next tried to elucidate the reason why the fluorescence ON/OFF threshold was shifted in different solvents. Judging from the solvent-dependent change of Φ_{fl}, the electron-transfer process becomes thermodynamically disadvantageous in lower polarity solvents. Therefore, we measured E_{ox} and E_{red} in every solvent.

3.1. Electrochemistry. Cyclic voltammetry was carried out to determine the redox potentials of BODIPY derivatives in various solvents (Table 2). BODIPY derivatives could not be used for the comparison of E_{ox} of the benzene moiety in each solvent, because the BODIPY fluorophore can be oxidized earlier than the benzene moiety. Therefore, we compared the half-wave potential of the corresponding benzene moiety of the BODIPY derivatives; for example, *p*-dimethoxybenzene was measured for compound **9**. As the solvent polarity decreased

(29) Rehm, D.; Weller, A. *Isr. J. Chem.* **1970**, *8*, 259−271.

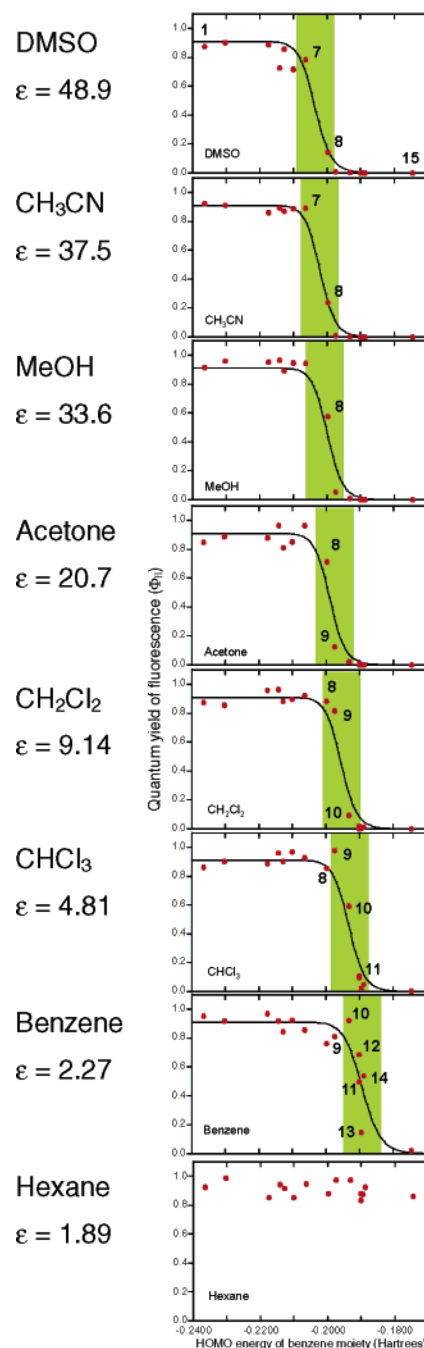


Figure 3. Relationships between the HOMO energy level of the benzene moiety and the Φ_{fl} of BODIPY derivatives. The curves represent the best fit to the Marcus equation (refs 26 and 27). Depending upon the dielectric constant (ϵ) of the solvents (ref 28), the fluorescence ON/OFF threshold (green column) gradually changes.

(from DMSO to CH_2Cl_2), the oxidation potential of the donor moiety increased (for example for 9,10-dimethylanthracene, from 1.00 V in DMSO to 1.15 V in CH_2Cl_2) and the reduction potential of the BODIPY fluorophore decreased (from −1.21 V in DMSO to −1.26 V in CH_2Cl_2). These results indicate that it is harder for the donor moiety to be oxidized and for the fluorophore to be reduced in less polar solvents.

3.2. Other Parameters in the Rehm−Weller Equation. In the case of BODIPY derivatives, all compounds showed almost the same absorption maxima and emission maxima, which indicated that E_{00} was essentially constant at 2.44 ± 0.02 eV.

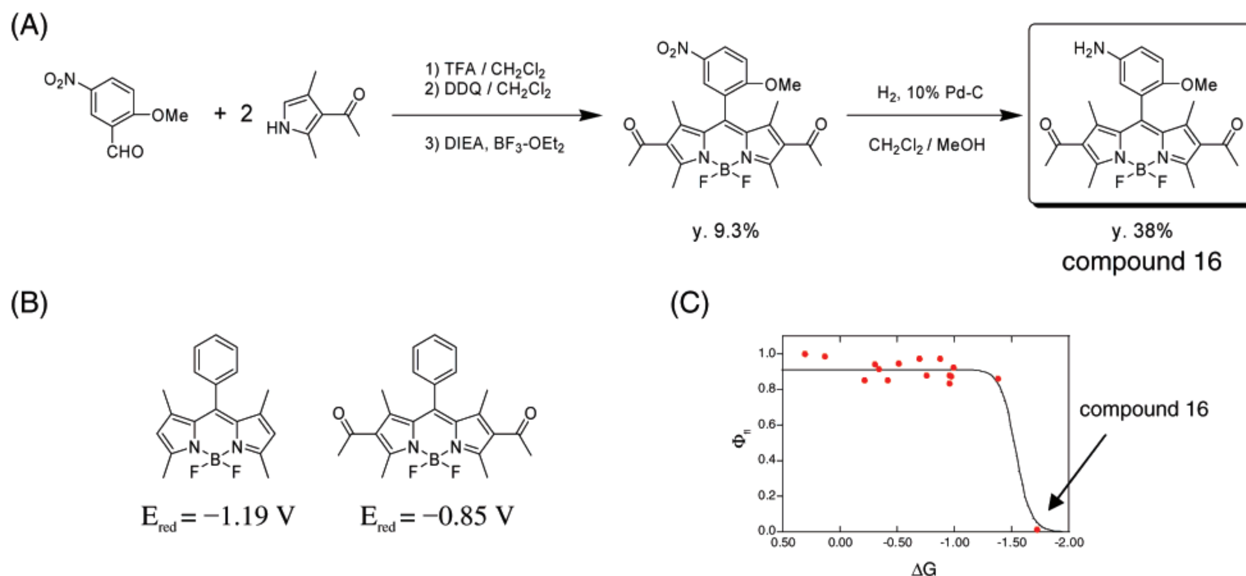


Figure 4. Electrochemical and fluorescence properties of compound **16**: (A) synthesis of compound **16**; (B) electrochemical properties of the fluorophore of BODIPY derivatives in acetonitrile (V vs SCE); (C) relationship between the fluorescence quantum efficiency (Φ_{fl}) in hexane and the free energy change (ΔG). ΔG was calculated from eq 1 ($E_{00} = 2.44$, $C = 0.72$; see text) in acetonitrile.

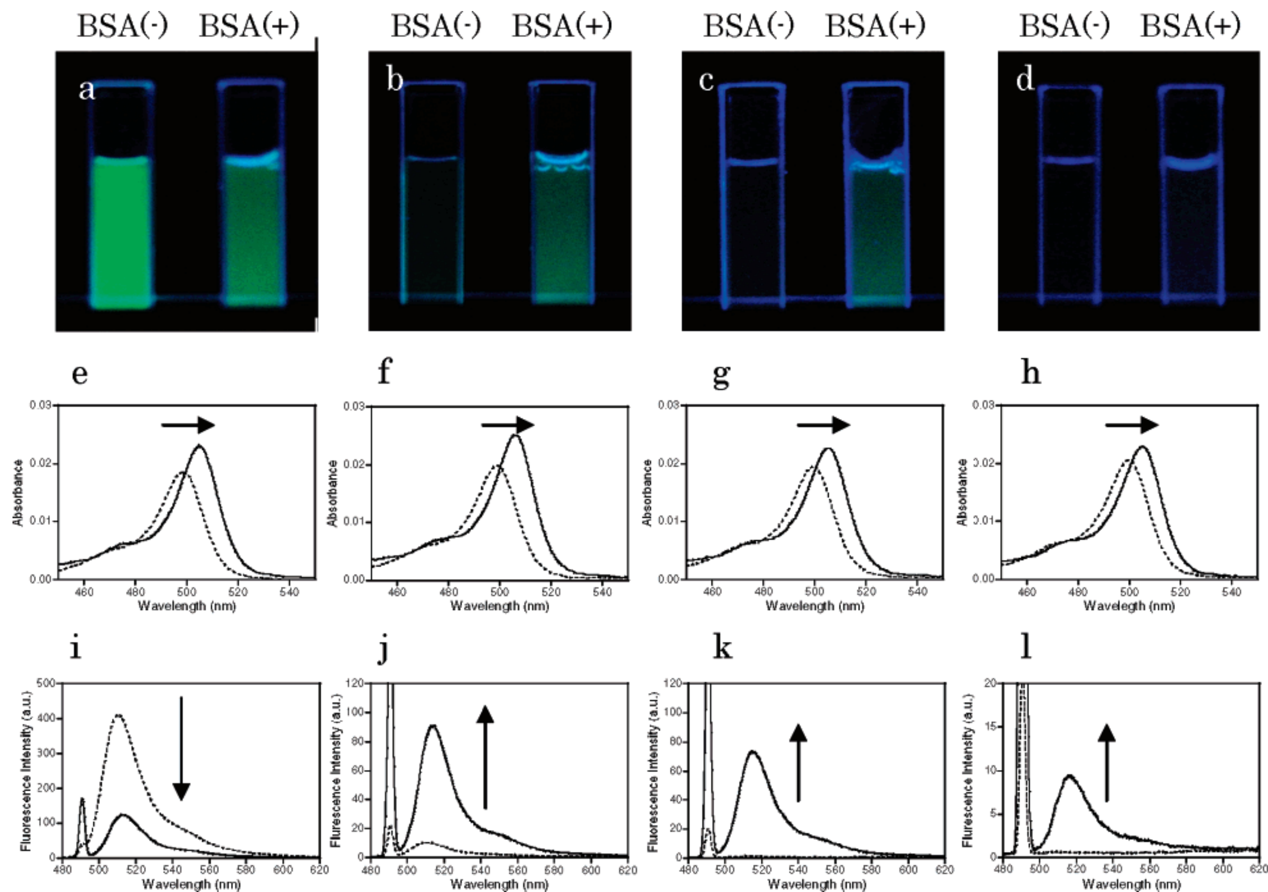


Figure 5. Fluorescence change of BODIPY derivatives (0.25 μM, containing 0.2% DMSO as a cosolvent) with or without BSA (20 μM) in sodium phosphate buffer (pH 7.4): fluorescence of compound **3** (a), compound **7** (b), compound **9** (c), and compound **11** (d) excited at 365 nm; absorbance spectra of each solution (e−h) without BSA (broken lines) or with BSA (solid lines); fluorescence spectra of each solution excited at 490 nm (i−l).

Previously, we showed that the electron-transfer process of fluorescein could be reasonably described by assuming C to have a constant value.[10] In a directly linked donor−acceptor system, the dielectric constant of the solvent becomes unrelated to C. So, we considered C to be a constant for the BODIPYs tested here.

These findings suggest that the redox potential influences ΔG_{eT} and determines the rate of electron transfer. Therefore, the solvent-dependent change of fluorescence ON/OFF threshold is concluded to be due primarily to the change of ΔG_{eT} caused by the influence of solvent polarity on the redox potentials. Electron transfer occurs more readily in high-polarity solvents.

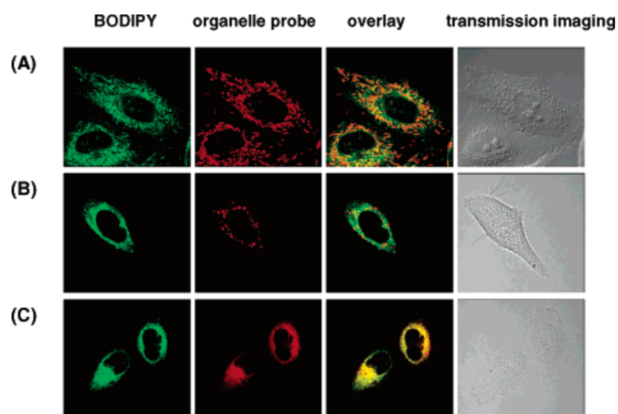


Figure 6. Fluorescence micrographs of HeLa cells colabeled for 10 min with 50 nM compound **3** (containing 0.1% DMSO as a cosolvent) in HBSS and various organelle-specific probes: (A) mitochondrial staining with 100 nM MitoTracker red CM-H2XRos; (B) lysosomal staining with 75 nM LysoTracker red DND-99; (C) staining of the Golgi apparatus with 5 μM BODIPY TR ceramide.

Table 3. Comparison of Spectroscopic Properties of BODIPYs with or without Serum Albumin

	compd **3**		compd **7**		compd **9**		compd **11**	
BSA	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)
λ_{abs} (nm)	498	505	498	505	499	505	499	505
λ_{em} (nm)	510	513	512	515	n.d.[a]	515	n.d.[a]	516
Φ_{fl}	0.871	0.330	0.020	0.218	0.002	0.187	0.001	0.023

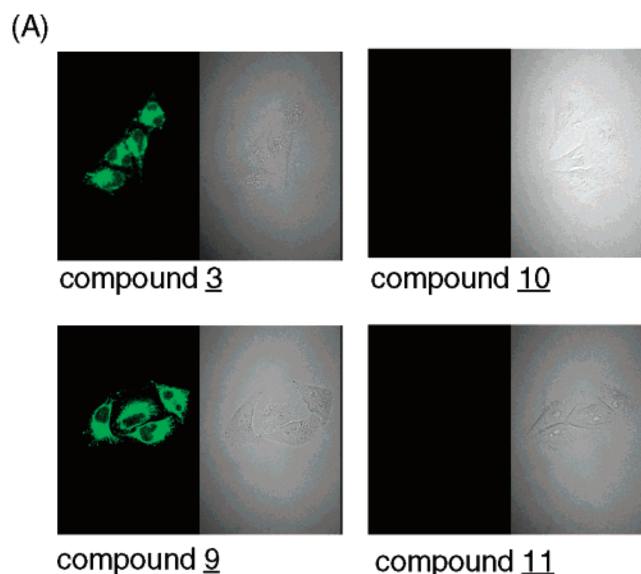
[a] n.d. = not detectable.

4. Quenching of Fluorescence in Hexane. To prepare an environment-sensitive fluorescence probe library of BODIPY derivatives having different fluorescence ON/OFF thresholds of solvent polarity, we required a compound whose fluorescence would be quenched in every solvent. Since the fluorescence of the 15 BODIPY derivatives described above was not quenched in hexane, we tried to design a compound whose fluorescence is quenched in hexane.

Because the ΔG_{eT} value in a nonpolar solvent becomes more positive, quenching of fluorescence in a nonpolar solvent requires a more negative value of ΔG_{eT}. According to the Rehm−Weller equation, the free energy change of the PeT process is determined not only by the electron-donating ability of the benzene moiety but also by the reduction potential and the excitation energy of the fluorophore.

So we synthesized a BODIPY derivative whose benzene moiety has a higher HOMO energy level as a PeT donor and whose fluorophore has acetyl moieties in the 2,6-positions of BODIPY as a readily reducible fluorophore (Figure 4A). The reduction potentials of the fluorophore were measured as −1.19 and −0.85 V versus SCE for 2,6H-BODIPY and 2,6Ac-BODIPY in acetonitrile, respectively (Figure 4B). Because the reduction potential of the BODIPY fluorophore was made more positive by introduction of the acetyl groups, Φ_{fl} of compound **16** became as small as 0.06 even in hexane (Figure 4C).

Thus, we successfully utilized our finding that the solvent dependence of fluorescence ON/OFF switching of BODIPY derivatives is due to the influence of environmental polarity on the PeT process to prepare a library of environment-sensitive fluorescence probes usable over a wide range of solvent polarity.



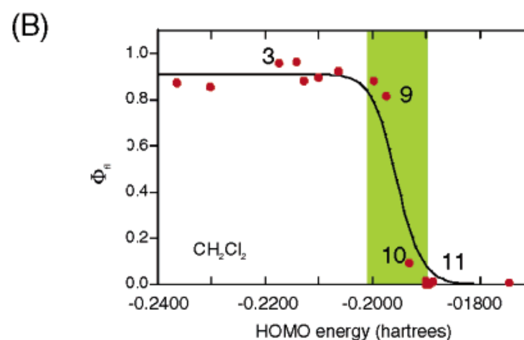


Figure 7. Fluorescence intensity change of BODIPY derivatives in HeLa cells. (A) HeLa cells were incubated with 50 nM BODIPY derivatives (containing 0.1% DMSO as a cosolvent) for 10 min at room temperature, and the fluorescence excited at 488 nm was measured. Fluorescence imaging and transmission imaging are shown. (B) The fluorescence ON/OFF threshold for HeLa cells loaded with BODIPY derivatives is compared with results from Figure 3.

5. Biological Applications of the Library of BODIPY Derivatives. 5.1. Serum Albumin. We examined the application of our library of BODIPY derivatives to assess the polarity of the surface of a protein (BSA) in vitro.

BODIPY derivatives were dissolved in sodium phosphate buffer (pH 7.4) with or without BSA. As shown in Figure 5, parts a−d, compounds **7** and **9** showed a marked fluorescence increase in the presence of BSA, i.e., almost no fluorescence without BSA and strong fluorescence with BSA. In contrast, compounds **3** and **11** showed almost no fluorescence change in the presence of BSA. The absorption maximum of each BODIPY (Figure 5, parts e−h) showed a red-shift in the presence of BSA, indicating that each BODIPY interacts with BSA with a binding constant of about 2×10^5 to 1×10^6 M^{-1} (see the Supporting Information), which is similar to those of conventional probes.[30,31]

The fluorescence properties of the BODIPY derivatives were shown in Figure 5, parts i−l, and Table 3. Fluorescence emission of compound **3** showed no increase, because there is no favorable PeT pathway in the absence or presence of BSA. The

(30) Haskard, C. A.; Li-Chan, E. C. Y. *J. Agric. Food Chem.* **1998**, *46*, 2671−2677.
(31) Cardamone, M.; Puri, N. K. *Biochem. J.* **1992**, *282*, 589−593.

reason for the fluorescence decrease is presumably an increase of the nonradiative pathway owing to attachment to the protein, as is generally observed with known fluorescence tags.[32] In the case of compounds **7** and **9**, fluorescence emission is quenched by PeT in the buffer, but at the surface of BSA, PeT is less favorable due to the hydrophobic environment, so fluorescence is increased. Compound **11**, in which PeT readily occurs in buffer, showed no fluorescence increase upon binding to BSA, because it showed almost no fluorescence even in a hydrophobic solvent such as $CHCl_3$.

Thus, fluorescence ON/OFF switching is caused by the detection of the protein surface polarity. The surface polarity of BSA is concluded to be similar to the polarity of acetone. Therefore, our environment-sensitive BODIPY library can be used for sensing the hydrophobicity at a protein surface.

5.2. Living Cells. Next, we examined the application of BODIPY derivatives to cultured living cells to estimate the polarity of the cell membrane.

To investigate the intracellular localization of BODIPY derivatives, we examined the colocalization of our BODIPYs with known organelle probes (Figure 6). HeLa cells were incubated first with organelle probes (MitoTracker red CM-H2XRos for 30 min at 37 °C, LysoTracker red DND-99 for 30 min at 37 °C, and BODIPY TR ceramide for 30 min at 4 °C then for 30 min at 37 °C), followed by BODIPY derivatives for 10 min at room temperature. BODIPY derivatives mainly colocalized with Golgi apparatus marker, and partially with mitochondrial and lysosomal markers. These results suggested that BODIPY derivatives accumulate generally on internal membranes in living cells.

We next examined the membrane polarity by measuring the fluorescence intensity of BODIPY derivatives loaded into HeLa cells (Figure 7). Cells loaded with compound **9** gave bright images, while those loaded with compound **11** gave dark images. These two compounds have almost the same methoxybenzene moiety, so it is plausible that they share almost the same localization. Comparison with the data in Figure 3 suggested that the polarity of the internal membranes is close to that of dichloromethane.

It is thought that the polarity of the lipid in membranes is similar to that of a long-chain alkane, so BODIPY derivatives may interact with membrane surface proteins.

These results demonstrate that our library of BODIPY derivatives has potential for biological applications.

Conclusion

We have shown that the fluorescence ON/OFF threshold of BODIPY derivatives is dependent upon solvent polarity, i.e., PeT-dependent fluorescence quenching occurred more easily in highly polar media than in less polar media. The oxidation potential of the donor moiety became more positive and the reduction potential of the fluorophore became more negative in less polar media so that the ΔG_{eT} value of PeT became larger. Hence, PeT is decreased in a nonpolar environment. Based on these findings, we developed a library of environment-sensitive fluorescence probes consisting of a series of BODIPYs bearing different electron donors. We showed that it could be used to estimate the polarity of the surface of BSA and internal membranes of HeLa cells.

Our environment-sensitive fluorescence probe library should be a useful tool to detect local polarity changes in a wide variety of biological samples, including proteins, membranes, and receptors.

Acknowledgment. This study was supported in part by a Grant from Hoansha Foundation to T.N., and also by research Grants (Grant Nos. 14103018, 16651106, 16689002, and 18038008 to Y.U.) from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, and by a Grant from the Kato Memorial Bioscience Foundation to Y.U.

Supporting Information Available: Characterization data for synthetic compounds, effect of cosolvent concentration, and determination of the binding constant. This material is available free of charge via the Internet at http://pubs.acs.org.

JA068551Y

(32) Lancet, D.; Pecht, I. *Biochemistry* **1977**, *16*, 5150−5157.