Fluorescent Probes

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A Ratiometric Fluorescent Probe Based on FRET for Imaging Hg^{2+} Ions in Living Cells**

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Mercury ions can easily pass through biological membranes and cause serious damage to the central nervous and endocrine systems.[1] Therefore, imaging of Hg^{2+} ions in living cells is crucial for the elucidation of their biological effects. Fluorescence spectroscopy has become a powerful tool for sensing and imaging trace amounts of samples because of its simplicity and sensitivity.[2] Thus, the development of fluorescent Hg^{2+} probes,[3] particularly those that have practical application in living cells,[4] has attracted much attention. Most reported examples of fluorescent sensing of Hg^{2+} ions in living cells function by the enhancement of fluorescence signals. However, as the change in fluorescence intensity is the only detection signal, factors such as instrumental efficiency, environmental conditions, and the probe concentration can interfere with the signal output.[5] Ratiometric sensors can eliminate most or all ambiguities by self-calibration of two emission bands.[6]

Ratiometric probes can be designed to function following two mechanisms: intramolecular charge transfer (ICT) and fluorescence resonance energy transfer (FRET). ICT probes have been frequently reported and some work well under physiological conditions. Two aspects which potentially influence the accuracy of ICT probes are: 1) Binding of the target ions promotes or inhibits ICT interactions, which results in remarkable shifts of the sensors' absorption maxima; but if multiple excitation wavelengths are used to match the different excitation maxima, their difference in efficiency may be a potential origin of inaccuracy. 2) Relatively broad fluorescence spectra are often observed for ICT fluorophores; in a significant number of cases the broad fluorescence spectra before and after binding target ions have a high degree of overlap (or in an extreme case, a broad spectrum with high intensity completely covers one with lower intensity), which makes it difficult to accurately determine the ratio of the two fluorescence peaks. Theoretically, the above problems can be avoided by using a FRET-based sensor for which the single excitation wavelength of a donor fluorophore results in emission of the acceptor at a longer wavelength.[7]

Herein we present a BODIPY-rhodamine (BODIPY = boron–dipyrromethene) FRET "off–on" system **3** as a ratiometric and intracellular Hg^{2+} sensor. A leuco-rhodamine derivative was chosen as a sensitive and selective chemosensor for Hg^{2+} ions. This was inspired by Tae and co-workers as well as other research groups,[8], who used these leuco derivatives with unconjugated structures as fluorogenic and chromogenic sensors. A highly efficient ring-opening reaction induced by Hg^{2+} generates the long-wavelength rhodamine fluorophore which can act as the energy acceptor. BODIPY[9] was chosen as the energy donor because its intense fluorescence is insensitive to environmental factors and its fluorescence spectrum matches well with the absorption spectrum of rhodamine. The choice of the connection between the donor and acceptor was equally important; a rigid and conjugated phenyl–ethynyl–phenyl spacer, which not only facilitates the through-bond energy transfer process[7a] but also greatly simplifies the synthesis of a relatively large molecule, was identified as an ideal bridge.

Both sensor **3** and ring-opened product **4** were efficiently synthesized (Scheme 1) and well characterized. An Hg^{2+}-induced process can change the emission maximum of the system from 514 nm (the characteristic peak of BODIPY) to 589 nm (the characteristic peak of rhodamine). This wavelength shift allows the ratiometric detection of Hg^{2+} ions both in ethanol/water solution and in living cells. To the best of our knowledge, this is the first successful intracellular application of a FRET-based ratiometric Hg^{2+} sensor.

The hydrophilicity of the rhodamine moiety and the lipophilicity of the BODIPY moiety mean that sensor **3** has the advantage of proper amphipathicity and can be dissolved in mixtures of organic solvents and water. Previous studies[4c,6b] have shown that the subtle equilibrium between hydrophilicity and lipophilicity is a very important factor for both cell permeability and intracellular fluorescent imaging. The UV/Vis spectrum of **3** (ethanol/water 80:20, pH 7.0) showed only the absorption profile of the donor (BODIPY), which has a maximum at 501 nm ($\log\varepsilon = 4.80$). Addition of Hg^{2+} ions immediately induced an increase in the absorption intensity at 560 nm, which corresponds to the absorption of rhodamine. The absorption stabilized after the amount of added Hg^{2+} ions reached 1 equiv (Figure 1) and a significant color change from green to pink could be observed easily by eye. This confirms that the addition of Hg^{2+} ions can promote

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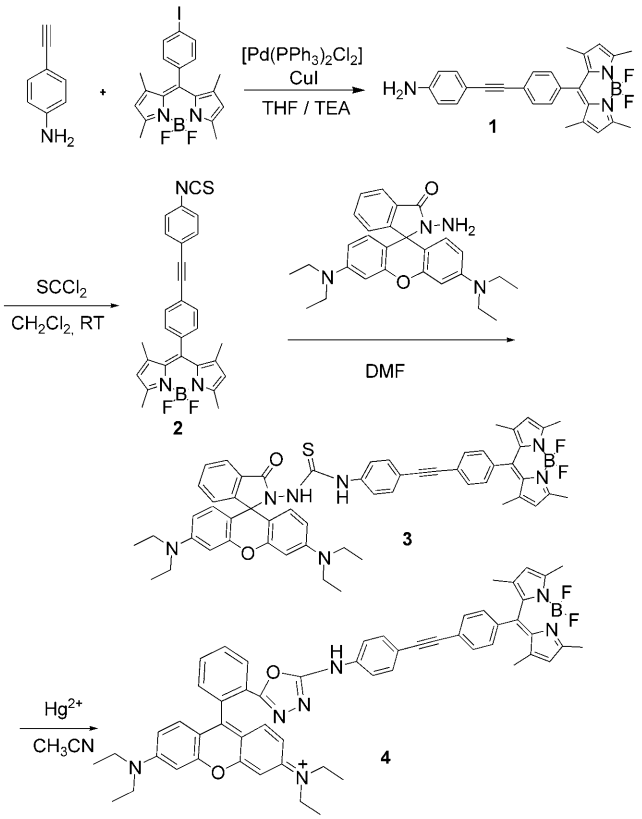

Scheme 1. Synthesis of compounds **3** and **4**.

the formation of the ring-opened compound **4**, which posesses a high molar extinction coefficient (Table 1).

Excitation of **3** at 488 nm resulted in the emission profile of BODIPY at 514 nm ($\Phi_f = 0.35$). A fluorescence titration with Hg^{2+} ions was conducted on a solution of **3** (0.3 μM) in ethanol/water (80:20) at pH 7.0. Upon addition of Hg^{2+} the BODIPY emission at 514 nm decreased, and a new emission band with a maximum at 589 nm (rhodamine) appeared and gradually increased in intensity (Figure 2a). These changes in

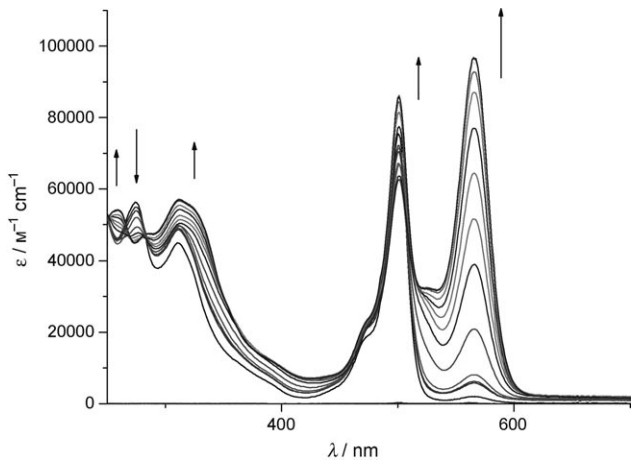

Figure 1. UV/Vis spectra of **3** in C_2H_5OH/H_2O (8:2) at pH 7.0 upon gradual addition of Hg^{2+} ions (mole equivalents = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, and 1.5).

Table 1: Spectroscopic data for compounds.

Compd.	λ_{abs} [nm]	logε [$M^{-1}cm^{-1}$]	λ_{em} [nm]	Φ_f
3	501	4.80	514	0.35[a]
	565	3.05	589	0.03[a]
4	501	4.90	514	0.001[a]
	565	4.99	589	0.20[a], 0.26[b]

[a] $\lambda_{ex} = 488$ nm. [b] $\lambda_{ex} = 543$ nm.

the fluorescence spectrum stopped and the ratio of the emission intensities at 589 nm and 514 nm (F_{589}/F_{514}) became constant when the amount of Hg^{2+} ions added reached 60 parts per billion (ppb; 1 equiv of the sensor, Figure 2b). The color of the fluorescence clearly changed from green to organge-red. It was clear that the FRET process was switched on by Hg^{2+} ions as excitation of BODIPY at 488 nm resulted in the emission of rhodamine with a maximum of 589 nm. The Förster energy transfer efficiency between BODIPY and rhodamine in compound **4** was calculated to be 99%, and the calculated R_0 value (the distance at which the energy transfer efficiency is 50%) was 58.9 Å (see the Supporting Information), which indicates the occurrence of a highly efficient

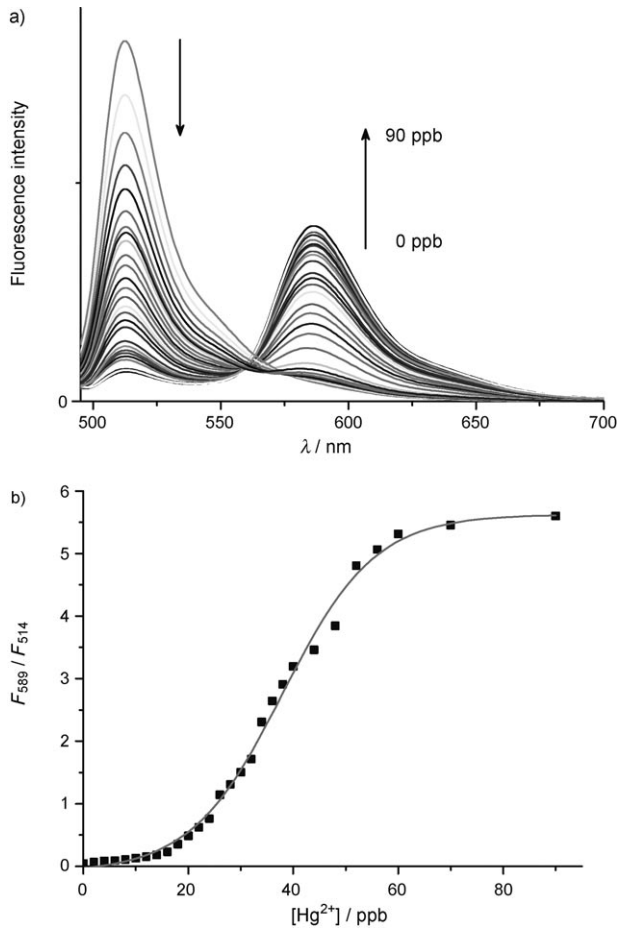

Figure 2. a) Fluorescence titration spectra of **3** (0.3 μM) in C_2H_5OH/H_2O (8:2) at pH 7.0 upon gradual addition of Hg^{2+} ions (in 2 ppb increments); b) Fluorescence intensity ratio changes (F_{589}/F_{514}) of **3** (0.3 μM) in C_2H_5OH/H_2O (8:2) at pH 7.0 upon gradual addition of Hg^{2+} ions. $\lambda_{exc} = 488$ nm.

FRET process. This FRET "off–on" sensing system has two distinct advantages. One is the large shift (100 nm) between donor excitation and acceptor emission, which rules out any influence of excitation backscattering effects on fluorescence detection. The other is the presence of two well-separated emission bands with comparable intensities, which ensures accuracy in determining their intensities and ratios.

The new FRET probe showed excellent selectivity toward Hg^{2+} ions. In fact, **3** did not give any observable response for many metal ions such as Mg^{2+}, Na^{+}, Mn^{2+}, Ni^{+}, Pb^{2+}, Al^{3+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, or K^{+} (Figure 3).

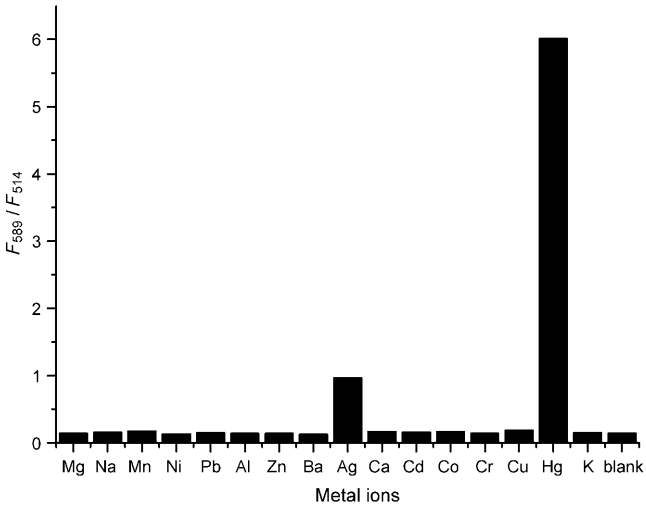

Figure 3. Fluorescence intensity ratio (F_{589}/F_{514}) of **3** (1 μM) in C_2H_5OH/H_2O (8:2) at pH 7.0 in the presence of 1 equiv of Mg^{2+}, Na^{+}, Mn^{2+}, Ni^{+}, Pb^{2+}, Al^{3+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, K^{+}, Ag^{+}, or Hg^{2+} ions.

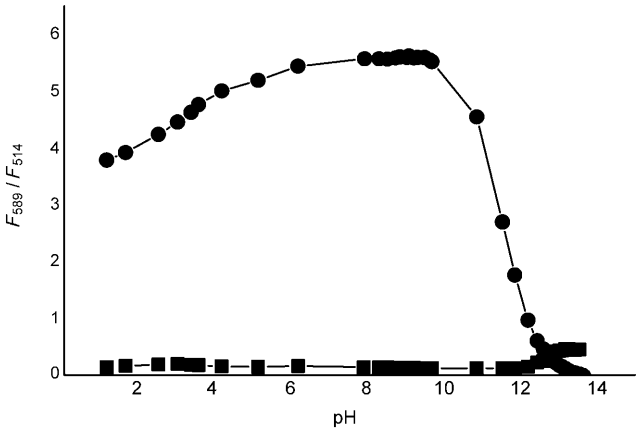

Figure 4. Fluorescence intensity ratio (F_{589}/F_{514}) of **3** (square) and **4** (circle) in C_2H_5OH/H_2O (8:2) vs. pH at 25 °C. $\lambda_{ex} = 488$ nm.

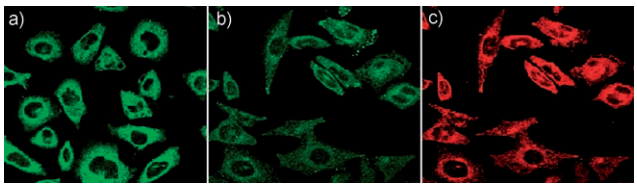

Figure 5. Confocal fluorescence images of MCF-7 (breast cancer) cells ($\lambda_{ex} = 488$ nm; Leica TCS-SP2 confocal fluorescence microscope, 20× objective lens). a) MCF-7 cells incubated with **3** (5 μM) for 30 min at room temperature, emission measured at (514 ± 15) nm. b, c) MCF-7 cells incubated with **3** (5 μM) and then further incubated with Hg^{2+} ions (5 μM), emission measured at (514 ± 15) nm (b) and (589 ± 15) nm (c).

Only Ag^{+} ions had a slight effect with a much longer equilibrium time (> 40 min) than that of Hg^{2+} ions (< 5 min).[8a] A pH titration showed that both **3** and **4** had stable fluorescence properties over a wide pH span of 4–10 (Figure 4), which suggests that sensor **3** is suitable for application under physiological conditions.

Fluorescence images of MCF-7 cells were observed under a Leica TCS-SP2 confocal microscope. The double-channel fluorescence images at (514 ± 15) and (589 ± 15) nm are shown in Figure 5. MCF-7 cells incubated with **3** (5 μM) for 30 minutes at room temperature showed a clear green intracellular fluorescence, which suggested that **3** was cell permeable. When cells stained with compound **3** were incubated with $HgCl_2$ (5 μM) in phosphate-buffered saline (PBS) for 30 minutes and washed, a partial quenching of the green fluorescence intensity (Figure 5b) and a remarkable increase in the red fluorescence intensity (Figure 5c) was observed. The intensity ratio data were obtained using commercial software (see Figure S17 in the Supporting Information). A similar phenomenon can be observed even if the concentrations of the probe **3** and $HgCl_2$ were further lowered to 1 μM. The cells remained viable and no apparent toxicity and side effects were observed throughout the imaging experiments (about 3–4 h). These experiments indicate that **3** can provide ratiometric detection for intracellular Hg^{2+} ions. Therefore, it could be a useful molecular probe for studying biological processes involving Hg^{2+} ions within living cells.

In summary, we have developed a FRET-based ratiometric probe **3** that can selectively detect amounts of Hg^{2+} ions on the ppb scale under physiological conditions. It exhibits a clear Hg^{2+}-induced change in the intensity ratio of the two well-separated and comparably strong emission bands of BODIPY and rhodamine. The significant changes in the fluorescence color can be observed by eye. Preliminary confocal laser scanning microscopy experiments show that **3** has practical application in living cells. Further investigations on its applications in life science are still underway.

Experimental Section

All chemicals were obtained from commercial suppliers and used without further purification. Tetrahydrofuran was distilled from sodium prior to use. Column chromatography was performed on silica gel (0.035–0.070 mm). ^1H NMR and ^{13}C NMR spectra were measured on a Bruker AV-400 spectrometer using TMS as an internal standard. Mass spectra were measured on HP 1100 LC-MSD and GC-Tof MS spectrometers. Fluorescence spectra were measured on a PTI-700 fluorescence lifetime spectrophotometer. Absorption spectra were determined on a PGENERAL TU-1901 UV/Vis spectropho-

tometer. Fluorescence quantum yields were determined in ethanol/water (8:2) relative to rhodamine 6G ($\varphi_f = 0.88$ in enthanol).[10] The given quantum yields are averaged from values measured with the absorption maximum 0.02–0.05.

1: A stirred solution of 4-ethynylaniline (62 mg, 0.53 mmol), BODIPY (200 mg, 0.44 mmol), CuI (3.5 mg, 0.022 mmol), and $[PdCl_2(PPh_3)_2]$ (6 mg, 0.044 mmol) in triethylamine (0.5 mL) and THF (2 mL) was heated for 2 h at 40 °C under an argon atmosphere. The solution was cooled and filtered and the resulting solid was washed with additional CH_2Cl_2 (5 mL). The filtrate was then evaporated under reduced pressure. The residual solid was purified on flash silica gel using dichloromethane as eluent to give **1** (120 mg) as a solid. Yield: 62 %. M.p. > 300 °C. $R_f = 0.3$ (SiO_2, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.62$ (d, $J = 8.0$ Hz, 2H, Ar-H), 7.36 (d, $J = 8.4$ Hz, 2H, Ar-H), 7.25 (d, $J = 8.0$ Hz, 2H, Ar-H), 6.66 (d, $J = 8.4$ Hz, 2H, Ar-H), 6.0 (s, 2H, pyrrole-H), 3.87 (s, 2H, NH_2), 2.56 (s, 6H, CH_3), 1.44 ppm (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 155.6$, 147.0, 143.1, 141.1, 134.2, 133.1, 132.0, 131.3, 128.1, 124.8, 121.3, 114.8, 112.0, 91.7. 86.7, 14.6 ppm (superposition of two signals); TOF MS calcd for $C_{27}H_{24}N_3BF_2$: 439.2031; found: 439.2040.

2: A solution of **1** (100 mg, 0.23 mmol) in dichloromethane (5 mL) was added slowly to a solution of thiocarbonyl chloride (0.069 mL, 0.92 mmol) in dichloromethane (12 mL) and triethylamine (1 mL) and the mixture was stirred for 15 min. Then the reaction mixture was washed with water, dried over $MgSO_4$, and evaporated under reduced pressure. The solid was purified on flash silica gel using dichloromethane /petroleum ether (50:50) as eluent. Yield: 78 mg, 71 %. M.p. 295–297 °C. $R_f = 0.8$ (SiO_2; dichloromethane/petroleum ether = 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J =$ 8.0 Hz, 2H, Ar-H), 7.54 (d, $J = 6.8$ Hz, 2H, Ar-H), 7.32 (d, $J = 8.0$ Hz, 2H, Ar-H), 7.24 (d, $J = 6.8$ Hz, 2H, Ar-H), 6.00 (s, 2H, pyrrole-H), 2.57 (s, 6H, CH_3), 1.44 ppm (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 155.8$, 142.9, 140.6, 136.9, 135.4, 132.8, 132.3, 131.4, 131.2, 128.3, 125.9, 123.6, 121.9, 121.4, 90.5, 89.6, 14.6 ppm (superposition of two signals); TOF MS calcd for $C_{28}H_{22}N_3BF_2S$: 481.1596; found: 481.1607.

3: A stirred solution of rhodamine B hydrazide[11] (45.6 mg, 0.10 mmol) and compound **2** (50 mg, 0.10 mmol) in DMF was heated at 50 °C for 6 h. The resulting solution was cooled and poured into water (5 mL). The resulting precipitate was filtered and purified on flash silica gel using dichloromethane /acetone (200:1) as eluent. Yield: 50 mg, 53 %. M.p. 200–202 °C. $R_f = 0.2$ (SiO_2; dichloromethane/acetone 200:1). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.02$ (d, $J = 7.2$ Hz, 1H, Ar-H), 7.62 (m, 4H, Ar-H), 7.56 (s, 1H, NH), 7.36 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.29 (m, 3H), 7.16 (d, $J = 7.6$ Hz, 2H, Ar-H), 6.95 (s, 1H, NH), 6.48 (d, $J = 7.0$ Hz, 2H, xanthene-H), 6.45 (s, 2H, xanthene-H), 6.32 (d, $J = 7.0$ Hz, 2H, xanthene-H), 6.0 (s, 2H, pyrrole-H), 3.35 (q, $J = 5.6$ Hz, 8H, CH_2CH_3), 2.56 (s, 6H, CH_3), 1.43 (s, 6H, CH_3), 1.16 ppm (t, $J = 5.6$ Hz, 12H, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 182.3$, 167.2, 155.8, 154.4, 150.1, 149.5, 143.0, 140.9, 138.1, 134.9, 134.4, 132.3, 131.7, 131.3, 129.2, 129.1, 128.2, 127.6, 124.8, 124.2, 124.1, 123.9, 121.4, 120.0, 108.5, 104.3, 98.5, 90.5, 88.6, 67.4, 44.5, 14.6 ppm (superposition of two signals), 12.6; UV/Vis (ethanol/water 8:2): λ_{max} (ε) = 501 nm (63095); fluorescence (ethanol/water 8:2, $\lambda_{exc} =$ 488 nm): $\lambda_{max} = 514$ nm; TOF MS calcd for $C_{56}H_{55}BF_2N_7O_2S$ $[M+H]^+$: 938.4121; found: 938.4450.

4: A mixture of compound **3** (30 mg, 0.032 mmol) and $HgCl_2$ (9 mg, 0.033 mmol) was stirred in acetonitrile for 15 min at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography on silica gel using dichloromethane/acetone (100:1). Yield: 28 mg, 97 %. M.p. 289–291 °C. $R_f = 0.1$ (SiO_2; dichloromethane/acetone 100:1). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 11.73$ (s, 1H, NH), 8.39 (d, $J = 6.2$ Hz, 1H, Ar-H), 7.87 (d, $J = 6.8$ Hz, 2H, Ar-H), 7.72 (t, $J = 6.2$ Hz, 1H, Ar-H), 7.62 (m, 3H), 7.39 (d, $J = 6.8$ Hz, 2H, Ar-H), 7.27(m, 1H, Ar-H), 7.23 (d, $J = 6.6$ Hz, 2H, Ar-H), 7.17(d, J=7.6 Hz, 2H, Ar-H), 6.81 (d, $J =$ 7.6 Hz, 2H, Ar-H), 6.76 (s, 2H, Ar-H), 6.0 (s, 2H, pyrrole-H), 3.58 (q, $J = 5.72$ Hz, 8H, CH_2CH_3), 2.55 (s, 6H, CH_3), 1.42 (s, 6H, CH_3), 1.32 ppm (t, $J = 5.72$ Hz, 12H, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 160.6$, 158.3, 157.9, 156.2, 155.6, 143.1, 141.2, 139.5, 135.2, 134.3, 132.2, 132.2, 131.6, 131.3, 130.8, 130.2, 130.0, 129.8, 129.5, 128.1, 125.1, 124.8, 123.9, 121.3, 118.1, 114.3, 114.1, 96.6, 91.8, 87.3, 46.1, 14.6, 14.1, 12.7 ppm; UV/Vis (ethanol/water 8:2): λ_{max} (ε) = 501 (79432), 565 nm (97723); luorescence (ethanol/water 8:2, $\lambda_{exc} =$ 488 nm): $\lambda_{max} = 589$ nm; TOF MS calcd. for $C_{56}H_{53}BF_2N_7O_2$: 904.4316, found: 904.4559.

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