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# Benzimidazole-BODIPY as optical and fluorometric pH sensor

Zhensheng Li [a], Lei-Jiao Li [b], Tingting Sun [a], Liming Liu [a], Zhigang Xie [a, *]

[a] State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, 5625 Renmin Street, Changchun 130022, PR China

[b] State Key Laboratory of Rare Earth Resource Utilization, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, PR China

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ABSTRACT

A new optical and fluorometric pH sensor has been obtained by attaching pH sensitive group benzimidazole at the 2-position of borondipyrromethene. Due to the electron-donor effect, benzimidazole group caused obvious bathochromic shift in the absorption and fluorescence spectra. Upon protonation, the solution exhibited drastic blue-shifted absorption and enhanced fluorescence, and the color changed from yellow to green simultaneously. The sensing mechanism was elucidated by quantum chemistry calculation approach. Cell experiments were carried out to verify the compound could selectively locate in the acidic lysosome. These results indicated the Benzimidazole-BODIPY could be used as an optical and "off-on" fluorescent pH indicator.



## 1. Introduction

The precise pH measurement attracts much attention of researchers for their potential application in environmental monitoring and biological systems. Among different analytic methods, fluorescent-based analyses are promising tools due to low cost, convenient operations, high sensitivity, excellent spatial and temporal resolution [1–4]. Although much work about pH sensor was reported, it remains challenging to design and synthesize novel fluorescent pH sensors with high sensitivity [5–10].

Borondipyrromethene (BODIPY) fluorophores possess outstanding photophysical properties, including high molar absorption coefficients and fluorescence quantum yields, narrow emission bandwidths, and high stability in physiological conditions [11,12]. Moreover, their spectroscopic characteristics can be fine-tuned by introducing appropriate substituents at different position of the BODIPY backbone. These unique advantages facilitate the design various fluorescent probes based on BODIPY in analyte-detection, bio-imaging and labeling [13–17]. Benzimidazole group has two acid–base equilibrium with $pK_a = 5.7, 12.6$, the former of which is ideally located within the physiological range of acidic organelles, such as lysosome (pH 4.5–5.5) and endosome (pH 4.5–6.8). So benzimidazole derivatives are usually employed to modify the fluorophore to develop intracellular pH sensors [18,19]. For example, Gökhan Sevinç et al. have reported 8-substituted benzimidazole-BODIPY pH sensor [20]. Unfortunately, the intense fluorescence was quenched drastically with addition of $H^+$. Moreover the protonation could not cause a substantial absorption change due to the orthogonal conformation.

Herein, we synthesized a novel 2-substituted BODIPY (**BDP-BIM**) which could be used as an optical and "turn-on" fluorescent pH sensor. The compound exhibited intense fluorescence in nonpolar solvents but not in polar solvents, while the photophysical spectra did not change apparently. On the other hand, the absorption and emission peaks of **BDP-BIM** showed obvious blue shift upon protonation of benzimidazole in aqueous solution with greatly increased fluorescence. The distinct spectra properties could be explained by electron effect as confirmed by density functional theory (DFT) calculation. It is expected that the designed compound can be used a dual pH and polarity probe due to 2-substituted benzimidazole moiety. Furthermore, the probe could respond to acid environment inside the cells and selectively label the lysosome.

## 2. Experiment section

### 2.1. General information

Solvents and reagents were purchased commercially as reagent grade and used as received unless otherwise mentioned.

* Corresponding author. Tel.: +86 431 85262775.
*E-mail address:* xiez@ciac.ac.cn (Z. Xie).

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Dichloromethane and N, N-dimethylformamide (DMF) were distilled over calcium hydride. The solvents used for spectroscopic measurements were of HPLC grade. β-Formyl-BODIPY was prepared according to the reported procedure [21]. The pH sensing experiment was carried in the $CH_3OH/H_2O$ (1:1 v/v) solution. All of the quantum calculations were performed using the Gaussian09 program package [22]. The ground state structure was optimized using the density functional theory (DFT) method with the hybrid-generalized gradient approximation (H-GGA) functional B3LYP.

### 2.2. Instruments

$^1H$ NMR and $^{13}C$ NMR spectra were recorded on a Bruker NMR 400 DRX Spectrometer using DMSO-$d^6$ as solvent. Electrospray Ionization (ESI) mass spectra were obtained on a Finnigan LCQ quadrupole ion trap mass spectrometer. UV–Vis absorption and emission spectra were obtained using a Shimadzu UV-2450 PC UV–Vis Spectrophotometer and a Hitachi F-4500 Fluorescence Spectrophotometer, respectively. The luminescence quantum yields in solution were measured by using rhodamine 6G with excitation wavelength 488 nm ($\Phi_F = 0.86$ in MeOH) as reference [23].

### 2.3. Confocal laser scanning microscopy (CLSM)

**BDP-BIM** was dissolved in DMSO with a concentration of 1.0 mM, and diluted to 5 μM in phosphate-buffered saline (PBS) solution. Cellular uptake by human cervical carcinoma (HeLa) cells was examined with CLSM. HeLa cells were seeded in 6-well culture plates (a clean cover slip was put in each well) at a density of $5 \times 10^4$ cells per well and allowed to adhere for 24 h. The medium was then replaced by **BDP-BIM** in PBS solution (containing 0.5% DMSO). After incubation for 0.5 h at 37 °C, the supernatant was carefully removed and the cells were washed three times with PBS. Subsequently, Lyso-Tracker Red was used to stain lysosomes for 45 min at 37 °C. After being washed with PBS, the cells were fixed with 1 mL of 4% formaldehyde each well for 10 min at room temperature and washed twice with PBS again. Samples were examined by CLSM using a Zeiss LSM 700 (Zurich, Switzerland).

### 2.4. Synthetic procedures

β-Formyl-BODIPY (0.5 mmol), *o*-phenylenediamine (0.5 mmol) and *p*-TsOH (0.1 mmol) were thoroughly mixed in DMF (10 mL). Then the solution was heated and stirred at 80 °C for 4 h. After cooling to room temperature, the reaction solution was quenched by addition of $Na_2CO_3$ aqueous (0.1 mmol, 20 mL), then the mixture was extracted with EtOAc (2 × 20 mL). The organic phase was dried over anhydrous sodium sulfate. Evaporation of solvent gave the crude product, which was purified by column chromatography over silica gel (hexane/ethyl acetate, 3/1) to afford the red solid product (yield: 82%). $^1H$ NMR (400 MHz, DMSO-$d^6$) δ 12.29 (s, 1H), 7.61 (d, $J = 6.5$ Hz, 4H), 7.46 (d, $J = 7.6$ Hz, 3H), 7.18 (s, 2H), 6.32 (s, 1H), 2.65 (s, 3H), 2.52 (d, $J = 5.1$ Hz, 3H), 1.50 (s, 3H), 1.39 (s, 3H).$^{13}C$ NMR (101 MHz, DMSO-$d^6$) δ 157.86, 152.88, 146.12, 144.84, 142.86, 139.57, 133.89, 131.92, 129.77, 129.55, 129.46, 127.78, 122.76, 122.45, 121.76, 14.47, 14.21, 13.43, 12.50. ESI-MS calculated ($[C_{26}H_{23}BF_2N_4]$) $m/z = 440.2$, found $m/z = 439.3$ $[M - H]^-$.

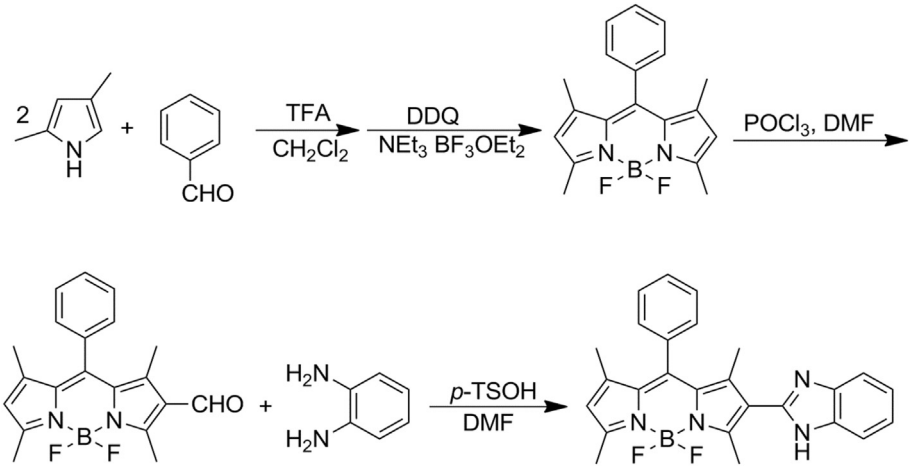
## 3. Result and discussion

### 3.1. Synthesis and characterization

Most fluorescent BODIPY-based sensors are modified on α or *meso* position [24,25]. β-Position functionalization strategies are less investigated. Formylation of the β-position of BODIPY via Vilsmeier–Haack reaction provides versatile functionalization approaches. **BDP-BIM** was synthesized by the facile route shown in Scheme 1. The electron-deficient BODIPY system facilitates the condensation of aldehyde group and benzene-1,2-diamine to obtain product in high yield. The compound was characterized by $^1H$ NMR, $^{13}C$ NMR, ESI-MS analysis and spectroscopic methods. The sharp signal at 12.29 ppm in the $^1H$ NMR spectrum (Fig. S1) was assigned to the active hydrogen of imidazole ring.

### 3.2. Spectroscopic properties

The spectra properties of **BDP-BIM** in various solvents were recorded as summarized in Table 1. As shown in Fig. 1, the absorption spectra inherit characteristics of typical BODIPY dyes, and the intense $S_0–S_1$ transition centers around 510 nm. Due to the 2-benzimidazole substituent, the shoulder peak ascribed to the 0–1 vibration band of $S_0–S_1$ transition attenuates significantly, meanwhile the maximum peaks display red shifts of 10–20 nm compared to that of unsubstituted BODIPY. The emission spectra exhibit similar red-shifted behaviors. These results confirm the "push–pull" electron interaction between the benzimidazole fragment and BODIPY core. Although the main absorption and




**Scheme 1.** The synthetic pathway of compound **BDP-BIM**.

**Table 1**
Spectroscopic data of BDP-BIM in various solvents.

| Compound | Media | $\lambda_{abs}$/nm | $\varepsilon_{max}$ ($dm^3\ mol^{-1}\ cm^{-1}$) | $\lambda_{em}$/nm | Stokes shift/nm | $\Phi_F$ |
|---|---|---|---|---|---|---|
| **BDP-BIM** | Hexane | 512 | 99,000 | 563 | 51 | 0.57 |
| | Toluene | 515 | 82,600 | 564 | 49 | 0.52 |
| | $CH_2Cl_2$ | 510 | 102,000 | 562 | 52 | 0.21 |
| | $CHCl_3$ | 511 | 73,900 | 558 | 47 | 0.34 |
| | THF | 513 | 80,100 | 568 | 55 | 0.18 |
| | DMF | 513 | 80,400 | 571 | 58 | 0.05 |
| | $CH_3OH$ | 506 | 86,600 | 548 | 42 | 0.10 |
| | $CH_3CN$ | 508 | 84,000 | 565 | 57 | 0.07 |

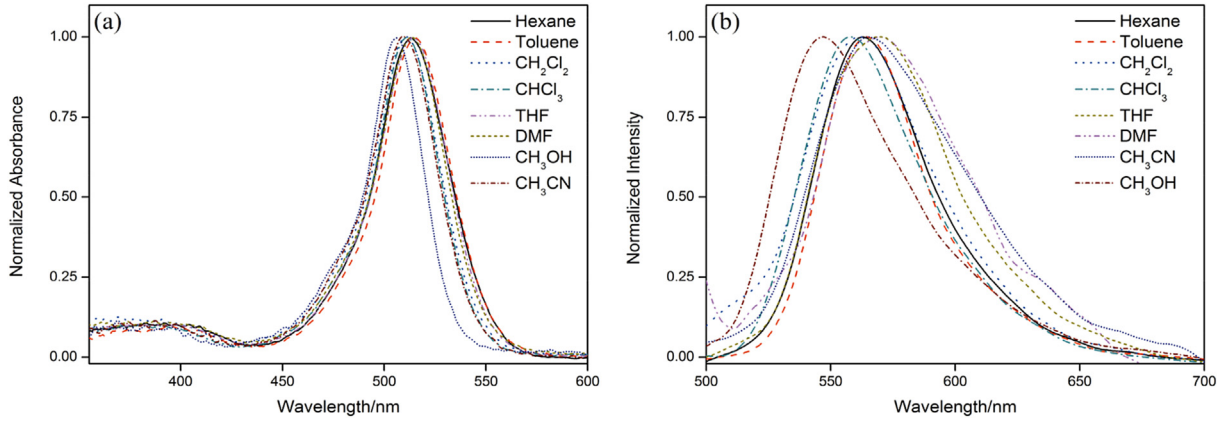



**Fig. 1.** Absorption (a) and fluorescence (b) spectra of **BDP-BIM** in various solvents.

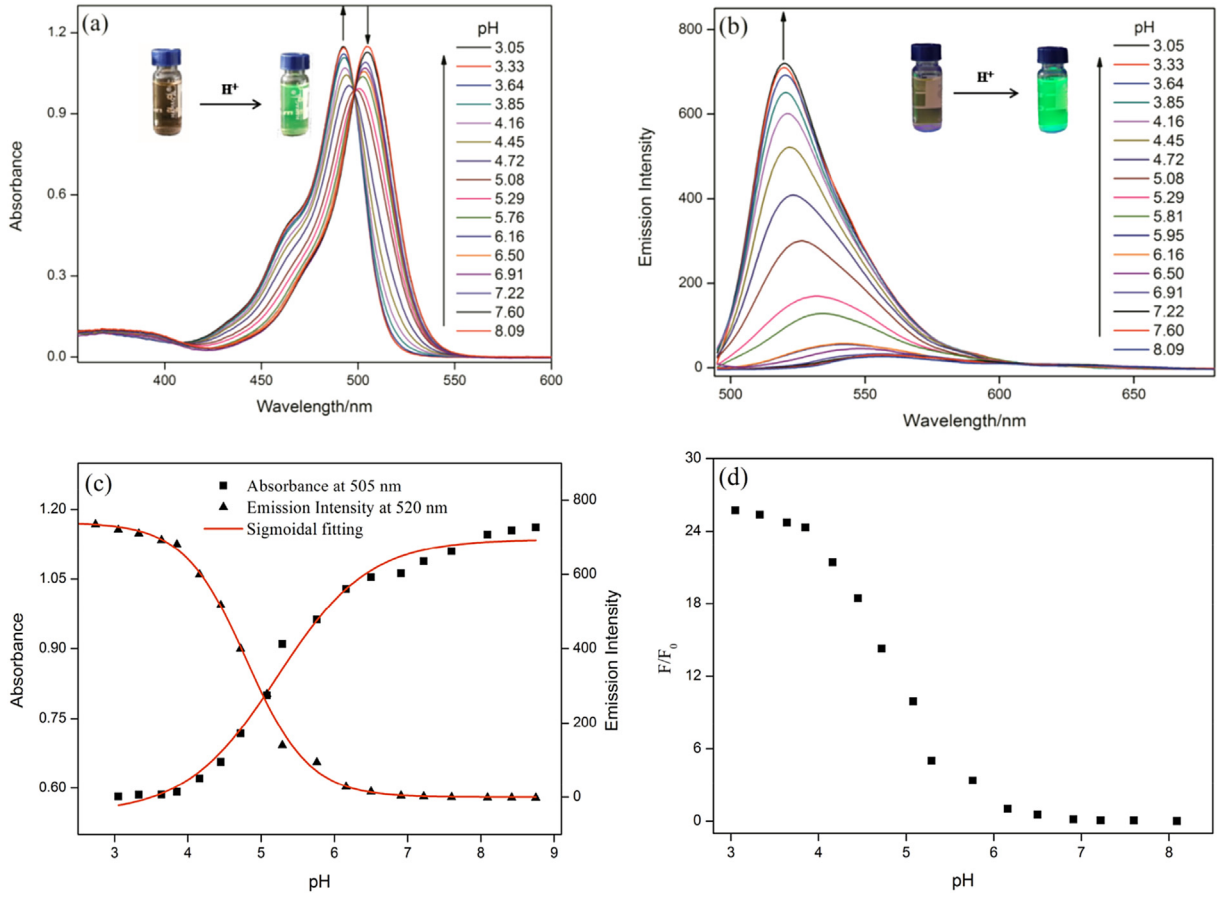



**Fig. 2.** The changes of absorption (a) and emission (b) spectra in the $CH_3OH/H_2O$ (1:1 v/v) solution upon addition of $H^+$; (c) the sigmoidal fitting of absorbance at 505 nm and emission at 520 nm; (d) fluorescence enhancement effect (F and $F_0$ are emission intensity at 520 nm in different pH intervals and at 560 nm in neutral condition, respectively).

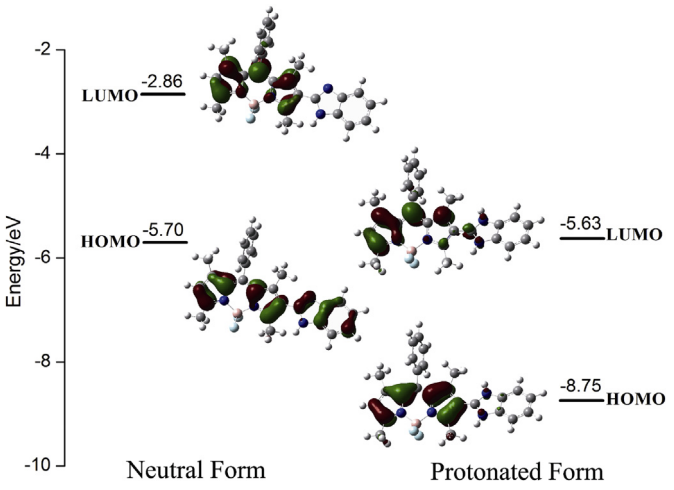



**Fig. 3.** Molecular orbital energy levels of **BDP-BIM** in neutral and protonated form.

emission show little solvent dependence, the fluorescence emission quantum yields vary dramatically in different solvent. For example, the $\Phi_F$ in nonpolar solvent such as hexane is measured to be 0.57, whereas it is 0.07 in $CH_3CN$. This polarity dependence is probably due to the positive solvatokinetic effect [26–28], facilitating "off-on" probing in polar media. In addition, the large hypochromatic-shifted spectra in $CH_3OH$ are ascribed to the polarity and protonation dual effect, which can be verified by the following pH titration experiment.

### 3.3. pH-dependent absorption and fluorescence spectra

The spectrophotometric pH titrations were carried out to examine the spectra changes. As shown in Fig. 2a, when pH value decreased from 8.09 to 3.05, the absorption at 505 nm declined. While a new peak at 490 nm increased gradually with the appearance of isobestic points at 498 and 409 nm. The 15 nm blue-shifted absorption attributed to the inhibition electron effect of benzimidazole. The color of solution changed from light yellow to bright green (in the web version), indicating the ability of optical detection of $H^+$ ion. The emission spectra changes depicted the same protonation–deprotonation process. At first, the **BDP-BIM** solution emitted weak fluorescence at 560 nm. With continuous addition of acid, the yellow emission vanished, and the new band at 520 nm increased significantly with a large fluorescent "off-on" ratio of 26 (Fig. 2d). The p$K_a$ and p$K_a^*$ values were determined to be 5.2 and 4.8 by the nonlinear sigmoidal fitting of titration data, respectively.

Benzimidazole possessed two acid–base equilibriums of p$K_a$ values of 5.7 (protonated-neutral) and 12.6 (neutral-anionic), therefore we added base to the neutral solution to examine the $OH^-$ response. Although the fluorescence of **BDP-BIM** was enhanced below pH 3, slightly hyperchromic effect and fluorescence quenching were observed with increasing alkaline concentration. These findings indicate that the improved emission intensity is related to the photoinduced electron transfer (PET) mechanism between the benzimidazole and BODIPY groups.

### 3.4. Density functional theory (DFT) calculation

In order to prove our speculation, theoretical calculations are performed with density functional theory by using Gaussian09 software package. The structures of the neutral and protonated BDP-BIM are optimized at the B3LYP/6-31 + G(d) level [29,30], followed by the analysis on the frontier orbitals. As shown in Fig. 3, HOMO of neutral **BDP-BIM** was localized on the BODIPY core and benzimidazole, while LUMO was mainly contributed by the BODIPY units. However, both HOMO and LUMO of protonated **BDP-BIM** were donated by the orbital from the BODIPY core, demonstrating that photoinduced electron transfer partly existed between BODIPY and benzimidazole moiety in neutral form, whereas this electron transfer was inhibited in protonated form. The recognition mechanism of this probe was completely opposite to that of 8-substituted analogy reported by Sevinç [20], resulting in our "light-up" probe. The energy band-gap was calculated to be 2.84 eV for neutral form and 3.12 eV for protonated form, reflecting increased transition energy, which was in well accordance with the blue-shift spectra.

### 3.5. Intracellular pH response

The probe with p$K_a$ value of 5.2 is expected to response to acidic media in the living cells. To assess whether **BDP-BIM** can be utilized as an organelle probe, the HeLa cells were labeled with our probe and Lyso-Tracker Red. The bright fluorescence was observed in the cells (Fig. 4a), indicating the efficient cell uptake and intracellular protonation of **BDP-BIM**. As shown in Fig. 4c, the green fluorescence of **BDP-BIM** can superimpose with red fluorescence (in the web version) of Lyso-Tracker Red mostly. This result confirmed that our probe could track acidic lysosome in the cells as well. The ability to sense acidic and near neutral pH in the cell using fluorescent probe could facilitate biological study associated with pH change in lysosome and cytosol.

## 4. Conclusion

In summary, we have developed an optical and "off-on" fluorescent pH sensor **BDP-BIM**. Upon protonation, the solution color changed from yellow to green with a large fluorescence "off-on" ratio of 26. The probing mechanism was different from the 8-

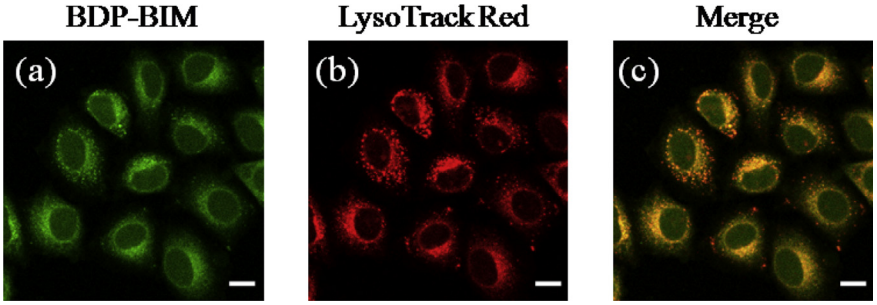



**Fig. 4.** Confocal laser scanning images of HeLa cells co-labeled with BDP-BIM (a) in PBS solution containing 0.5% DMSO and Lyso-Tracker Red (b), (c) merged image from (a) and (b). Scale bar = 20 μm.

substituted analog and confirmed by quantum calculation. Intracellular co-localization experiment demonstrated the sensor could selectively label acidic organelle lysosome.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2016.01.029.

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