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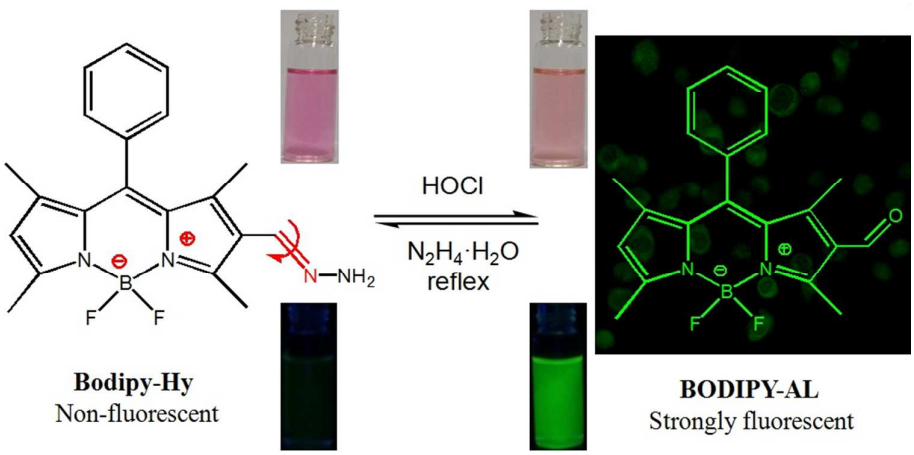

A novel "turn-on" fluorogenic probe for sensing hypochlorous acid based on BODIPY

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†Electronic Supplementary Information (ESI) available: Details of synthesis of BODIPY and BODIPY-AL, characterization of Bodipy-Hy, conditional experiments, and Thin Layer Chromotography (TLC), See DOI: 10.1039/x0xx00000x

Abstract: A highly selective and sensitive boron-dipyrromethene (BODIPY) based fluorescent probe (Bodipy-Hy) for the detection of hypochlorous acid (HOCl) was designed and easily synthesized by condensation reaction (C=N) of BODIPY aldehyde (BODIPY-AL) and hydrazine hydrate, which contain a newer group compared with other similar probes. With the specific HOCl-promoted oxidation grade of the C=N bond increasing, the fluorescence intensity of Bodipy-Hy are gradually increased more than 11-fold. And the fluorescent quantum yield enhances from 0.06 to 0.62. A linear increase of fluorescence intensity could be observed under the optimum conditions with increasing HOCl concentration over a wide linear range 0-22.5 μM, then obtained a lower detection limit of 56 nM based on $3\times\delta_{blank}/k$. Moreover, the probe can also be successfully applied to imaging of HOCl in living cells with low cytotoxicity.

Introduction

Hypochlorous acid (HOCl), one of the biologically important Reactive oxygen species (ROS), plays an essential role in diverse normal biochemical functions and abnormal pathological processes[1]. Endogenous HOCl is mainly formed from H_2O_2 and chloride ions by the catalysis of enzyme myeloperoxidase (MPO) in leukocytes including macrophages, monocytes and neutrophils[2, 3]. When a microbe invade human tissue, leukocytes engulf the invading microbes by phagocytosis. Endogenous HOCl can damage various biomolecules, including DNA, lipids, and proteins, and then kill the invading microbes[4]. Although HOCl functions mainly in the prevention of microorganism invasion, the uncontrolled levels of HOCl caused by MPO have been implicated in several human diseases including lung injury, neuron degeneration, cardiovascular diseases, renal disease and even cancers[5-7]. Because of the pathophysiological importance of hypochlorous acid, it is essential to develop imaging techniques for HOCl. Among the most powerful imaging tools, fluorescence probes have been made more attractive among these methods owing to their operational simplicity, high sensitivity, low-cost and real-time detection[8, 9]. More importantly, they are able to achieve visualization analysis of HOCl fluctuations in cells and in vivo through fluorescence imaging[10-22].

In recent years, a variety of fluorescent probes for HOCl are mainly based on the strong oxidation property of HOCl. Functional groups, sensitive to

hypochlorite oxidation, such as p-methoxyphenol, p-alkoxyaniline, selenide, thiol, oxime and dibenzoylhydrazine have been extensively utilized in the probe design. Commonly, compounds containing unbridged C=N bonds are usually non-fluorescent, where C=N isomerization is the predominant decay process of excited states[23]. Whereas if compounds, containing a cyclic C=N bond, but complexing with a guest species to inhibit the rotation of the C=N bond or removed the C=N bond by chemical reaction, are strongly-fluorescent[24]. Therefore many fluorescent probes are designed by the C=N isomerization mechanism in order to detect metal ions through complexation of metal ions[25-27]. Also several fluorescent sensors are designed by the removal of the C=N bond[24, 28].

As a continuation of our research efforts devoted to fluorescent probes for metal ions recognition. In this work, we have used a BODIPY (boron-dipyrromethene) dye which is a class of well-known fluorophores with widespread applications as the mother molecule due to their valuable characteristics, such as large absorption coefficient and high fluorescence quantum yield leading to intense absorption and fluorescence bands[29]. Therefore, we have designed and synthesized a novel and low-cost BODIPY derivate (Bodipy-Hy) for selective and sensitive detection of HOCl over other ROS and common metal ions in phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution. Bodipy-Hy displays weak fluorescence with a quantum yield of Φ_F=0.06 due to the C=N bond isomerization. The strong fluorescence of

BODIPY-AL (Φ_F=0.62) is restored after the oxidation of C=N bond by HOCl. Furthermore, Bodipy-Hy shows excellent cell membrane permeability and can also be applied to image HOCl in living cells.

Experimental

Apparatus

^{1}H and ^{13}C NMR spectra were measured on a Bruker DMX-300 spectrometer operating at 400 MHz. The MS spectra were performed on Bruker ESQUIRE HPLC-MS AB 4000Q. UV-Vis absorption spectra were recorded on a U-4100 spectrophotometer. Fluorescent spectra were recorded on a Hitachi F-7000 FL spectrofluorometer. FT-IR spectra were measured on Thermo Nicolet AVATAR360 spectrometer. An Olympus Zeiss 710 laser scanning confocal microscopy was used for fluorescence image of cells. The Jingke pH measurements were measured by use of a PHS-3D digital pH-meter.

Materials

2,4-dimethyl-1H-pyrrole was purchased from Shanghai chemical plant, benzoyl chloride, hydrazine hydrate and triethylamine were purchased from Tianjin reagent plant. Boron trifluoride diethyl etherate was purchased from Sinopharm Chemical Reagent plant. The solution of metal ions was prepared from their nitrate salts and chloride salts of analytical grade. The solvents were used as received without further purification. Distilled water was used throughout.

Cells culture

PC12 cells were seeded in glass bottom culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5 % fetal bovine serum (FBS) and 15 % horse serum at 37 °C with 5 % CO_2 atmosphere until harvesting for the experiment. When harvesting, the DMEM was drawn out from the culture dishes, and the dishes were rinsed three times with 10 mM phosphate buffer saline (PBS) and then treated with 4 mL trypsinase solution containing 0.25 % EDTA for 3 min in the incubator. The cells were centrifuged at 3000 rpm for 5 min, then removed the supernatant.

Cytotoxicity assay

The methyl thiazolyl tetrazolium (MTT) assay was used to measure the cytotoxicity of Bodipy-Hy in PC12 cells. PC12 cells were seeded into a 96-well cell-culture plate. Various concentrations (10, 20, 30, 40, 50 μM) of Bodipy-Hy were added to the wells. The cells were incubated at 37 ^{o}C under 5 % CO_2 for 24 h. 10 μL MTT (5 mg mL^{-1}) was added to each well and incubated at 37 ^{o}C under 5 % CO_2 for 4 h. Then the culture medium was removed and the cell layer was dissolved in DMSO (100 μL). Thermo Multiskan Ascent microplate reader was used to measure the absorbance at 570 nm for each well.

Synthesis of Bodipy-Hy

As depicted in Scheme 1, BODIPY was synthesized according to the literature procedure[30]. BODIPY-AL was synthesized from BODIPY by using well known Vismeier Haack's formylation reaction[31] (see ESI†). To a 100 mL round-bottomed flask, hydrazine hydrate (16 mmol, 1 mL) were dissolved in 10

mL absolute ethanol. After stirred about 10 min at 60 ^{o}C under N_2 atmosphere, drop-by-drop addition of 20 mL absolute ethanol solution of BODIPY-AL (1.7 mmol, 0.6 g) was begun. And then three drops glacial acetic acid were added. The mixture was stirred and refluxed for 4 h at 80 ^{o}C under N_2 atmosphere. Following the completion of the reaction, the solvent was removed under reduced pressure and the residue was dissolved in 100 mL dichloromethane. The organic phase was washed with 100 mL water for three times and dried with anhydrous sodium sulfate. The product was purified by column chromatography (petroleum ether-dichloromethane) to give a solid (0.25 g, 40 %). Mass spectrometry: m/z, calcd: 366.18, found: 367.1 ($[M+H]^+$), 389.3 ($[M+Na]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.71 (s, 1 H), 7.55-7.42 (m, 3 H), 7.29-7.27 (m, 2 H), 6.09 (s, 1 H), 2.85 (s, 3 H), 2.60 (s, 3 H), 1.61 (s, 3 H), 1.40 (s, 3 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 155.36, 155.00, 141.90, 136.73, 136.11, 135.03, 133.95, 133.44, 132.05, 131.30, 129.66, 128.44, 126.21, 121.74, 17.77, 14.80, 14.51, 12.18.

Results and discussion

UV-Vis absorption response of Bodipy-Hy with HOCl

The absorption spectra of Bodipy-Hy (10 μM) was firstly explored in phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution in the presence of 20 equiv. of different ROS, anions and common metal ions and the results are shown in Fig. 1. The probe Bodipy-Hy (10 μM) exhibited a very strong absorption at 516 nm, also absorption spectra didn't changed significantly in

the presence of 20 equiv. of different ROS, anions and common metal ions except HOCl. As shown in Fig. 4, when the increasing concentration of HOCl was added, a new absorption band at 494 nm was gradually appeared indicating a possible structural change of the BODIPY core[28] and also the blue shift of the absorption wavelength was reflected in a change in the colour of the solution from pink to light orange.

Fluorescence spectral responses of Bodipy-Hy with HOCl

Changes of fluorescence emission spectra of Bodipy-Hy (10 μM) caused by various ROS, anions and common metal ions (200 μM) in phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution were record in Fig. 2. Bodipy-Hy itself showed a weakly fluorescence emission (Φ_F=0.06). The addition of HClO induced a significant enhancement of the fluorescence emission spectra (Φ_F=0.62). However, representative species such as H_2O_2, ·OH, $^1O_2$, $^-O_2$, TBHP, TBO·, NO·, K^+, Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, F^-, I^-, CO_3^{2-}, AcO^-, NO_3^-, NO_2^-, SO_4^{2-}, exhibited almost no changes in the fluorescence spectra indicating that Bodipy-Hy was highly selective toward HOCl.

Conditional experiments

Effect of fraction of water on the interaction of Bodipy-Hy with HOCl in 0.1 M phosphate buffer-ethanol solution was investigated. Among various fraction of water tests, a combination of H_2O-ethanol (v/v, 1:1) proved to be highly efficient for the sensing process (Fig. S1, ESI†). Therefore, we choose H_2O-ethanol (v/v, 1:1) as our test system.

For practical application, the fluorescence intensity response of Bodipy-Hy in the absence and presence of HOCl in different pH values were evaluated in Fig. S2, ESI†. Increased fluorescence intensity (Φ_F=0.22) of Bodipy-Hy was observed at strong acidic condition, which was likely due to the H^+-induced hydrolysis of the C=N bond. But it remained stable and weakly fluorescent (Φ_F=0.06) in a comparatively wide pH range from 5.00 to 10.60. On the other hand, the fluorescence response of the probe towards the addition of HOCl was indeed pH dependent. Bodipy-Hy displayed an efficient fluorescence response to HOCl in the pH range of 5.0-10.0, the fluorescence enhancement was significantly greater at physiological pH 7.2 which indicated that Bodipy-Hy was highly suitable for biological applications.

Fig. 3 shows the reaction of Bodipy-Hy with HOCl was particularly fast and the fluorescence intensity reached its maximum value at about 3 min. Therefore, a 3 min reaction time and a medium of 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution were selected in subsequent experiments in order to make the reaction of Bodipy-Hy with HOCl sufficiently.

Linearity

To further investigate the interaction of HOCl with Bodipy-Hy, the fluorescence titration experiment was carried out in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution. As shown in Fig. 5, the free Bodipy-Hy showed a weakly fluorescence emission intensity (Φ_F=0.06) which

can be attributed to the C=N bond isomerization. As envisioned, the increase in the fluorescence emission intensity was proportional to the concentration of HOCl over a range (0-22.5 μM) with a good linear correlation and the minimum amount of HOCl that can be detected under these conditions was evaluated to be 56 nM based on $3\times\delta_{blank}/k$ (where δ_{blank} is the standard deviation of the blank solution and k is the slope of the calibration plot). The regression equation is Y=147.96573+66.40288X (R=0.9962) (Fig. S3, ESI†). The fluorescence emission intensity reached its maximum when 30 μM of HOCl was added, with an enhancement factor over 11-fold. The relative fluorescence quantum yields were determined to be 0.62 with Rhodamine B (Φ_F=0.97) in ethanol as a standard and calculated using the following equation[32].

$$\Phi_x = \Phi_s \left(\frac{F_x}{F_s}\right)\left(\frac{A_s}{A_x}\right)\left(\frac{\lambda_{exs}}{\lambda_{exx}}\right)\left(\frac{n_x}{n_s}\right)^2$$

Where subscripts X and S refer to the unknown and the standard, Φ stands for quantum yield, F represents integrated area under the emission curve, A is the absorbance intensity at the excitation wavelength, λ_{ex} exhibits the excitation wavelength, n is index of refraction of the solution.

Proposed mechanism

The proposed mechanism of Bodipy-Hy with HOCl was shown in scheme 2. The result of the reaction-based sensing process could be easily monitored using Thin Layer Chromotography (TLC) (Fig. S4, ESI†). After the reaction of Bodipy-Hy with HOCl, a green fluorescent compound was appeared which indicated that a new BODIPY derivate (BODIPY-AL) was formed.

Subsequently, ^{1}H NMR spectra was used to demonstrate the formation of BODIPY-AL, in which the hydrazine group was converted to an aldehyde one (Fig. 6).

Tolerance of Bodipy-Hy to HOCl over other interferent

The competitive experiment was implemented to analyze the influence of other ROS and metal ions on the reaction of Bodipy-Hy with HOCl. As shown in Fig. 7, the change of fluorescence emission intensity (Φ_F=0.62) caused by HOCl with background species together such as H_2O_2, ·OH, $^{1}O_2$, $^{-}O_2$, TBHP, TBO·, NO·, K^+, Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, F^-, I^-, CO_3^{2-}, AcO^-, NO_3^-, NO_2^-, SO_4^{2-} was similar to that caused by HOCl alone. The results indicated that the recognition of HOCl by Bodipy-Hy was hardly affected by other coexisting ROS, anions and metal ions.

Laser scanning confocal microscopy experiments of PC12

Depending on the promising properties of Bodipy-Hy, we next questioned its potential for monitoring HOCl in living cells. We investigated the cytotoxicity of Bodipy-Hy by MTT assay with PC12 cells. As shown in Fig. 8, the cellular viability was estimated to be greater than 90 % after 24 h, which indicated that Bodipy-Hy (< 50 μM) has low cytotoxicity. Furthermore, PC12 cells were incubated at 37 °C first with Bodipy-Hy (10 μM) for 30 min which exhibited non-fluorescence (Fig. 9b), followed by the addition of HOCl (20 μM) and then incubated for another 30 min. After three times washed with 0.1 M phosphate buffer solution, bright green fluorescence was observed in PC12

cells (Fig. 9e). More importantly, throughout the cell imaging process the cells were undamaged and showed a healthy spread and adherent morphology (Fig. 9a, c, d, f). The above facts indicated that Bodipy-Hy showed excellent cell membrane permeability and can be efficiently used for in vitro imaging of HOCl in living cells.

Conclusion

In summary, we have designed and synthesized a novel "turn-on" and low-cost BODIPY derivate (Bodipy-Hy) for highly selective and sensitive detection of HOCl over other ROS and common metal ions in phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution. The above results show that Bodipy-Hy with HOCl have lower detection limit and a wide linear range under physiological conditions. Besides the rapid and specific response to HOCl, confocal fluorescence microscopy imaging demonstrated that this probe can also be applied to monitor HOCl in living cells.

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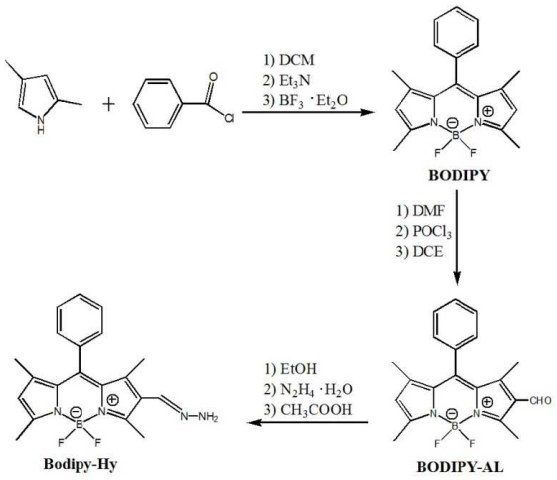


Scheme 1. Synthesis of Bodipy-Hy

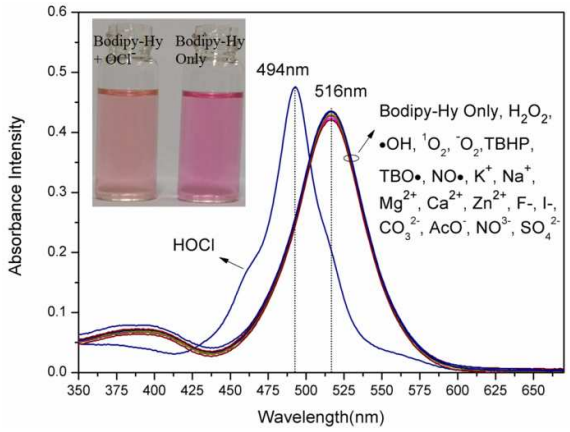


Fig. 1. UV-Vis absorbance spectra of Bodipy-Hy (10 μM) in phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution upon addition of different ROS and metal ions (200 μM).

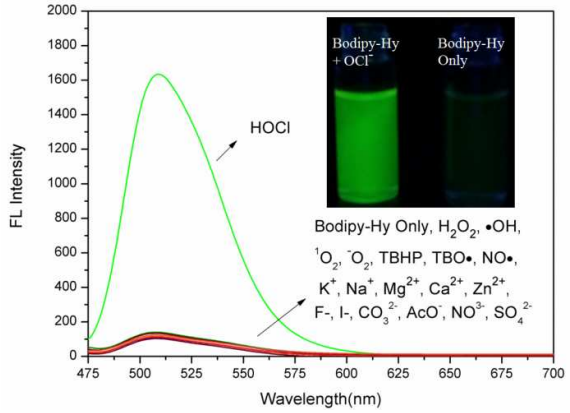


Fig. 2. Fluorescence spectra of Bodipy-Hy (10 μM) in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution upon addition of different ROS and metal ions (200 μM). (λ_{ex}=465 nm, λ_{em}=510 nm at 25 °C).

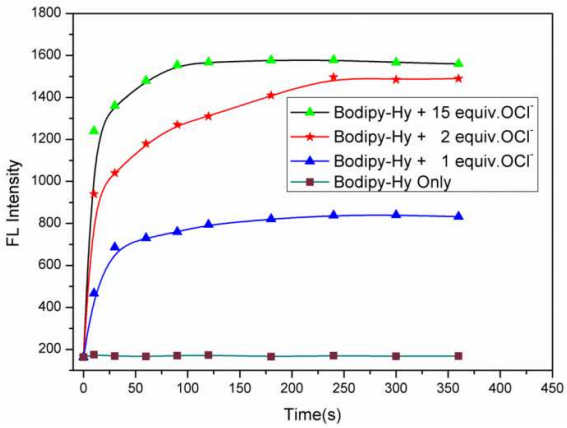


Fig. 3. The time courses of fluorescence intensity of Bodipy-Hy (10 μM) with different concentrations of HOCl (0, 10, 20, 150 μM) in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution (λ_{ex}=465 nm, λ_{em}=510 nm at 25 °C).

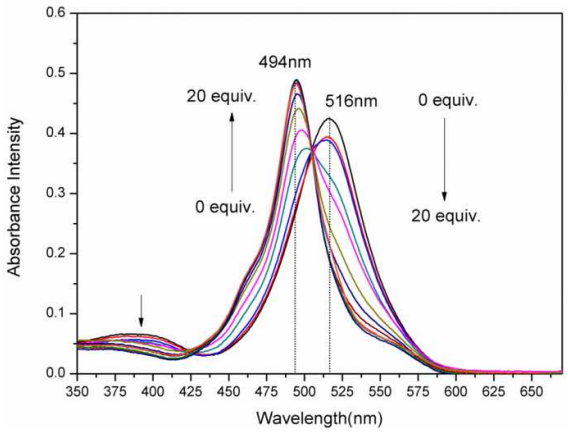


Fig. 4. Absorbance spectra of reaction solution of Bodipy-Hy (10 μM) in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution with different concentrations of HOCl.

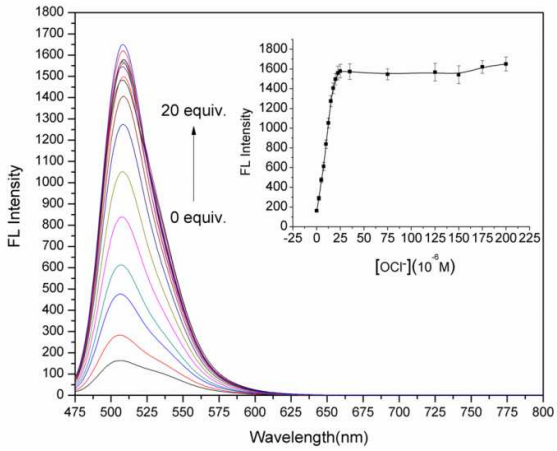


Fig. 5. Fluorescence intensity changes of Bodipy-Hy (10 μM) against HOCl concentration from 0 to 22.5 μM in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution (λ_{ex}=465 nm, λ_{em}=510 nm at 25 °C).

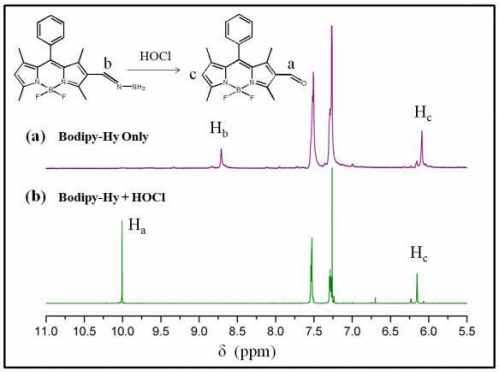
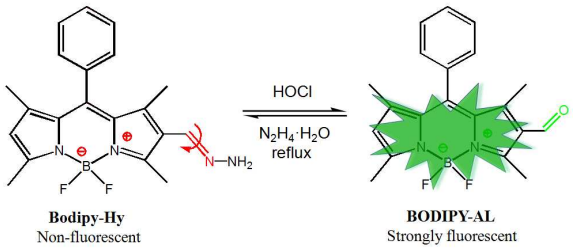


Fig. 6. Partial ^{1}H NMR spectra of (a) Bodipy-Hy, (b) Bodipy-Hy + HOCl.



Scheme 2. Proposed mechanism of Bodipy-Hy with HOCl.

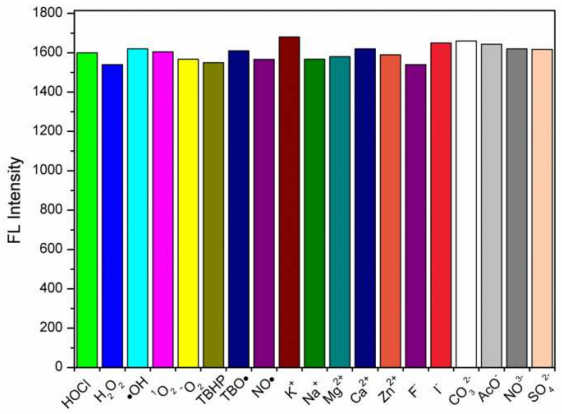


Fig. 7. Fluorescence intensities of Bodipy-Hy (10 μM) in 0.1 M phosphate buffer-ethanol (pH 7.20, v/v, 1:1) solution upon addition of HOCl (200 μM, 20 equiv.) in the presence of background species (200 μM, 20 equiv.) (λ_{ex}=465 nm, λ_{em}=510 nm at 25 °C).

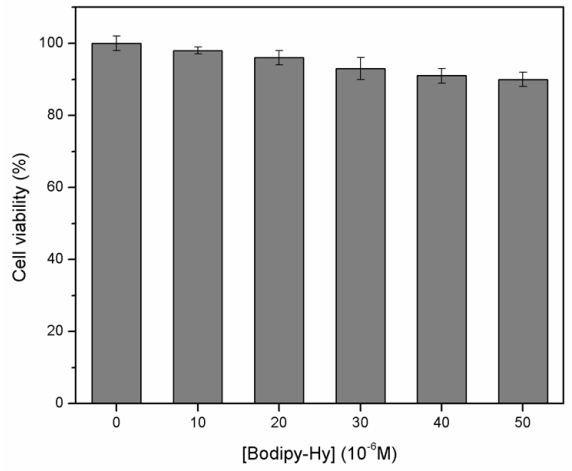


Fig. 8. Viabilities of the PC12 cells incubated with different concentrations of Bodipy-Hy for 24h.

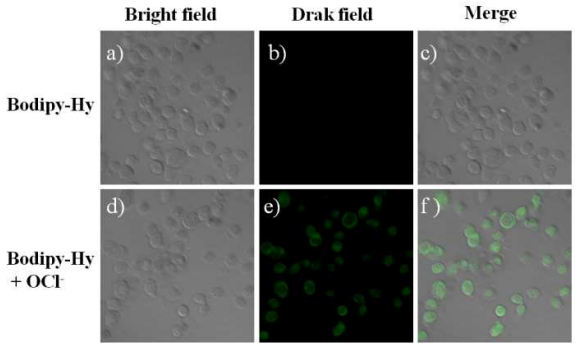


Fig. 9. Confocal fluorescence images of PC12 cells. (a) and (d) Bright-field image; (b) and (e) fluorescence image; (c) and (f) Overlay image. (a-c) PC12 cells incubated with probe Bodipy-Hy (10 μM) for 30 min. (d-f) Then incubated with HOCl (10 μM) for 30 min (λ_{ex}=488 nm).