Analytical Methods

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An Efficient Ratiometric Fluorescent Excimer Probe for Hypochlorite based on a Cofacial Xanthene-bridged Bispyrene

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ARTICLE TYPE

An Efficient Ratiometric Fluorescent Excimer Probe for Hypochlorite based on a Cofacial Xanthene-bridged Bispyrene

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In this paper, by employing a rigid xanthene scaffold as a bridge, we for the first time report a cofacial bispyrene derivative **DPH** as an efficient ratiometric fluorescent excimer probe for hypochlorite (OCl^-). The probe is comprised of a rigid xanthene scaffold and two parallel pyrenes, which are linked by an OCl^--sensitive dicarboxylic acid hydrazide group. The introduction of OCl^-, however, will induce the oxidation of the dicarboxylic acid hydrazide moiety into a diimide group, and the subsequent hydrolysis of the diimide, to give 1-pyrenecarboxylic acid which exhibits a monomer emission at about 380 nm, with its intensity increasing with the addition of increased concentration of OCl^-. Meanwhile, the excimer emission intensity is gradually decreased. Such a ratiometric fluorescent response of the probe affords a high sensitivity to OCl^-, with a linear response concentration range from 1 to 300 μM, and a detection limit of 0.35 μM for OCl^-. It also shows a high selectivity for OCl^- with no interference observed from other common anions and small-molecules. Moreover, it can also act as a colorimetric probe for OCl^- due to the cyan-to-blue fluorescence color change. It has been preliminarily used for practical detection of OCl^- in river water samples with satisfying results.

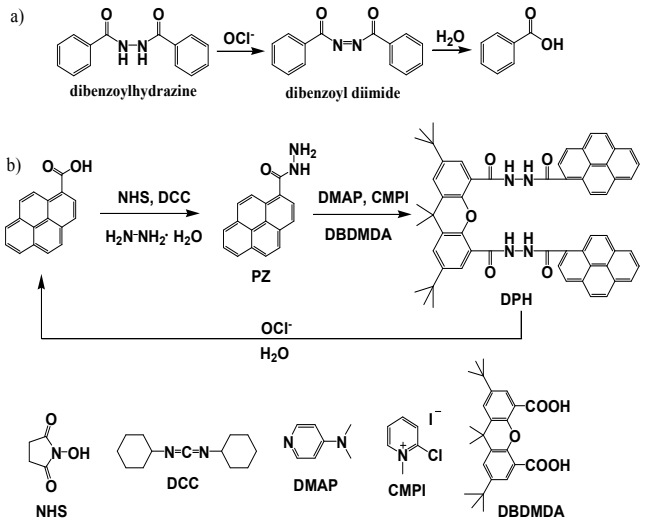
Introduction

Anions play important roles in biological and chemical systems, so there is a rising interest in the design of probes that are able to selectively recognize anions. [1-3] Hypochlorite (OCl^-) is one of the most important reactive oxygen species (ROS) in biological processes and plays a crucial role in vivo due to its antibacterial properties. [4-6]Additionally, hypochlorite is widely used in our daily life as a disinfectant or as a bleaching agent. However, water disinfected with high concentrations of hypochlorite may result in eye or nose irritation and stomach discomfort. Excessive production of OCl^- can lead to numerous diseases and damage to tissues, such as rheumatoid arthritis, cardiovascular diseases, atherosclerosis, renal disease, lung injury and cancers. [7-13] So far, much attention has been paid to the studies of the biological application and harmful effects of OCl^-. Thus the development of efficient methods for direct detection of OCl^- is of considerable importance, and has become a current focus in chemical research.

Among the available techniques for detect of analytes, fluorescent methods have been extensively pursued due to their simplicity, versatility, highly sensitive, fast analysis and non-destructive advantages. In the past two decades, quite a few fluorescent probes based on target-triggered single emission intensity changes, or ratiometric intensity changes of two emissions, have been developed for detection of various analytes. [1-3, 14-33] Probes based on single emission intensity changes may be affected by a variety of factors such as instrumental efficiency and environmental conditions, as well as the concentration of probe molecule. Fortunately, these interferences can be eliminated by employing ratiometric fluorescent probes, [19-33] which involve the observation of ratiometric changes of the fluorescent intensities at two wavelengths upon addition of targets, and could provide built-in correction for eliminating environmental effects. Several strategies, including internal charge transfer (ICT), [19-23] fluorescence resonance energy transfer (FRET), [24-31] and two fluoroionphores designs, [32-33] have been adopted to design ratiometric fluorescent probes. Unfortunately, for most FRET systems, to achieve the largest energy transfer efficiency, sufficiently large spectral overlap is necessary between the donor emission and the acceptor absorption, which would limit the wavelength difference between the two emission peaks (e.g., the difference for the classical fluorescein-rhodamine dye pair is fixed at ~65 nm). Further improving the fluorescence intensity detection will need use dye pairs with less spectral overlap, which will result in reduced energy transfer efficiency in some extent, and a decreased signal-to-background ratio (SBR) for the probe. Thus, new strategy for design of ratiometric probes with large SBR is desired.

As a classic fluorophore, pyrene has been well known for its monomer and excimer emissions. [34-35] Pyrene monomer exhibits a characteristic emission at about 380 nm. However, when two pyrenes are brought into close proximity in a cofacial manner, a broad excimer emission centered at about 490 nm will be observed due to the interaction of an excited pyrene (Py*) unit with a ground-state pyrene (Py) unit, along with a concomitant

decrease of the monomer emission intensity. [36-37] This unique characteristic of pyrene provides a novel strategy for design of efficient ratiometric fluorescent probes with a large wavelength difference of 110 nm between the two emission peaks with large SBR and high sensitivity. However, even though pyrene has been widely applied to design fluorescent small molecular probes for various targets, [38-45] most of them are based on the change of its single monomer emission. Since the excimer emission of pyrene will be observed only when two pyrenes exist in a cofacial manner within an enough short distance. As a result, the development of satisfactory ratiometric fluorescent probes based on the switch between the monomer and excimer emission of pyrene is practicable but still an attractive and challenging.



Scheme 1 (a) Schematic of the OCl^--induced oxidation of dibenzoylhydrazine into dibenzoyl diimide. (b) Chemical structure and synthetic route of **DPH**.

It is reported that OCl^- can specifically oxidize the dibenzoylhydrazine to be cleaved into dibenzoyl diimide, which can further undergo a decomposition process in some nucleophilic solvents (Scheme 1a). [46] In this work, by employing this specific reaction as a recognition event, and a rigid xanthene scaffold as a bridge, we for the first time report a cofacial bispyrene derivative **DPH** (2, 7-diterbutyl-9, 9-dimethylxanthene-4, 5-dipyrenebenzoylhydrazine) as an efficient ratiometric fluorescent excimer probe for OCl^- (Scheme 1b). The probe is comprised of a rigid xanthene scaffold and two parallel pyrenes, which are linked by an OCl^--sensitive dicarboxylic acid hydrazide group. The two pyrene units in the probe molecule will form a cofacial excimer structure due to the rigidity of xanthene scaffold and the steric hindrance of the pyrene. In this case, a strong excimer emission at about 490 nm and a weak monomer emission at about 380 nm could be observed. The introduction of OCl^-, however, will induce the oxidation of the dicarboxylic acid hydrazide moiety into a diimide group, and the subsequent hydrolysis of the diimide, to give 1-pyrenecarboxylic acid which exhibits a monomer emission at about 380 nm, with its intensity increasing with the addition of increased concentration of OCl^-. Meanwhile, the excimer emission intensity at about 490 nm is gradually decreased. Such a fluorescent probe shows two well-separated emission bands for ratiometric sensing of OCl^- with high sensitivity and high selectivity, with a detection limit of 0.35 μM observed for OCl^-. Moreover, it can also be acted as a colorimetric probe for OCl^- due to the cyan-to-blue color change between the excimer and the monomer of pyrene. The practical application of the new probe was then evaluated by determination of recovery of spiked OCl^- in river water samples with satisfactory result.

Experimental section

Reagents and Apparatus

Solvent were supplied by commercial suppliers, and were used without further purification. Water used in all experiments was doubly distilled and purified by a Milli-Q system. All fluorescence measurements were performed on a Hitachi F4500 fluorescence spectrometer. LC-MS analyses were carried out by using an Agilent 1100 HPLC/MSD. Column chromatography was conducted over silica gel (200-300 mesh) and thin layer chromatography (TLC) was carried out using silica gel 60 F254, both of which were obtained from the Qingdao Ocean Chemicals (Qingdao, China). ^1H and ^{13}C NMR spectra were respectively measured on a Bruker DRX-400 spectrometer operating at 400 and 100 MHz. The pH value of buffer is detected by Metteler teledo delta 320 pH meter. N-Hydroxysuccinimide (NHS), 2-chloro-1-methylpyridiniumiodide (CMPI), Dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 1-pyrenecarboxylic acid, 2,7-diterbutyl-9,9-dimethylxanthene-4,5-dibenzioc acid (DBDMDA) are all obtained from Alfa Aesar company. The stock solutions of OCl^-, Ac^-, H_2O_2, SiO_3^{2-}, CN^-, SCN^-, PO_4^{3-}, $P_2O_7^{4-}$, HS^-, SO_3^{2-}, SO_4^{2-}, $S_2O_8^{2-}$, NO_2^-, NO_3^-, F^-, Cl^-, ClO_2^-, ClO_3^-, ClO_4^-, Br^-, I^-, IO_4^-, $Cr_2O_7^{2-}$, MnO_4^- were prepared by dissolving their sodium salts in distilled water, respectively.

Compounds **DPH** were efficiently synthesized following the synthetic methodology shown in Scheme 1b.

Synthesis of PZ

To a 50 mL flask, 1-pyrenecarboxylic acid (246 mg, 1.0 mmol), DCC (412 mg, 2.0 mmol) and NHS (230 mg, 2.0 mmol) were dissolved in CH_2Cl_2 (10 mL).The mixture were stirred at room temperature for 2 hours. When pyrene formic acid completely converted to NHS-ester intermediate (monitoring by TLC), hydrazine monohydrate (85%) was then added, and the resulting reaction mixture was stirred at room temperature for another 2 hours. Then the solution was diluted with CH_2Cl_2 (20 mL), washed with water (10 mL×3), the organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by chromatography on a silica gel column with a mixture of CH_2Cl_2/EtOH (50:1) as the mobile phase, to afford **PZ** as a pale yellow solid (150 mg, 58%). ^1H NMR (400 MHz, DMSO) δ (ppm): 4.910(s, 2H), 8.121(t, J=18.0 Hz, 2H), 8.227(d, J=8.8 Hz 1H), 8.273(d, J=12.4, 2H), 8.346(t, J=18.8 Hz, 3H), 8.535(d, J=9.2Hz, 1H), 9.925(s, 1H). ^{13}C NMR (100 MHz, DMSO) δ (ppm): 123.813, 123.974, 124.607, 124.881, 125.545, 125.827, 126.011, 126.781, 127.391, 128.261, 128.520, 130.397, 130.656, 130.893, 131.854. MS: [M+]=260.2

Synthesis of DPH

PZ (78 mg, 0.3 mmol), DBDMDA (42 mg, 0.1 mmol), DMAP (122 mg, 1.0 mmol), CMPI (123 mg, 0.5 mmol) were dissolved in dry CH_2Cl_2 (10 mL), and the reaction mixture was refluxed for

4 hours under N_2 atmosphere. Then the solution was diluted with CH_2Cl_2 (100 mL), washed with water (10 mL×3), the organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by chromatography on a silica gel column with a mixture of CH_2Cl_2/EtOH (50:1) as the mobile phase, which afforded target probe **DPH** as a pale yellow solid (49 mg, 54%). ^{1}H NMR (400 MHz, DMSO) δ (ppm): 1.36(s, 18H), 1.42(s,6H), 6.68(s,4H), 6.88(s, 8H), 7.43(d, J=8Hz, 1H), 7.65(d, J=8Hz, 1H), 7.91(m, J=24Hz, 5H), 8.04(d, J=8Hz, 1H), 8.19(d, J=8Hz, 1H), 8.57(d, J=8Hz, 1H). ^{13}C NMR (100 MHz, DMSO) δ (ppm): 30.722, 31.439, 34.673, 34.780, 120.093, 123.404, 123.640, 123.877, 124.617, 125.197, 125.685, 125.868, 126.471, 126.806, 127.424, 128.325, 128.416, 128.607, 129.148, 130.079, 130.461, 130.628, 131.849, 139.478, 145.360, 146.229, 151.775, 165.339, 168.429. MS: [M$^-$]=893.1.

Spectrophotometric Experiments

Both the UV-vis absorption and fluorescence measurement experiments were conduct in ethanol aqueous solution. The fluorescence emission spectra were measured at excitation wavelength 344 nm and emission wavelength range from 360 to 600 nm. The excitation and emission slits width set up at 5.0 nm. A 10 μM stock solution of **DPH** was prepared by dissolving **DPH** in ethanol. A stock standard solution of OCl^- (0.01 M) was prepared by dissolving an suitable amount of NaOCl in water, which was further diluted to standard solution of 2.0×10^{-6} - 2.0×10^{-4} M stepwise with HEPES buffer solution (pH=7.4). The solution was kept at 4°C and protected from light for further use. The complex solution of OCl^-/**DPH** was prepared by adding 500 μL of the stock solution of **DPH** and 500 μL of the stock solution of OCl^- in a 10 mL volumetric flask. Then obtaining mixture of concentrations were 5×10^{-6} M for **DPH** and 1×10^{-6} M for OCl^-. The mixture was placed at room temperature for 0.5 hour in order to ensure **DPH** to react with hypochlorite anion completely. A blank solution of **DPH** was prepared under the same conditions without OCl^-.

Results and discussion

Optimized Design and Synthesis of the DPH Probe

To observe the excimer emission of pyrene, two pyrenes should exist in a cofacial manner within an enough short distance. It is reported that OCl^- is able to specifically oxidize dibenzoylhydrazine into dibenzoyldiimide, which can further be hydrolyzed into two benzoic acids. On the base of this reaction, we have designed a new ratiometric fluorescent probe **DPH** for OCl^- (Scheme 1). The probe is comprised of a rigid xanthene scaffold and two parallel pyrenes, which are linked by an OCl^--sensitive dicarboxylic acid hydrazide group. The two pyrene molecules in the probe molecule will form a cofacial excimer structure due to the rigidity of xanthene scaffold and the steric hindrance of the pyrene, which results in an excimer emission at about 490 nm. The introduction of OCl^-, however, will induce the oxidation of the dicarboxylic acid hydrazide moiety into a diimide group, and the subsequent hydrolysis of the diimide, to give 1-pyrenecarboxylic acid (see Mass spectrum in supporting information: the reaction of DPH with OCl^- give a product with a molecular weight of 246, which is the same as 1-pyrenecarboxylic acid) which exhibits a monomer emission at about 380 nm, with its intensity increasing with the addition of increased concentration of OCl^-. Meanwhile, the excimer emission intensity at about 490 nm will gradually decrease, and therefore affords ratiometric fluorescence response for OCl^-.

Spectrophotometric Response of the DPH Probe

The absorption spectrum of **DPH** is shown in Figure 1. Free **DPH** shows the typical pyrene monomer absorption band at about 340 nm. In addition, a remarkable low-energy band could be observed at about 450 nm, which should be attributed to the pyrene excimer, demonstrating the two pyrenes in **DPH** existence in a cofacial manner within an enough short distance. Upon the addition of 20 equiv of OCl^- to the solution of **DPH**, no obvious change was observed around the pyrene monomer absorption. However, the pyrene excimer absorption at about 450 nm disappeared, indicating the destroying of the pyrene excimer. This result confirms that OCl^- could promote the change of cofacial excimer manner of two pyrene units to pyrene monomer in solution.

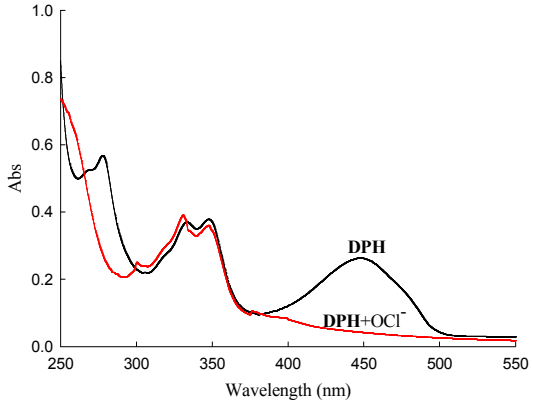


Fig. 1 UV-vis spectra of chemosensor **DPH** (5 μM) in absence (black) and presence (red) of OCl^- (20 equiv) in EtOH.

To investigate the analytical performance of the probe to OCl^-, the fluorescence changes of probe **DPH** to OCl^- in EtOH/HEPES (pH=7.4, 1:1) were then recorded. Figure 2a show the fluorescence spectra of the probe **DPH** exposed to aqueous EtOH solution containing different concentrations of OCl^-, respectively. The spectrum of **DPH** without OCl^- exhibits an obvious broad emission at 495 nm of pyrene excimer, and weak characteristic emission of pyrene monomer at 375 nm. The two pyrene units in the probe molecule form a cofacial excimer structure due to the rigidity of xanthene scaffold and the steric hindrance of the pyrene, which achieves the interaction of one excited pyrene (Py*) unit with the other ground-state pyrene (Py) unit under optical excitation to afford a strong excimer emission at about 490 nm. However, upon the introduction of OCl^- in the buffered sensing system, the excimer emission of pyrene at 495 nm decreases, and emission band corresponded to the pyrene monomer with a maximum at 375 nm increases (Figure 2a), indicating that the OCl^- can prompt the transfer of pyrene excimer to its monomer form. Experimental results showed that the addition of OCl^- to

the probe **DPH** could result in a remarkable increase of fluorescence signal at 375 nm and the decrease of that at 495 nm. The emission intensity ratio, I_{375}/I_{495}, was gradually increased from 0.14 to 21.36 with the concentration of OCl^- changing from 0 to 300 μM, corresponding to a largest SBR of 152.6 due to the two well-separated emission bands. The large SBR of the probe is favorable for a high sensitivity for OCl^- detection. The probe exhibits a linear response concentration range for OCl^- from 0 to 300 μM (Figure 2b), with a detection limit of 0.35 μM (3σ/slope).

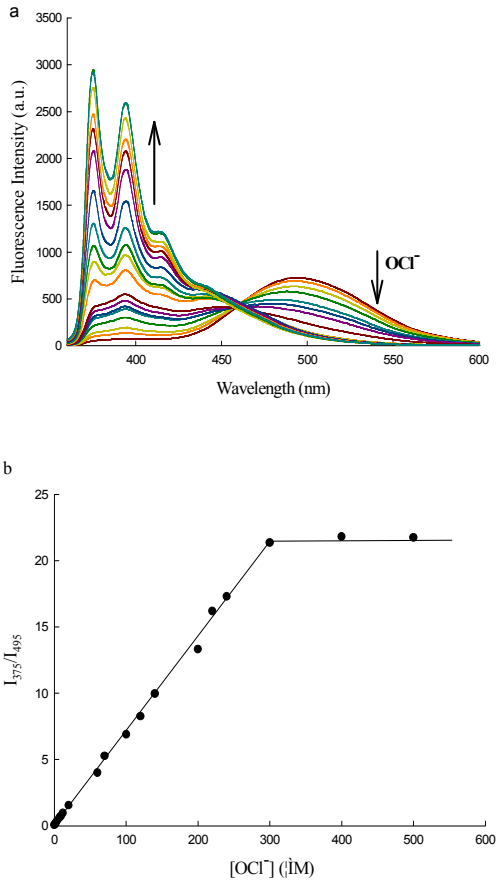


Fig. 2 (a) Ratiometric fluorescence emission spectra of **DPH** (5 μM) in the presence of different concentrations of OCl^- (1×10^{-6}-5×10^{-4} M) in buffer (pH=7.4) aqueous EtOH solution. (b) Calibration curve of the **DPH**. The curve was plotted with the fluorescence intensity ratio (I_{375}/I_{495}) vs OCl^- concentration. (λ_{ex} =344 nm)

Selectivity is a very important parameter to evaluate the performance of a new fluorescent probe. In the present work, the oxidization of OCl^- was employed as recognition event for the design of probe **DPH**, which should theoretically possess a higher selectivity toward OCl^- than that of coordination-based ones. We tested the selectivity of the probe **DPH** by recording the fluorescent emission with excitation at 344 nm (Figure 3). The addition of 50 μM of OCl^- into the sensing system could induce a significant enhancement of fluorescence ratio at I_{375}/I_{495} , while various other anions (Ac^-, SiO_3^{2-}, CN^-, SCN^-, PO_4^{3-}, $P_2O_7^{4-}$, HS^-, SO_3^{2-}, SO_4^{2-}, $S_2O_8^{2-}$, NO_2^-, NO_3^-, F^-, Cl^-, ClO_2^-, ClO_3^-, ClO_4^-, Br^-, I^-, IO_4^-, $Cr_2O_7^{2-}$, MnO_4^-) and H_2O_2 did not induce obvious changes of fluorescence ratio. To test the practical applicability of our probe for OCl^-, competition experiments were also carried out. 100 μM of competing anions are added to 50 μM of OCl^- and the fluorescence change of the probe are then detected, with results shown in Figure 3 (the gray bar portion). Before and after the addition of other interfering anions and small molecule, the probe showed almost unchanged responses to OCl^-.

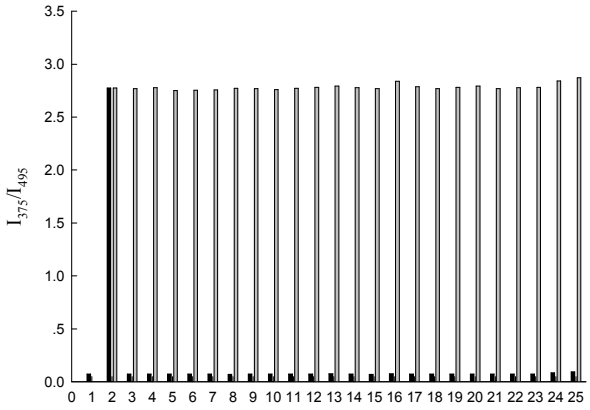


Fig. 3 Fluorescence response of **DPH** (5μM) to 50 μM of OCl^- or 100 μM of other anions (the black bar portion) and to the mixture of 100 μM of other anions with 50 μM of OCl^- (the gray bar portion). 1, blank; 2, OCl^-; 3, Ac^-; 4, H_2O_2; 5, SiO_3^{2-}; 6, CN^-; 7, SCN^-; 8, PO_4^{3-}; 9, $P_2O_7^{4-}$; 10, HS^-; 11, SO_3^{2-}; 12, SO_4^{2-}; 13, $S_2O_8^{2-}$; 14, NO_2^-; 15, NO_3^-; 16, F^-; 17, Cl^-; 18, ClO_2^-; 19, ClO_3^-; 20, ClO_4^-; 21, Br^-; 22, I^-; 23, IO_4^-; 24, $Cr_2O_7^{2-}$; 25, MnO_4^-.

Naked eye observation is a powerful mean for fast qualitative analysis. **DPH** can also act as a colorimetric probe for naked eye detection of OCl^-. We found that blank **DPH** (5.0 μM) in buffer (pH=7.4) aqueous EtOH solution was colorless along with a cyan fluorescence emission (Figure 4), indicating the two pyrenes existence in a cofacial manner within an enough short distance to form a pyrene excimer. Various other anions (NO_3^-, Ac^-, SO_4^{2-}, IO_4^-, CN^-, F^-, ClO_3^- had been chosen as samples) and H_2O_2 did not induce obvious color and fluorescence color changes. However, the introduction of OCl^- could induce a pale yellow of the solution and a blue fluorescence color, which corresponded to the pyrene monomer characteristics.

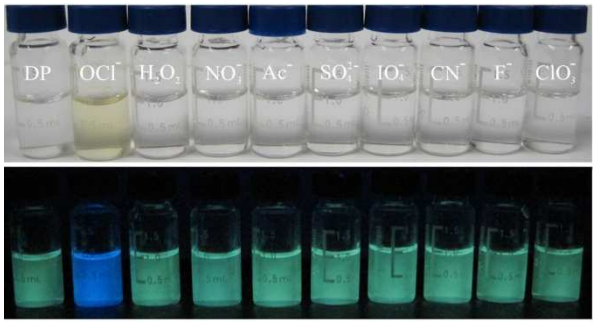


Fig. 4 Change in color (top) and fluorescence (bottom) of compound **DPH** (5.0 μM) in buffer (pH=7.4) aqueous EtOH solution of OCl^-, some anions and small molecule.

Because of the protonation of the probe and anions in the acidity condition, the pH value of the environment around the fluorescent probe for anions usually shows somewhat of effect on

its performance. In order to verify the capability of the probe for detection of OCl^- under physiological condition, we investigated the effect of pH on its response to OCl^-. The experiments were carried out at a pH range from 5.0 to 9.0. As shown in figure 5, for free **DPH**, the fluorescence intensity ratio at I_{375}/I_{495} almost did not vary with the pH value in the range of 5.0-9.0, suggesting that the excimer form was stable in this pH range. Upon the addition of OCl^-, the emission intensity ratio at I_{375}/I_{495} of probe **DPH** almost did not vary with the pH value in the range of 6.5-7.5, suggesting that the oxidation reaction is conducted perfectly at neutral condition, and the probe is favorable for detection of OCl^- under physiological condition.

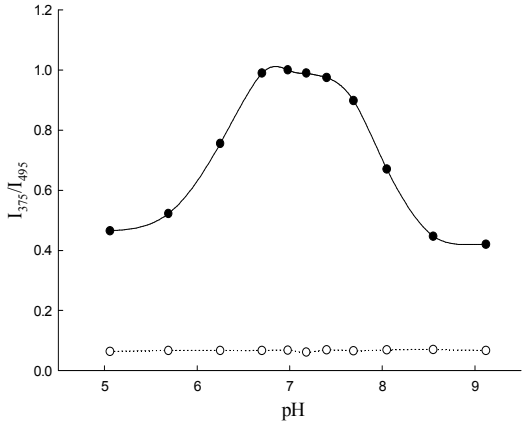


Fig.5 Effect of pH on the fluorescence intensity at 375 nm to that at 495 nm of free **DPH** (5 μM) (dashed line) and after addition of 12 μM OCl^- (solid line).

The practical application of the new probe was then evaluated by determination of recovery of spiked OCl^- in river water samples. The river water samples were obtained from Xiang river (Changsha, China). The samples collected were simply filtered and showed that no OCl^- was present. OCl^- stock solution at different concentrations was spiked in these samples, and the **DPH** was then employed to detect its concentration, with analytical results shown in Table 1.

Table 1 Recovery study of spiked OCl^- in river waters with proposed sensing system

Sample	OCl^- spiked (M)	OCl^- recovered (M) mean[a] ± SD[b]	Recovery (%)
River water 1	0.0	0.0	-
River water 2	2.0×10^{-6}	$(1.93\pm 0.04)\times 10^{-6}$	96.5
River water 3	5.0×10^{-6}	$(4.91\pm 0.05)\times 10^{-6}$	98.2
River water 4	2.0×10^{-5}	$(2.01\pm 0.03)\times 10^{-5}$	100.5
River water 5	1.0×10^{-4}	$(9.73\pm 0.08)\times 10^{-5}$	97.3

[a] Mean of three determinations. [b] SD: standard deviation.

One observed that the results obtained in real water samples show good recovery values, which confirmed that the proposed probe was applicable for practical OCl^- detection in real samples with other potentially competing species co-existing.

Conclusions

In summary, we have designed and synthesized a novel ratiometric fluorescent probe **DPH** for OCl^- by using pyrene as a fluorophore. The probe is comprised of a rigid xanthene scaffold and two parallel pyrenes, which are linked by an OCl^--sensitive dicarboxylic acid hydrazide group. Such a fluorescent probe shows two well-separated emission bands for ratiometric sensing of OCl^- with high sensitivity and high selectivity. It can also be acted as a colorimetric probe for OCl^- due to the cyan-to-blue fluorescence color change, and the practical application of the new probe is satisfactory. Furthermore, the design mechanism of our probe might provide a universal platform for developing excellent ratiometric fluorescent excimer probes with cleavable active bonds for other analytes.

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Notes and references

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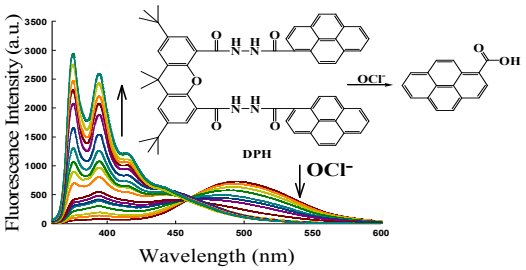

We report a cofacial bispyrene derivative as an efficient ratiometric fluorescent excimer probe for hypochlorite (OCl^-).