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PAPER

A ratiometric fluorescent probe for cyanide based on FRET†

Xin Lv, Jing Liu, Yunlong Liu, Yun Zhao, Maliang Chen, Pi Wang and Wei Guo*

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On the basis of FRET from 4-(*N*,*N*-dimethylamino)benzamide to fluorescein, a new ratiometric fluorescence probe bearing a hydrazone binding unit was developed for highly selective and sensitive detection of CN^- in aqueous solution.

Introduction

Anion recognition is an area of growing interest in supramolecular chemistry due to its important role in a wide range of environmental, clinical, chemical, and biological applications.[1] Among the various anions, cyanide is one of the most important anions due to it being widely used in synthetic fibers, resins, herbicides, and the gold-extraction process.[2] Unfortunately, the cyanide anion is extremely detrimental and can be absorbed through the lungs, gastrointestinal track and skin, leading to vomiting, convulsion, loss of consciousness, and eventual death.[3] Thus, there is a need for an efficient sensing system for cyanide, to monitor the cyanide concentration from contaminant sources.

In the past few years, a variety of colorimetric and fluorescent probes for cyanide have been reported. One of the common approaches is utilizing cyanide complexes or addition with Zn^{2+}–porphyrin,[4a] Ru^{2+}–pyridine,[4b] boronic acid derivatives[4c] or CdSe quantum dots.[4d] Other strategies have also been used, such as hydrogen bonding interactions,[5] copper–cyanide affinity[6] and single-electron transfer reaction.[7] Recently, nucleophilic addition of cyanide has also been adopted for sensing cyanide. This type of recognition mode takes advantage of a particular feature of the cyanide ion: its nucleophilic character, and enables the recognition system with some characteristic features such as CN^--specific response and little competition from the aqueous media. Based on this idea, nucleophilic addition of cyanide to oxazine, pyrylium, squarane, trifluoroacetophenone, acyltrazene, acridinium, salicylaldehyde and carboxamide has been reported,[8] in which the interference by other anions, such F^- and AcO^-, can be efficiently minimized.

Fluorescence spectroscopy has become a powerful tool for sensing and imaging trace amounts of samples because of its simplicity and sensitivity.[9] However, most of the reported examples of fluorescent sensing of CN^- function by fluorescence quenching or fluorescence enhancement.[4b,4d,6a,8e,8h,8j–8l,10] 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. The ratiometric method, measuring the ratio of fluorescence intensities at two wavelengths, provides an alternative approach that can overcome the drawbacks of intensity-based measurements by built-in correction of two emission bands and seems to be more favorable for sensing target ions in comparison with fluorescence intensity-based probes.

In general, 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. However, these spectra-shift type probes, in many cases, show relatively broad emission spectra with a high degree of overlap before and after binding target ions, which makes it difficult to accurately determine the ratio of the two fluorescence peaks. Theoretically, the problem can be avoided by using a FRET-based sensor for which the single excitation wavelength of a donor results in emission of the acceptor at a longer wavelength. However, up to now, although some ratiometric fluorescence probes for CN^- have been reported in the literature,[11] to our knowledge, ratiometric fluorescence probes for CN^- based on FRET are considerably limited.[11e]

Herein, we present a 4-(*N*,*N*-dimethylamino)benzamide–fluorescein FRET "off-on" system **1** as a ratiometric fluorescence probe for CN^-. The probe was designed to involve three parts: a 4-(*N*,*N*-dimethylamino)benzamide energy donor, a salicylaldehyde hydrazone binding unit,[12] and a fluorescein spirolactone unit (potential fluorescein monoanion energy receptor). It is expected that nucleophilic attack by cyanide toward the activated hydrazone functionality, followed by fast proton transfer of the acidic phenol proton to the developing nitrogen anion of this compound, will trigger ring-opening in the fluorescein spirolactone unit to generate a long-wavelength fluorescein monoanion fluorophore[13] that can act as the energy acceptor (Fig. 1). Although the efficiency of FRET is affected by the distance between the donor and the acceptor and the relative orientation of transition dipoles of both the donor and acceptor, it is mainly determined by the extent of the spectral overlap between the donor emission and the acceptor absorption.[14] Thus, 4-(*N*,*N*-dimethylamino)benzamide was chosen as the energy donor in our work, because its fluorescence

School of Chemistry and Chemical Engineering, Shanxi University, Taiyuan 030006, China. E-mail: guow@sxu.edu.cn; Tel: +86 351 7011600

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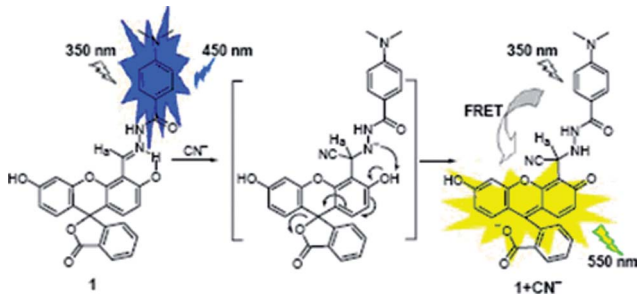


Fig. 1 A plausible mechanism of response of **1** to CN^-.

spectrum matches well with the absorption spectrum of fluorescein monoanion, fulfilling a favorable condition for FRET (Fig. 2). In the absence of CN^-, the fluorescein moiety adopts a closed, non-fluorescent spirolactone form. As a result, FRET is suppressed, and only the blue emission of the donor is observed upon excitation of the 4-(*N*,*N*-dimethylamino)benzamide chromophore. Binding to CN^- induces opening of the fluorescent fluorescein monoanion moiety, corresponding to intense absorption in the 4-(*N*,*N*-dimethylamino)benzamide emission region. Spectral overlap is enhanced, and excitation of the 4-(*N*,*N*-dimethylamino)benzamide chromophore results in strong emission of fluorescein monoanion owing to FRET.

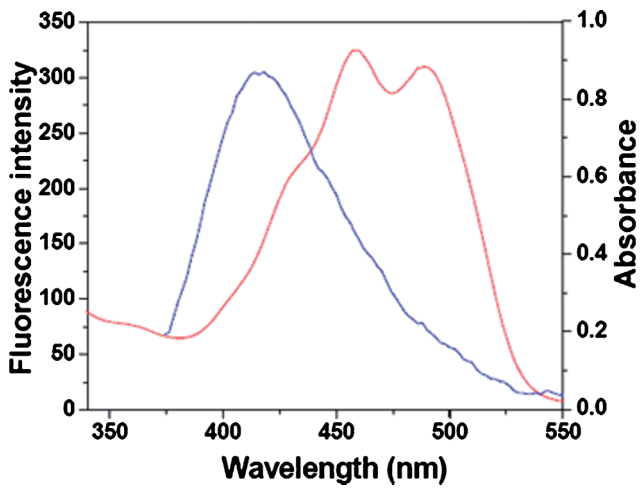
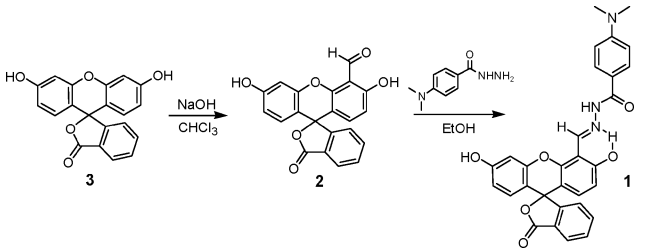


Fig. 2 Spectral overlap of 4-(*N*,*N*-dimethylamino)benzoylhydrazine emission (blue) with fluorescein absorption (red).[15]

Results and discussion

Probe **1** is synthesized *via* a Reimer–Tiemann formylation reaction of fluorescein followed by condensation with 4-(*N*,*N*-dimethylamino)benzoylhydrazine in refluxing ethanol (Scheme 1). The experimental details and characterization data for **1** are given in the Experimental section and ESI.† CN^- detection using probe **1** was found to be sensitive to water content. Increasing the water content resulted in a decrease in the fluorescence intensity of the **1**–CN^- complex. In view of the solubility and sensitivity of **1**, a DMF–water (9 : 1, v/v) solution was used in the subsequent investigation. The time course studies of the absorption and fluorescence responses in DMF–water (9 : 1, v/v) solution revealed that the binding process of CN^- to **1** was very fast at room



Scheme 1 Synthesis of compound **1**.

temperature, and could complete within 5 s (Fig. S1†), suggesting the rapid detection ability of **1** for CN^-.

Changes in the UV-vis spectra for **1** in the absence and presence of CN^- in DMF–water solution (9 : 1, v/v) are shown in Fig. 3. The UV-vis spectrum of **1** shows mainly the absorption profile of the donor (4-(*N*,*N*-dimethylamino)benzamide group), which has a maximum at 350 nm. In addition, a weak absorption band at 515 nm is also observed, which resulted from the trace ring-opened form of the fluorescein unit in **1**. This result suggests that **1** exists mainly in the fluorescein spirocyclic form. The characteristic peak of the spirocycle carbon (C-9) shift of **1** in the alkyl region (near 81 ppm) in the ^{13}C NMR also supports this consideration (Fig. S10†). When CN^- was introduced into the sensing system, the band at 350 nm was attenuated and the band at 515 nm sharply increased with the increase in the CN^- concentration, indicating that the addition of CN^- can promote the formation of the ring-opened form of **1** from the spirocyclic form. The absorption stabilized after the amount of added CN^- reached 20 equiv. and a significant color change from colorless to brown could be easily observed by eye. The absorption titration profile at 515 nm *versus* concentration of CN^- is shown in Fig. 3 inset. The association constant for CN^- was estimated to be 4.78×10^4 M^{-1} ($R^2 = 0.9974$) on the basis of nonlinear fitting of the titration curve, assuming 1 : 1 stoichiometry.[16] Job plot analysis of the UV-vis titrations revealed a maximum at about 0.5 mole fraction (Fig. S2†), in accord with the proposed 1 : 1 binding stoichiometry.

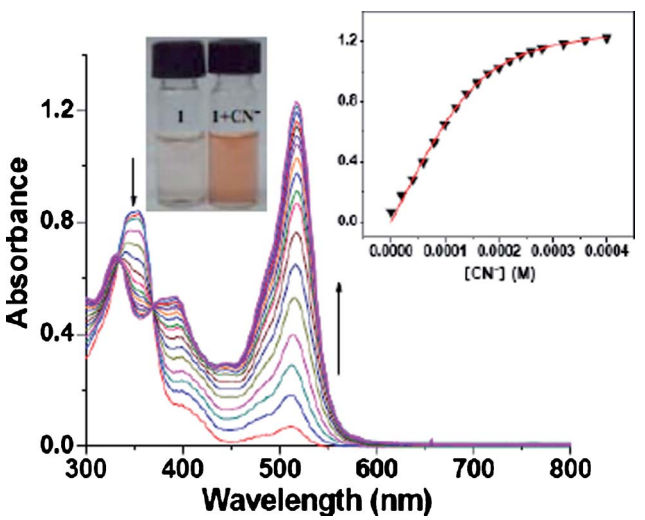


Fig. 3 Change in absorption spectra of **1** (20 μM) measured in DMF–water (9 : 1, v/v) upon addition of CN^-. Inset: absorption titration profile (at 515 nm) *versus* concentration of CN^- for **1**.

Fig. 4 shows the fluorescence spectra of **1** exposed to DMF–water solution (9 : 1, v/v) containing different concentrations of CN^-. The spectrum of **1** without CN^- exhibits a blue emission at 450 nm of donor (4-(*N*,*N*-dimethylamino)benzamide group) and a weak yellow emission at 530 nm of energy acceptor. Similar to the case of absorption spectrum of **1**, the latter can be ascribed to the trace ring-opened form of fluorescein unit in **1**. Upon addition of CN^-, the donor emission at 450 nm decreased, and the acceptor emission band gradually increased in intensity with a substantial red shift from 530 nm to 550 nm, indicating that the configuration transformation of the fluorescein moiety from the spirocyclic form to a ring-opened form. These changes in the fluorescence spectrum stopped and the ratio of the emission intensities at 550 nm and 450 nm (I_{550}/I_{450}) became constant when the amount of CN^- added reached 26 equiv., and the emission intensity ratio, I_{550}/I_{450}, varied from 0.60 to 16.6. The color of the fluorescence changed from blue to yellow. It was clear that the FRET process was switched on by CN^- as excitation of the 4-(*N*,*N*-dimethylamino)benzamide group at 350 nm resulted in the emission of fluorescein with a maximum of 550 nm. In addition, the excitation spectra obtained by collecting emission data of **1**–CN^- adduct (**1** + 20 equiv. CN^-) at 550 nm shows the donor and the acceptor bands (Fig. S3†), respectively, indicating that both the transitions participate in the emission process and an efficient energy transfer can occur from the 4-(*N*,*N*-dimethylamino)benzamide donor to the fluorescein monoanion receptor. Of note, the large wavelength shift (200 nm) between the donor excitation and the acceptor emission can eliminate any influence of excitation backscattering effects on the fluorescence assay. When examining the emission changes at 550 nm upon gradual addition of CN^-, a good linear working range from 0–90 μM was observed (Fig. S4†). The detection limit was measured to be 4.4×10^{-7} M at S/N = 3. According to the World Health Organization (WHO), cyanide concentrations lower than 1.9 μM are acceptable in drinking water.[17] This means that our proposed fluorescent method based on probe molecule **1** is sensitive enough to monitor cyanide concentrations in drinking water.

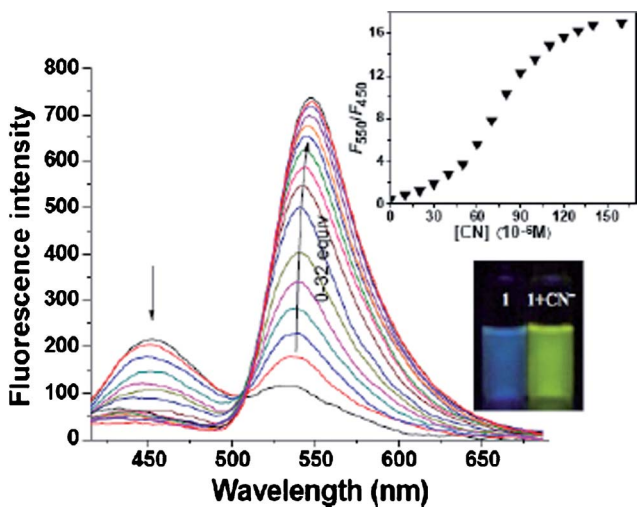


Fig. 4 Fluorescence titration spectra of **1** (5 μM) in DMF-water (9 : 1) upon gradual addition of CN^-. Inset: Fluorescence intensity ratio changes (I_{550}/I_{450}) of **1** upon gradual addition of CN^-. λ_{ex} = 350 nm. Slits: 5 nm/5 nm.

The spectral response of **1** in the absence and presence of CN^- at different pH values was also evaluated (Fig. 5). At pH values lower than 3, no obvious characteristic absorption of the fluorescein chromophore could be observed regardless of the presence and absence of CN^-, indicating that no reaction occurs between **1** and CN^-. This is possibly because the protonation of CN^- decreases the actual concentration of CN^- in the sample solution. Between pH 4 and 8, free **1** showed a weak absorption band at 515 nm due to the trace ring-opened form of the fluorescein unit in **1**. However, upon addition of CN^-, **1** responded stably to CN^- without any interference by protons. At pH > 8, the spectral response also occurred upon addition of CN^-, but the absorbance of free **1** increased with increasing pH. This could be ascribed to the deprotonation of the phenol OH group in **1** in basic conditions, resulting in the ring-opening of the spirolactone moiety of **1**. The results indicate that **1** can successfully react with CN^- and allows CN^- detection at a range of pH values (pH 4–8).

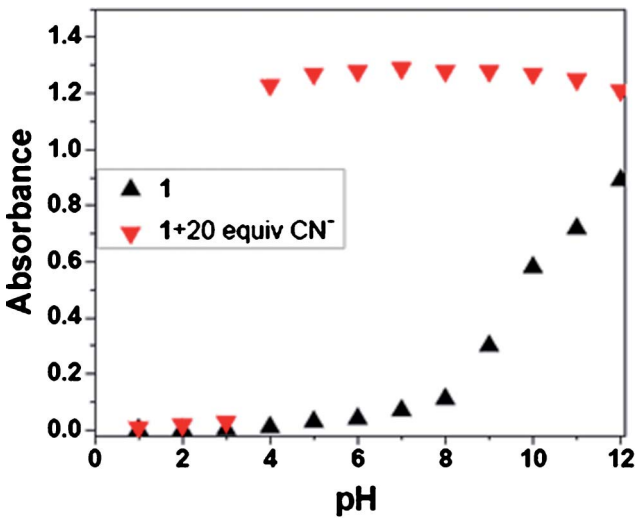


Fig. 5 Changes in absorption (515 nm) of **1** (20 μM) in DMF–water (9 : 1, v/v) measured with and without CN^- (20 equiv.) as a function of pH.

Subsequently, the selectivity of **1** for CN^- was evaluated (Fig. 6). Changes in the fluorescence spectra of **1** caused by CN^- and miscellaneous anions generally used in the literatures, including F^-, Cl^-, Br^-, I^-, AcO^-, N_3^-, SCN^-, ClO_4^-, NO_3^-, SO_4^{2-}, and $H_2PO_4^-$ as their sodium salts, are recorded in Fig. 6a. Most of the competitive anions did not lead to significant fluorescence changes, whereas F^-, AcO^- and $H_2PO_4^-$ had a slight interference due to the deprotonation reaction of **1** by these weakly basic anions to partly trigger the ring-opening of the fluorescein unit. For anions, such as Cl^-, Br^-, SO_4^{2-} and NO_3^-, which can be present in higher concentration in practice, no significant interference was observed even if their concentration reached 10 mM (Fig. S5†). For more basic anions, such as PO_4^{3-} and CO_3^{2-}, the interference is significant due to the deprotonation reaction (Fig. S6†). In addition, the competition experiments were also measured by addition of 25 equiv. of CN^- to the aqueous solutions of **1** in the presence of 25 equiv. of miscellaneous anions except for PO_4^{3-} and CO_3^{2-} (Fig. 6b). The competitive anions had no obvious interference with the detection of CN^-, indicating the system was hardly affected by these coexistent anions.

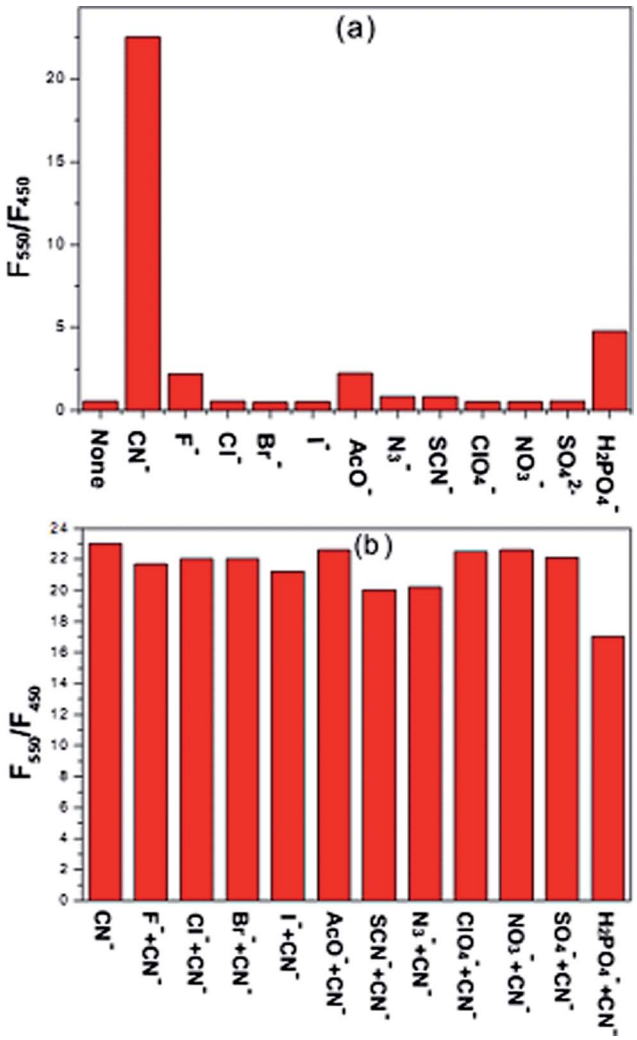


Fig. 6 (a) Fluorescence responses of **1** (5 μM) upon addition of 25 equiv. of various other anions in DMF–water (9 : 1) solution. (b) Fluorescence responses of **1** (5 μM) to CN^- (25 equiv.) containing 25 equiv. of various anions. λ_{ex} = 350 nm. Slit: 5 nm/5 nm.

With regards to the mechanism, the nucleophilic attack of CN^- to hydrazone functionality in **1** followed by the ring-opening of the spirocyclic moiety of fluorescein (Fig. 1) is likely to be responsible for the spectral change. The result obtained from a Job plot supports the formation of a 1 : 1 **1**–CN^- adduct (Fig. S2†). In addition, a clear isosbestic point at 368 nm and a well-defined isoemissive point at 506 nm in the absorption and fluorescence spectra, respectively, upon addition of CN^- to the solution of **1** indicates that the binding of **1** with CN^- produces a single component. Furthermore, we investigated the ^{1}H NMR spectra of **1** in the presence of CN^- and compared it with that of the sensor itself (Fig. 7). The imine proton (H_a) at around δ 9.22 ppm was dramatically shifted upfield toward δ 5.60 ppm upon CN^- addition, indicating that the CN^- functions as a nucleophile. Also, upon addition of CN^- to the DMSO-d_6 solution of **1**, the signal of C-9 (spirocyclic carbon atom, 81 ppm) disappeared (Fig. S7†), which supports the ring-opening of the spirocyclic structure of **1** due to the nucleophilic attack of the CN^- anion.[18] The solid evidence of the **1**–CN^- interaction mode comes from the MS spectra of the adduct of **1** with CN^-, in which the peaks at *m*/*z*

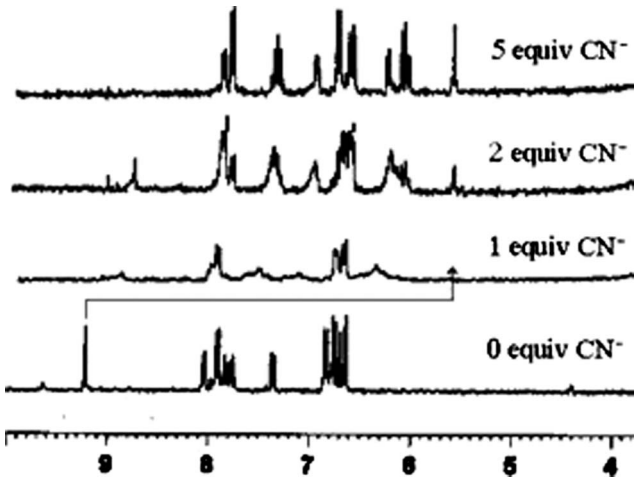


Fig. 7 ^{1}H NMR spectral change of **1** in DMSO-d_6 upon addition of cyanide anions.

547.0 (calcd = 547.1) corresponding to [**1** + CN^-]$^-$ was observed (Fig. S8†). All of these results are consistent with our proposed mechanism.

Conclusions

In summary, we have developed a FRET-based ratiometric probe **1** that can selectively and sensitively detect CN^- in aqueous solution. It exhibits a clear CN^--induced change in the intensity ratio of the two well-separated emission bands of 4-(*N*,*N*-dimethylamino)benzamide group and fluorescein. The significant changes in the color and fluorescence can be observed by the naked eye.

Experimental section

Materials and general methods

All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on an Agilent 8453 spectrophotometer. Fluorescence spectra were taken on Varian Cary Eclipse fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer.

Synthesis of compound 2

Fluorescein-monoaldehyde **2** was synthesized according to the previous report.[19] Fluorescein (4 g, 12 mmol), 10 mL $CHCl_3$, 6 mL MeOH and 0.06 g 15-crown-5 were placed in a 100 mL flask. Then 20 g 50% NaOH solution was carefully added while the reaction temperature was maintained at 55 °C. The mixture was stirred at this temperature for 5 h. After cooling, the mixture was acidified with 10 M H_2SO_4. The precipitates were collected and

dried *in vacuo*. Chromatography on silica gel (EtOAc : CH_2Cl_2, 15 : 85) and recrystallization in CH_3OH afforded 1.5 g product as pale yellow solid. Yield: 34%. ^{1}H NMR ($CDCl_3$) δ (ppm): 12.10 (s, 1H), 10.59 (s, 1H), 8.00 (d, J = 7.5, 1H), 7.63 (m, 2H), 7.12 (d, J = 7.2, 1H), 6.85 (d, J = 9.0, 1H), 6.76 (s, 1H), 6.58 (m, 3H).

Synthesis of compound 1

A solution of 4-(*N*,*N*-dimethylamino)benzoylhydrazine (54 mg, 0.3 mmol) and fluorescein-monoaldehyde **2** (108 mg, 0.3 mmol) in ethanol (50 mL) was heated under reflux for 3 h. After cooling, the precipitated crystals were filtered and washed with ethanol to give pure **1** as yellow solid (130 mg, 83%). Mp: > 250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.22 (s, 1H), 8.05 (d, J = 7.2, 1H), 7.73–7.98 (m, 4H), 7.37 (d, J = 7.5, 1H), 6.64–6.85 (m, 8H), 3.06 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.6, 161.4, 158.5, 151.8, 151.2, 150.2, 148.3, 141.8, 134.6, 129.2, 128.3, 125.0, 123.8, 123.1, 117.1, 112.2, 110.0, 108.4, 105.0, 101.0, 81.5; MALDI-TOF MS: calcd. for $(M + H)^+$ 522.166, found 522.109 $(M + H)^+$; Anal. calcd for $C_{30}H_{23}N_3O_6$: C, 69.09; H, 4.45; N, 8.06. Found: C, 69.11; H, 4.48; N, 8.17%.

Procedure for ion sensing

Deionized water was used throughout all the experiments. All anions were prepared from their sodium salts. A stock solution of **1** (5 mM) was prepared in DMF. The stock solution of **1** was then diluted to the corresponding concentration (20 μM, 5 μM) with the solution of DMF–water (9 : 1, v/v). The sodium cyanide stock solution of 1.0×10^{-1} M was diluted to 1.0×10^{-2} M and 1.0×10^{-3} M with deionized water for spectra titration studies. Spectra data were recorded in an indicated time after the addition.

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