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ARTICLE TYPE

Fluorescent Probe based on Hydroxylnaphthalene 2-Cyanoacrylate : Fluoride Ions Detection and its Bio-imaging in Live Cell

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A novel rationmetric fluorescent probe based on hydroxylnaphthalene 2-cyanoacrylate was developed to act as a luminescent probe for biological fluoride ions detection. The probe is responsive and highly selective for the fluoride ion over other common anions; it also exhibits a very low detection limit of 8.54 μM. The deprotonation of phenol moieties led to fluorescent change of probe. The biological applications of probe were also evaluated and it was found to exhibit low cytotoxicity, good water solubility, and membrane permeability. Probe was, therefore, employed as a sensing probe for the detection of fluoride ions in living cells.

Introduction

The design and synthesis of fluorescent probes that allow selective and sensitive detection of anions have been paid increasing attention in recent years[1]. On the one hand, fluorescent techniques offer the feasibility of fast, facile and highly sensitive detection of target analytes. On the other hand, these anions have caused adverse health and environmental problems. For example, fluoride ion has unique chemical properties and widely exists in toothpaste and pharmaceutical agents and is used for prevention of dental caries, enamel demineralization while wearing orthodontic appliances, and treatment for osteoporosis[2]. It also has been proven that fluoride ions can cause dental and bone fluorosis and diseases such as gastric and kidney disorders, urolithiasis and even death[3]. Therefore, fluoride detection in drinking water and living organs has attracted much attention[4].

Hence, there are many efforts devoted to the development of fluorescent chemical sensors for F^-. A variety of fluorescent F^- sensors based on hydrogen bonding interactions[5] and fluoride ion-induced chemical reactions [6], have been synthesized. However, the number of fluoride probes suitable for cell-imaging applications is still very limited due to the stringent requirements a probe has to meet for applications such as high selectivity for F^- in water, high cell permeability, and low/no toxicity. Thus, development of selective fluorescent fluoride probe under physiological conditions is of our great interest.

It should be noted that a variety of moieties such as amides[7], sulfonamide [8], (thio)ureas[9], phenol[10], and thiohydantoin[11] can recognize and bind with fluoride ions via hydrogen bonding. At low concentrations, F^- ions can form hydrogen bonding with the above mentioned species, whereas at relatively high concentrations they can act as a strong base to promote the deprotonation process via Brønsted acid–base interactions.

Using the same principle, we synthesized a new aqueous fluorescent fluoride ion sensor containing phenol groups (1) based on naphthalene 2-cyanoacrylate, because 2-cyanoacrylate dyes are cell permeability and have been used for amyloid[12] fluorescent detection and intracellular imaging. The experimental results show that this new sensor has excellent properties for detection of F^-.

Experiments Section

Materials

Unless otherwise stated, all the chemicals used in this study were purchased from Aldrich or Tokyo Kasei Kogyo Co., Ltd. (TCI, Tokyo, Japan). 2-(2-(2-methoxyethoxy)ethoxy)ethyl 2-cyanoacetate was synthesized according to reference 11. 6-hydroxy-2-naphthaldehyde was purchased from Sigma-Aldrich company.

The stock solutions of anions for selectivity experiments were prepared respectively by dissolving NaF, $NaNO_3$, NaBr, KCl, $NaHSO_4$, K_2CO_3, NaS_2, KI, NaN_3, Na_3PO_4, KSCN and CH_3COONa in doubly distilled water. PI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), fetal bovine serum (FBS), penicillin and streptomycin were ordered from Invitrogen (Carlsbad, CA). Prostate Cancer PC3 and HeLa cell were kindly provided by nanfang hospital. DMEM was used for PC3 cells' culture, and RPMI 1640 was used for HeLa cells' culture.

Instruments

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker Avance/DMX 400-MHz NMR spectrometer with DMSO-d_6 as solvents and tetramethylsilane as an internal reference. IR spectra were measured using a Shimadzu FTIR-8100 spectrophotometer. Melting point (mp) was measured on a

Yanaco micro-melting point apparatus. Elemental analysis was performed on an Eager 300 elemental microanalyzer. UV–vis spectra were recorded in a quartz cell (thickness: 1 cm) at room temperature using a JASCO J-820 spectropolarimeter. Fluorescence spectra were measured on FLS-920 Edinburgh Fluorescence Spectrophotometer. Fluorescent images were acquired on an Olympus FV1000 confocal laser-scanning microscope with a 60 objective lens.

Synethsis of probe 1

Into a 100 mL flask was added (3.44 g, 20 mmol) of 6-hydroxy-2-naphthaldehyde in 30 mL of dry THF. 2-(2-(2-methoxyethoxy)ethoxy)ethyl 2-cyanoacetate (4.62 g, 20 mmol) and *N*,*N*-dimethylpyridin-4-amine (0.5g, 4.1 mmol) were then added into the flask. The reaction mixture was refluxed for 24 h. The crude mixture was concentrated under reduced pressure and the product was purified via flash column chromatography (50% EtOAc in hexane). Yellow solid was obtained in 94% yield (7.24 g). Mp 137.0~138.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6):δ 3.22 (s, 3H, CH3), 3.37~3.44(m, 4H, CH2CH2O), 3.52~3.56(m, 10H, OCH2CH2, CH2CH2O, CH2), 3.60~3.63(t, J =4.52Hz, 2H, CH2), 3.74~3.76 (m, 2H, CH2O), 4.40~4.43 (m, 2H, COOCH2), 7.23 (s, 2H), 7.85~7.88(d, J =8.82Hz, 1H, Ar), 7.88~7.90(d, J =9.51Hz, 1H, Ar), 8.13~8.16(dd, J = 1.39, J = 8.32Hz, 1H, Ar), 8.42 (s, 1H, Ar), 8.45 (s, 1H, CH=C), 10.45(s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 57.98(OCH_3), 65.32(OCH_2), 68.06(OCH_2), 69.59(OCH_2), 69.73(OCH_2), 69.90(OCH_2), 71.24(OCH_2), 99.49, 109.19, 116.09(CN), 120.03, 124.76, 131.52, 135.22, 137.22, 155.27, 158.80, 162.34(COO). IR (cm^{-1}, KBr): 3330, 2962, 2878, 1898, 1719, 1617, 1589, 1517, 1481, 1351, 1191, 1144,930, 817, 744, 631. Elemental analysis: Anal. Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63; O, 24.91. Found: C, 65.82; H, 6.08; N, 3.71.

Absorption and fluorescence analysis

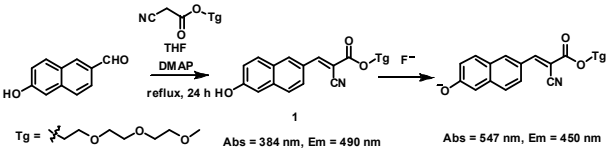
UV/Vis spectra were measured at room temperature on a JASCO J-820 with 1.0 cm glass cells. Fluorescence emission spectra were obtained at room temperature on a FLS-920 Edinburgh Fluorescence Spectrophotometer, with a Xenon lamp and 1.0 cm quartz cells. With a pipette gun (Eppendorf), taking 10 μL of the probe **1** (DMSO, 30 mM) and 100μL of NaF (DI water, 30 mM), then the solution was diluted to 10 mL with PBS buffer (pH 7.4, 10 mM) before measurement. The concentration of probe **1** was 30 μM throughout the analysis experiments except that otherwise pointed out. The fluorescence intensity was measured with the excitation wavelength 388 nm except as otherwise noted, and the excitation and emission slits were set to 1 and 1 nm, respectively.

Cell cytotoxicity assay

Cytotoxicity studies were performed using MTT assay. PC3 or HeLa Cells (10^6 cell/mL) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL well^{-1}. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 4 h. The probe was diluted to different concentrations of solution with medium and added to each well after the original medium has been removed. PC3 or HeLa Cells were incubated with probe concentrations for 24 h. The concentrations of the probe were 0 to 20 μM. 200 μL MTT solution (5.0 mg/mL, HEPES) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader.

Culture of PC3 cells for intracellular imaging

The prostate cancer (PC3) cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37□ in a 5% CO_2 atmosphere. Cells were seeded on 6 well chambered cover glass at a density of 1.5×10^6 for 24 h. Probe dissolved in DMSO (0.5 μL, 10 mM) were added to the cells medium (500 μL) at 10 μM final concentrations. After incubating for 30 min, excess probes were removed by gentle rinsing with cold phosphate buffered saline (PBS, pH 7.4) three times. The cells pre-treated with **1** were then treated with NaF (100 μM) in DMEM medium and incubated for further 30 min at 37ºC and washed with PBS buffer. To observe the subcellular distributions of the probes, the cells were treated with a PI (10 μg /mL) for additional 30 min. The media was removed and the cells were washed three times with PBS buffer (pH 7.4). Fluorescence images were collected by sequentially line scanning with an Olympus FV1000 confocal laser-scanning microscope. Fluorescence images are recorded at both green (470 ± 20 nm) and red channels (570 ± 20 nm).



Scheme 1 Design and synthesis of the probe **1**

Results and Discussion

Probe **1** was prepared by reaction of 6-hydroxy-2-naphthaldehyde with 2-cyanoacetate in THF (Scheme 1). The structure of probe **1** was confirmed from its spectroscopic and analytical data (see ESI† for details).

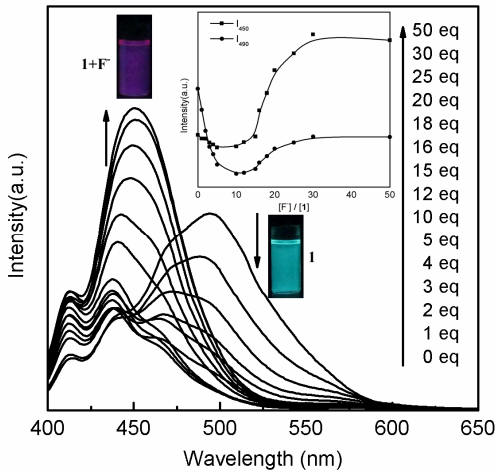


Fig 1 Fluorescence spectra of probe **1** (30 μM) upon addition of NaF (0~50 equiv.) in PBS buffer (pH 7.4, 10 mM, containing 1% DMSO). Each spectrum was recorded after 5 min. λ_{ex} = 388 nm. Slits: 1/1 nm

We first evaluated the capability of probe **1** to detect fluoride ion in PBS buffer. The titration of NaF to the probe **1** was performed in PBS buffer (pH 7.4, 10 mM, containing 1% DMSO) (Fig 1). The free probe displayed a green emission with the maximum at 490 nm; upon addition of F^-, the emission intensity at 490 nm gradually decreased with the simultaneous appearance of a new emission peak at 450 nm, indicating that the chemical reaction interrupted the π-conjugation of probe **1**. Essentially, the ratio of the emission intensities (I_{490}/I_{450}) became constant when the amount of F^- added reached 30 equiv. Furthermore, the fluorescent ratiometric values of I_{490}/I_{450} have an excellent linear relationship with the concentrations of F^- in the range of 0 ~ 10 equiv. The detection limit for F^- was determined as 8.54 μM based on S/N = 3 (Fig. S4 ESI). In the presence of 10 equiv. of F^-, a ca. 6.1-fold enhancement in the ratiometric value of I_{490}/I_{450} (from 0.51 to 3.11) is achieved compare to the F^- free solution.

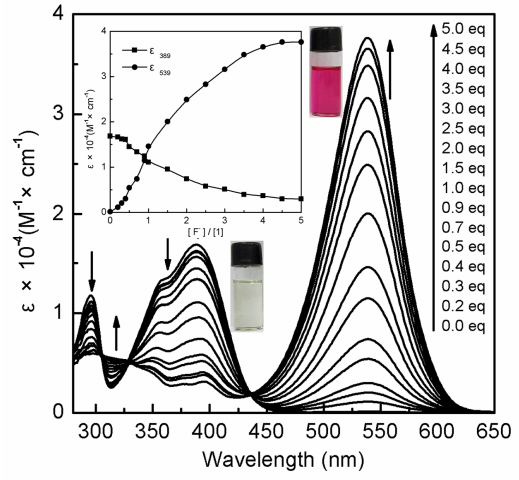


Fig 2 UV-Vis spectra of probe **1** (30 μM) upon addition of increasing concentrations of NaF (0~5 equiv) in PBS buffer (pH 7.4, 10 mM, containing 1% DMSO).

Meanwhile, the UV-Vis spectrum absorption titration experiment was also performed under the same conditions (Fig 2). The free probe was essentially colorless in PBS buffer. However, introduction of NaF induced a color change from colorless to pink. The UV-vis spectrum of probe **1** exhibited a strong absorption band around at 298nm and 382 nm. Upon gradual addition of fluoride ions to a solution of probe **1** in PBS buffer, the absorption band at 298nm and 382 nm decreased and a new band at 548 nm appeared instantly, showing a large red shift of 166 nm. Isosbestic points at 304nm, 328nm, and 438nm were observed, suggesting the chemical transformation. The spectral change almost stops upon addition of 5.0 equiv of F^-.

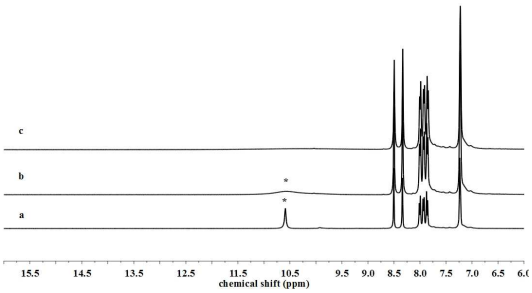


Fig 3 Partial ^{1}H NMR spectra of probe **1** upon addition of F^- in DMSO-d_6.(a) $[F^-]/[\mathbf{1}] = 0$, (b) $[F^-]/[\mathbf{1}] = 0.5$, (c) $[F^-]/[\mathbf{1}] = 1$.

The changes in the fluorescence and absorption spectra of probe **1** are ascribed to the deprotonation of the phenol moiety upon addition of F^-. To confirm this assumption, ^{1}H NMR F^- titration experiment was carried out. It was found that the phenol OH proton signal (10.45 ppm) was disappeared upon addition of 1.0 equivalent of fluoride ions (Fig 3). These observations clearly supported the deprotonation of phenol OH group to form the phenoxide ion.

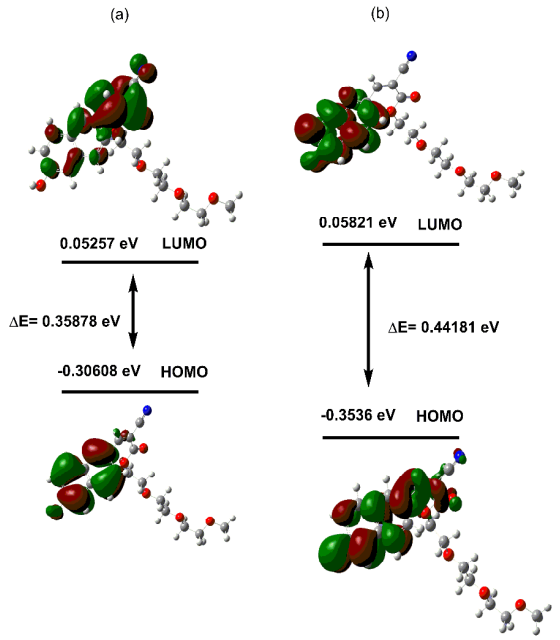


Fig 4 Frontier Molecular orbital amplitude plots of LUMO and HOMO of probe **1** before (a) and after (b) depronation by Guassian B3LYP/6-31G^{++} method.

Computational insight into the probe **1** before and after deprotonation was obtained using Gaussian 09W software package and its accompanying graphical interface program GaussView 5.0, at the B3LYP/6-31G^{++} level of theory. Fig 4 shows the HOMO and LUMO orbitals of the probe **1** before and after deprotonation. The optimized structures for the ground state (S0) and lowest lying singlet excited state (S1) of probe **1** before and after deprotonation clearly show a high degree of conjugation between the naphthalene and phenoxide ion and a hampered conjugation in between the naphthalene and phenol group. This significant difference in the π-conjugation results in an increased HOMO–LUMO energy gap of probe **1** after deprotonation compared to before deprotonation, which is responsible for the observed change in the absorption and emission spectrum of probe **1**.

Based on the excellent ratiometric fluorescent properties observed with F^-, the selectivity of the probe for F^- over other anions was examined in PBS buffer (pH 7.4, 10 mM, containing 1% DMSO). As shown in Fig 5, addition of the representative anions did not lead to any significant fluorescence changes of probe **1**. In contrast, upon treatment of probe **1** with F^-, a

dramatic fluorescence response was observed. We also examined the absorption spectrum of probe **1** in the presence of various anions (Fig S6). A new red-shifted absorption band appeared at 548 nm upon the addition of CH_3COO^- and F^-. In contrast, the absorption spectrum of probe **1** changed only slightly with the addition of other anions. The high selectivity of probe **1** towards F^- was also observable by the naked eye. When probe **1** was excited at 365 nm using a UV lamp in the presence of various species, only F^- caused an obvious fluorescence change (Fig S5 ESI).

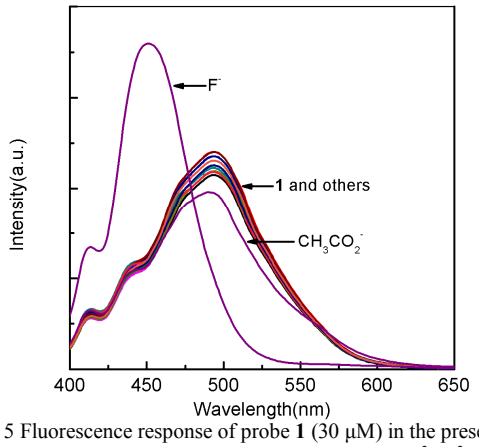


Figure 5 Fluorescence response of probe **1** (30 μM) in the presence of various anions including F^-, NO_3^-, Br^-, Cl^-, HSO_4^-, CO_3^{2-}, S^{2-}, I^-, N_3^-, PO_4^-, SCN^- and CH_3COO^-(100 μM for each) in PBS buffer (pH 7.4, 10 mM, containing 1% DMSO)..

In addition, to evaluate the cytotoxicity of probe **1**, we performed standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using HeLa and PC3 cells at concentration from 0~20μM of probe **1** for 24 h, respectively. The result clearly showed that probe **1** was nontoxic to the cultured cells under the experimental conditions (Fig. S7 ESI).

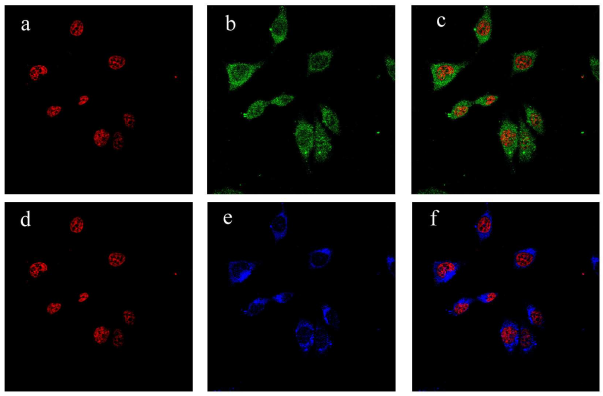


Fig. 6 Fluorescence response of the probe **1** to F^- in living PC3 cells. (a) fluorescence imaging of PC3 cells incubated with probe **1** (10 μM) for 30 min, then PI (10 μg /mL) for 30 min from the red channel(nuclear staining); (b) fluorescence imaging of (a) from the green channel; (c) overlay of (a) and (b); (d) fluorescence imaging of PC3 cells incubated with **1** for 30 min, and further incubated with NaF (100 μM) for 30 min, then PI (10 μg /mL) for 30 min from the red channel(nuclear staining); (e) fluorescence imaging of (d) from the green channel; (f) overlap of (d) and (e).

We used PC3 cells as a model cell to study the subcellular distributions of probe **1** by a confocal fluorescence microscope. To achieve intracellular images with satisfactory signal-to-noise ratios, the probes concentration of 10 μM was chosen for cell internalization study. Herein cultured PC3 cells were incubated with probe **1** (10 μM) for 30 min at 37 °C. Then a commercially nuclear-selective staining probe PI (10 μg/mL) was also used to co-stain the cells. As predicted, confocal fluorescent microscopic images showed that probe **1** with green fluorescence were cell membrane permeable and localized in the cytoplasm region (Fig 6). These results clearly showed that probe **1** had good cell membrane permeability.

In a control experiment, the cells were pretreated with probe 1 for 30 min, and further incubated with F^- (100 μM) for 30 min, eliciting an obvious fluorescence decrease in the green channel and a fluorescence increase in the blue channel (Fig 6e), in agreement with the F^- induced ratiometric fluorescence response, thus demonstrating the ability of the developed probes for use as tools to visualize F^- in live cells. The overlay of fluorescence and bright-field images revealed that the fluorescence signals were localized in the cytoplasm (Fig 6c and f). Thus, all results indicated that probe **1** was cell membrane permeable, nontoxic, and capable of detecting F^- in living cell.

Conclusions

In summary, we have reported a new fluorescent probe for F^- detection that exhibits a robust response both in vitro and in live cells. Detection of F^- is realized through distinct fluorescence changes resulting from deprotonation of phenol group via F^-. The probe displays advantages such as being easy-to-make, excellent ratiometric fluorescent response, and high selectivity. Preliminary biological experiments indicate its potential to probe F^- in biological systems.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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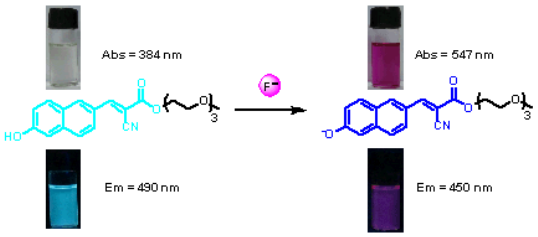
Graphical Contents



A novel rationmetric fluorescent probe based on hydroxylnaphthalene 2-cyanoacrylate shows high selectivity for fluoride ion and bio-imagines in living cell.