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Selective detections of Hg^{2+} and F^- by using tailor-made fluorogenic probes

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Graphic Abstract

Abstract:

By ingeniously using a (imino)coumarin-precursor, three reactive fluorogenic probes of **MP**, **FP**, and **FMP** have been fabricated in a single facile synthetic route. **MP** and **FP** are able to respectively act as selective “turn-on” fluorescent probes for detecting Hg^{2+} and F^- in buffer solution *via* specific analyte-induced reactions. Linear ranges for the detection of Hg^{2+} and F^- are 0-10 μM and 0-100 μM with the limits of detection (LODs) of $4.0 \times 10^{-8} \text{ M}$ and $1.14 \times 10^{-6} \text{ M}$ ($3\delta/\text{slope}$), respectively. **FMP** is able to work as a molecular “AND” logic gate-based fluorogenic probe for monitoring the coexistence of Hg^{2+} and F^- *via* a multistep reaction cascade. The analytes-induced sensing mechanisms have been determined by using high-performance liquid chromatography analysis (HPLC). In addition, three probes show negligible toxicity under the experimental conditions, and are successfully used for monitoring Hg^{2+} and F^- in living cells with good cell permeability. The success of the work demonstrates that ingenious utility of specific analyte-induced reactions and conventional concepts on the appropriate molecular scaffold can definitely deliver tailor-made probes for various intended sensing purposes.

Keywords: Coumarin; Chemosensor; Mercury; Fluoride; “AND” logic gate

1. Introduction

Mercury, one of the most toxic metal ions, can lead to the dysfunction of brain, kidney and stomach even at a low concentration because of the strong affinity of Hg^{2+} with thiophilic nature in proteins and enzymes [1]. Also, it can accumulate in astrocytes and prevent glutamate uptake, and then cause excitotoxic injury to central nervous system [2]. According to the Environmental Protection Agency (EPA) standard of the United States, the maximum allowable level of inorganic Hg^{2+} ion in drinking water is 2 ppb [3]. Hence, the rapid and accurate detection of mercury is always the research priority topic in environmental monitoring [4]. Except for the detection of toxic heavy metal ions, the recognition of anions in various samples have been also put more and more attention [5-8]. That is because that anions are ubiquitous and play important roles in chemical and biological processes, and excessive anions such as fluoride, phosphate, nitrate, cyanide are convicted as pollutants [9]. Take F^- as an example, although it has beneficial effects in dental health and osteoporosis treatment, it also can lead to severe diseases including dental and skeletal fluorosis, osteosarcoma, nephrotoxic changes and urolithiasis in humans

1 when people are exposed to high level of fluoride [10, 11]. According to the guidelines of EPA
2 (United States), the maximum contaminant level in drinking water is 4 ppm and the secondary
3 standard is 2 ppm for preventing the harmful of osteoporosis and dental fluorosis [11]. Due to
4 the high sensitivity and selectivity of fluorogenic detection, a great deal of attention has been
5 devoted to the development of novel molecular probes either for cations or anions [12-16].
6 Meanwhile, multi-functional sensors towards multi-target analysis were also frequently
7 fabricated because they are more effective and economical than one-to-one analysis [17, 18].

8 To date, a large number of chemodosimeters have been strategized for detecting Hg^{2+} based
9 on specific Hg^{2+} -induced reaction, including desulfurization [19, 20], desulfurization followed
10 by cyclization [21-23], thioacetal deprotection [24-26] and coordination-induced ring-opening
11 [27]. Meanwhile, the most common fluorogenic sensors for detecting F^- mainly based on the
12 several principles, including fluoride-triggered desilylation and intermolecular hydrogen
13 bonding between receptor and fluoride ions [15, 28]. All these mechanisms could be employed
14 to construct “turn-on” fluorescent sensors for single analyte on the appropriate fluorophore
15 scaffolds. However, it is still an atelic challenge to construct a single “turn-on” probe for two
16 distinct analytes. Only a few reported probes are able to sense Hg^{2+} and F^- synchronously [17,
17 29-36]. For instance, Li and co-authors reported a probe with quenching response towards both
18 Hg^{2+} and F^- [32]. Kumari and co-authors reported a probe, which exhibited green and yellow
19 fluorescence response towards Hg^{2+} and F^- , respectively [36]. However, both cases are not able
20 to detect the coexistence of Hg^{2+} and F^- . Indeed, the coexistence of Hg^{2+} and F^- is very common
21 in the environmental or biological samples. Recently, Kumar and co-authors presented a
22 colorimetric molecular probe which is able to monitor the coexistence of Hg^{2+} and CH_3COO^-
23 / F^- [17]. In this case, a colorimetric “AND” logic gate was constructed with Hg^{2+} and CH_3COO^-
24 / F^- as two inputs and the absorbance at 590 nm as the output, respectively. To our knowledge,
25 fluorogenic “AND” logic gate-based probes for the detection of Hg^{2+} and F^- are still lack of
26 study.

27 As well-known, fluorescent “AND” logic gate has inherent characteristic to provide
28 fluorescent output in the coexistence of multiple analytes. Therefore, “AND” logic gate-based
29 probe should be a solution to handle multiple signals and one fluorescent output. Specifically,
30 to monitor the coexistence of Hg^{2+} and F^- , a sensing system with Hg^{2+} and F^- as two inputs and
31 a “turn-on” fluorescence signal as an output is needed. Romieu has summarized that “AND”
32 logic based fluorogenic probes could be constructed based on the multistep reaction cascades,
33 where *in situ* formation of fluorophore can be mediated by two distinct analytes [37]. However,

so far, there are only few examples have been reported by literatures. As mentioned in that review, (imino)coumarin-precursors of 7-amino-/7-hydroxycoumarins might be an ideal platform to construct such logic probes. It is because that, in the (imino)coumarin precursors, several sites could be masked by trigger-recognition units, which are reactive towards multi-distinct analytes [37]. Herein, we skillfully take use of this structural feature of such precursor to develop an “AND” logic fluorogenic probe for Hg^{2+} and F^- . Benefited from the ingenious use of reasonable synthesis protocol, we certainly obtained “AND” logic fluorogenic probe **FMP** as designed (Fig. 1). Meanwhile, we also achieved two “turn-on” fluorogenic probes of **FP** and **MP** for F^- and Hg^{2+} , respectively. Further studies on the photophysics and sensing behaviors of all these probes demonstrate that *in-situ* formation of (imino)coumarin triggered by the F^- and Hg^{2+} work as expected. In particular, **FMP** not only exhibits a good fluorescence response for the coexistence of Hg^{2+} and F^- , but also could mimic the “AND” logic gate with Hg^{2+} and F^- as the two inputs. The cellular studies further confirm that this “AND” logic gate-based fluorogenic probe can perform the concomitant detection of the coexistence of Hg^{2+} and F^- in the biological samples as well.

Fig. 1. “AND” logic gate based on the fluorogenic reaction of **FMP** with Hg^{2+} and F^- .

2. Experimental

2.1. Chemical and instruments

Most of the chemicals and organic solvents are A.R. grade and were purchased from Aladdin and Sigma. The reference compound ethyl 7-diethylaminocoumarin-3-carboxylate (**Cou**, CAS number: 28705-46-6) were purchased from TCI. Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Thermo Fisher scientific. The deionized water was used in all the experiments.

Proton nuclear magnetic resonance (^1H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) were recorded on a Varian 300 MHz spectrometer. Electrospray ionization mass spectra (ESI-MS) were recorded on a Wasters SQ Detector. High performance liquid chromatography (HPLC) were run on a Gilson Unipoint system with a Gemini C18 column under neutral condition using gradient CH_3CN /water buffered with NH_4OAc as eluent. The pH was measured on a PHS-3C meter. The fluorescent spectra measurements were investigated by Hitachi F-4500 fluorescence spectrometer, respectively. For all fluorescence measurements, the excitation wavelength was 405 nm with excitation and emission slit widths of 5 nm, respectively.

2.2. Synthesis of compounds

Synthesis of **1**

A mixture 4-(diethylamino)-2-hydroxybenzaldehyde (1 g, 5.2 mmol), 4-dimethylamino pyridine (DMAP, 10 mg, 0.08 mmol) and triethylamine (576 mg, 5.7 mmol) in dichloromethane (20 mL) was stirred at 0°C for 10 min under a nitrogen atmosphere. To the mixture was added *tert*-butyldimethylsilyl chloride (860 mg, 5.7 mmol) in dichloromethane (10 mL) dropwise at same temperature. The mixture was stirred at room temperature for 12 h under a nitrogen atmosphere. After the reaction was completed, the mixture was treated with a saturated NaHCO_3 solution (20 mL). The aqueous layer was extracted with dichloromethane (20 mL $\times$ 3). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 , and concentrated in vacuum. The residue was purified by silica gel flash chromatography with heptane/EtOAc (10:1) to give off-white solid **1** (1.52 g, 95%). ^1H NMR (300 MHz, CDCl_3) δ 10.14 (s, 1H), 7.69 (d, J = 8.9 Hz, 1H), 6.32 (dd, J = 9.0, 2.4 Hz, 1H), 5.97 (d, J = 2.3 Hz, 1H), 3.38 (q, J = 7.1 Hz, 4H), 1.20 (t, J = 7.1 Hz, 6H), 1.02 (s, 9H), 0.27 (s, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 187.67, 161.27, 153.80, 130.19, 116.74, 105.82, 100.84, 44.97, 25.96, 18.59, 12.83, -3.99. MS (ESI-MS): Calcd for $\text{C}_{18}\text{H}_{29}\text{NO}_2\text{Si}$ [$\text{M}+\text{H}^+$] 308.20; Found, 308.56.

Synthesis of **FP**

To a solution of titanium tetrachloride in dichloromethane (1.0 M, 6.05 mL) was added dropwise ice-cold dry THF (10 mL) and a solution of pyridine (1.00 mL) in dry THF (1.63 mL), then added a solution of diethylmalonate (520 mg, 3.25 mmol) and compound **1** (1 g, 3.25 mmol) in 10 mL dry THF slowly. The resulting mixture was stirred at 0°C overnight, then quenched with water and extracted with dichloromethane. The combined organic extracts were washed with saturated NaHCO₃ and water, brine, dried over anhydrous Na₂SO₄, and concentrated in vacuum. The residue was purified by silica gel flash chromatography with heptane/EtOAc (30:1 to 10:1) to give yellow compound **FP** (586 mg, 50%). ¹H NMR (300 MHz, CDCl₃): δ 8.13 – 8.04 (m, 1H), 7.30 (d, J = 9.0 Hz, 1H), 4.34 (q, J = 7.1 Hz, 2H), 4.25 (q, J = 7.1 Hz, 3H), 3.34 (q, J = 7.1 Hz, 5H), 1.31 (dt, J = 8.8, 7.2 Hz, 8H), 1.17 (t, J = 7.1 Hz, 6H), 1.04 (s, 9H), 0.24 (s, 6H). ¹³C NMR (75 MHz, CDCl₃): δ 168.63, 165.56, 157.65, 151.32, 137.75, 130.24, 118.56, 105.82, 101.72, 61.40, 60.96, 44.77, 26.01, 18.51, 14.50, 14.28, 12.88, -4.05. MS (ESI-MS): Calcd for C₂₄H₃₉NO₅Si [M+H⁺] 450.26; Found, 450.77.

*Synthesis of **FMP***

To a solution of **FP** (0.5 g, 1.11 mmol), ethyl mercaptoacetate (67.0 mg, 11.1 mmol) in 50 mL of dichloromethane was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 41.3 μL, 0.2 mmol). The resulting mixture was stirred at room temperature overnight. Solvents were evaporated in vacuum and the crude product was purified directly by silica gel chromatography with heptane/EtOAc (10:1) to give light yellow oil **FMP** (527 mg, 83%). ¹H NMR (300 MHz, CDCl₃): δ 7.06 (d, J = 8.7 Hz, 1H), 6.19 (dd, J = 8.6, 2.5 Hz, 1H), 6.10 (d, J = 2.6 Hz, 1H), 4.23 (q, J = 7.1 Hz, 3H), 4.09 (q, J = 7.1 Hz, 2H), 3.97 (q, J = 7.1 Hz, 2H), 3.28 (q, J = 7.1 Hz, 5H), 3.14 (d, J = 4.5 Hz, 2H), 1.57 (s, 1H), 1.28 (t, J = 7.1 Hz, 3H), 1.23 (t, J = 7.1 Hz, 3H), 1.13 (t, J = 7.0 Hz, 6H), 1.07 (s, 9H), 1.02 (t, J = 7.1 Hz, 3H), 0.31 (d, J = 2.6 Hz, 6H). ¹³C NMR (75 MHz, CDCl₃): δ 170.93, 168.01, 167.25, 154.89, 148.71, 130.22, 115.06, 104.76, 102.52, 61.89, 61.56, 57.50, 44.83, 26.40, 18.79, 14.42, 14.14, 13.01, -3.57. MS (ESI-MS): Calcd for C₂₈H₄₇NO₇SSi [M+H⁺] 570.28; Found, 570.94.

*Synthesis of **MP***

To a stirred solution of **FMP** (15.0 mg, 0.026 mmol) in THF (3 mL) was added tetrabutylammonium fluoride THF solution (TBAF) (1 M, 26 μL, 0.026 mmol) at room temperature. The mixture was stirred for 5 hours, then water (7 mL) was added to the mixture and extracted with dichloromethane. The combined organic extracts were then dried over anhydrous Na₂SO₄. Solvents were evaporated in vacuum and the crude product was purified directly by silica gel

chromatography with heptane/EtOAc (10:1) to give yellow compound **MP** (10 mg, 85%). ¹H NMR (300 MHz, CDCl₃): δ 7.03 (d, J = 8.5 Hz, 1H), 6.72 (s, 1H), 6.21 (q, J = 2.3 Hz, 2H), 4.86 (d, J = 10.9 Hz, 1H), 4.26 (q, J = 7.1 Hz, 2H), 4.14 (q, J = 7.1 Hz, 2H), 4.06 – 3.93 (m, 2H), 3.29 (q, J = 7.1 Hz, 4H), 3.11 (q, J = 15.2 Hz, 2H), 1.29 (dt, J = 13.1, 7.1 Hz, 6H), 1.13 (t, J = 7.0 Hz, 6H), 1.05 (t, J = 7.1 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃): δ 171.11, 167.92, 167.71, 156.55, 149.70, 130.84, 110.52, 105.57, 101.27, 62.60, 62.44, 62.34, 58.01, 44.87, 44.41, 33.68, 14.66, 14.33, 13.26. MS (ESI-MS): Calcd for C₂₂H₃₃NO₇S [M+H⁺] 456.20; Found, 456.69.

2.3. Sensing studies of ions

Stock solution of three probes **MP**, **FP** and **FMP** (1 mM) was prepared in absolute EtOH. 10 mM of each metallic salt was prepared in deionized water and 10 mM of each anion salt was prepared in deionized water, respectively. The buffer solution is EtOH-HEPES (20 mM, pH = 7.4, 1:1, v/v).

20 µL probe (**MP**, **FP** and **FMP**) was placed in 1 mL tubes for dilution with the buffer solution to afford 20 µM aqueous solution. The mixture of probe/analyte was prepared in the buffer solution by adding the stock solution of probes (20 µL) and metal ions (5 equiv.) in 1 mL test tubes to afford 20 µM probes and 100 µM analytes, respectively.

2.4. Cytotoxicity assays

To investigate the cytotoxicity of three probes (**MP**, **FP** and **FMP**), an MTT (5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay was carried out over a 24 h period. HeLa cells were passed and plated in 96-well plates over night before the experiment. On the second day, three probes (0, 5, 10, 20, 30, 40, 60 and 80 µM) were added to the wells for incubation of 24 h at 37 °C under 5% CO₂. Twenty-four hours later, the medium was removed and then 50 µL of MTT (KeyGENBioTECH) solution (5 mg/mL) was added to the cells for another incubation of 4 h under the same condition. Then 150 µL DMSO was added to the cells after the MTT solution was removed. After agitated on the orbital shaker for 5 min, the absorbance of each well at 570 nm was measured. The cell viability (%) was calculated according to the following equation:

$$\text{cell viability\%} = \text{OD}_{570}(\text{sample}) / \text{OD}_{570}(\text{control}) \times 100\%,$$

where OD₅₇₀ (sample) represents the optical densities of the wells treated with various concentrations of three probes and OD₅₇₀ (control) represents that of the wells treated with

DMEM plus 10% FBS. The percentage of cell survival values are relative to untreated control cells.

2.5. Cell fluorescence imaging

The living HeLa cells (human cervical cancer cell) were provided by the School of Medicine, Tongji University (Shanghai, China) and cultured in DME supplemented with 10% FBS. One day before imaging, cells were seeded on confocal glass dishes. The next day, the HeLa cells were treated with 10 μ M three probes (**MP**, **FP** and **FMP**) for 30 min at 37 °C under 5% CO₂, then washed with PBS buffer solution (phosphate buffered saline, KeyGEN BioTECH) three times to remove the probes. And bright field and fluorescence images were taken with a leica TCS SP5 Laser Scanning Confocal Microscope. Then cells were treated with Hg²⁺ (25 μ M) and F⁻ (25 μ M) and their coexistence for another 30 min. The cells were rinsed with PBS three times again, after which the confocal images were performed under the same Confocal Microscope. The excitation wavelength is 405 nm.

3. Results and discussion

3.1. Synthesis and characterization of **MP**, **FP**, and **FMP**

Scheme 1. Synthesis of probes **MP**, **FP** and **FMP**

Recently, we reported a Cu⁺ fluorogenic sensor, in which a precursor of (imino)coumarin was caged by a specific Cu⁺ ionophore. After Cu⁺-ionophore binding, specific cleavage reaction were promoted to release the precursor, which *in-situ* formed (imino)coumarin for giving “turn-on” fluorescence [38]. Actually, if we use tert-butyldimethylsilyl as the cage moiety, a fluoride probe can be achieved in the similar scaffolds [39]. Inspired by these works, we proposed **FP**

for specific fluoride detection (in Scheme 1). Meanwhile, as pointed above, the precursor of (imino)coumarin is an interesting scaffold, where there are several sites can be masked by trigger-recognition units. For a probe for Hg^{2+} detection, a mercapto moiety can be employed to mask C=C double bond in the precursor coumarinic acid; and specific Hg^{2+} -promoted desulfurization will release coumarinic acid, which can undergo facile *in situ* formation of fluorescent (imino)coumarin [40]. This work spurred us to envision a molecule **MP**, where an ethyl mercaptoacetate was used to mask the C=C. We then conceived that this mercapto moiety could mask C=C double bond in **FP** to give **FMP** as well, which can satisfy the requirements of “AND” logic gate probe of F^- and Hg^{2+} . **FMP** then can enable the detection of coexistence of F^- and Hg^{2+} through the multi-step *in situ* formation of **Cou**. All these together, as shown in Scheme 1, we ingeniously achieved three probes in a single facile synthetic route, where the conversions of each step are acceptable.

3.2. Sensing mechanism and kinetic studies of **MP**, **FP** and **FMP** towards Hg^{2+} and F^-

As proposed above, the sensing behaviors of three probes are based on the Hg^{2+} -promoted desulfurization and F^- -triggered desilylation, which lead to the formation of fluorescent **Cou**. To experimentally confirm this mechanism, high performance liquid chromatography (HPLC) analysis were performed for three isolated probes and their resultants treated with their corresponding analytes. In addition, commercial **Cou** was used as the reference compound to determine if the sensing process will produce fluorescent **Cou** as proposed. As shown in Fig. 2, **FP**, **MP**, and **FMP** exhibit RT (retention times) at 4.16, 2.87 and 4.27 min, respectively. Upon addition of 5 equiv. of Hg^{2+} , F^- and their coexistence, **FP**, **MP** and **FMP** were almost consumed and new compound with RT at 2.49 min was generated (Fig. 2c, 2e, 2g), whose RTs are identical to that of **Cou** (Fig. 2a). These results strongly supported the proposed sensing mechanisms work well for the newly-designed sensing system (depicted in Fig. 1).

Fig. 2. HPLC chromatograms of: a) **Cou** as a reference; b) the isolated **FP**; c) **FP** with addition of F^- (5 equiv.) for 1 hour; d) the isolated **MP**; e) **MP** with addition of Hg^{2+} (5 equiv.) for 1 hour; f) the isolated **FMP**; g) **FMP** with addition of F^- (5 equiv.) and Hg^{2+} (5 equiv.) for 1 hour. The samples were analyzed by HPLC with gradient elution: 10-100% acetonitrile buffered with 10 mM NH_4OAc , flow rate 1.8 mL/min.

The stability of the probes and reaction kinetics are very important parameters for the practical application of the probes. To evaluate these parameters, the kinetic experiments were carried out by monitoring the fluorescence intensities at 470 nm in the absence and presence of Hg^{2+} and F^- . As shown in Fig. S1, it is obvious that the three probes are all stable during the measuring time. Upon addition of 10 equiv. of Hg^{2+} , the fluorescence intensity at 470 nm of **MP** enhanced gradually and remained unchanged within 10 min. In contrast, the rate of the reaction between **FP** and F^- is slower, which takes 40 min. Consequently, ca. 40 min are also need for **FMP** to complete sensing the coexistence of Hg^{2+} and F^- . Therefore, in the subsequent experiments, all spectra measurements were performed after probes exposed to analytes for 1 hour.

3.3. Selective sensing of Hg^{2+} ions by using **MP**

The fluorescent sensing ability of **MP** toward Hg^{2+} was investigated in EtOH-HEPES (20 mM, 1:1, v/v) buffer solution. First, pH-dependent experiments were carried out for ensuring the suitable pH ranges for Hg^{2+} detection. As shown in Fig. S2, isolated **MP** (20 μ M) exhibited

no obvious fluorescence in the pH range of 3.0-9.0. Upon addition of Hg^{2+} (20 μM), however, remarkably enhanced fluorescence was observed at 470 nm no matter the test solution is under acidic, neutral or alkaline conditions. It indicates the fluorescence response of **MP** towards Hg^{2+} is pH insensitive. Considering the better fluorescence properties of **MP** in neutral solution and its biological application in relevant pH range of 5.5-7.5 [41], the pH of 7.4 is chosen as the optimum condition in the following fluorescence experiments.

The titration experiments of **MP** towards Hg^{2+} were also performed in HEPES buffer solution. **MP** exhibited non-fluorescence in the absence of Hg^{2+} . With increasing concentration of Hg^{2+} , the fluorescence emission intensity at 470 nm enhanced gradually. The emission intensity stabilized after the amount of Hg^{2+} reached 10 μM with a defined emission point. As shown in Fig. 3, the fitting curve shows there is a good linear relationship ($R^2 = 0.9911$) between fluorescence intensity (470 nm) and the concentration of Hg^{2+} (0-10 μM). And the limit of detection (LOD) was determined to be $4.0 \times 10^{-8} \text{ M}$ ($3\delta/\text{slope}$), which indicates **MP** is sensitive enough for the quantitative detection of Hg^{2+} in the environmental samples. And the LOD, solution systems and biological application of **MP** were compared with those in the literature as listed in Table 1 [27, 42-49]. Obviously, **MP** has its own advantages with comparison in the detection limit and potential application in cellular imaging for Hg^{2+} .

Fig. 3. Fluorescent titration of **MP** (20 μM) with 0 to 2.5 equiv. of Hg^{2+} .

Table 1. The comparison of LOD with some reported Hg²⁺ probes.

Refs.	LOD	Solution system	Biological application
[27]	15.7 nM	MeOH-HEPES (0.4 M, pH 7, 6:4, v/v)	Yes
[42]	0.522 μM	DMF-HEPES (10 mM, pH 7.4, 3:1, v/v).	No
[43]	60.7 nM	CH ₃ CN-HEPES (25 mM pH 7.2, 2:1, v/v).	Yes
[44]	46.5 nM	HEPES (50 mM, pH 7.4)	Yes
[45]	16 nM	CH ₃ CN-HEPES (20 mM, pH 7.4, 7:3, v/v)	No
[46]	55.95 nM	DMSO medium	No
[47]	0.646 μM	water	Yes
[48]	30 nM	CH ₃ CN-HEPES (50 mM, pH 7.4, 1:1, v/v)	Yes
[49]	4.2 μM	CH ₃ CN-PBS (1 mM, pH 7.4, 4:1, v/v)	Yes
This work	40 nM	EtOH-HEPES (20 mM, pH = 7.4, 1:1, v/v)	Yes

Further, to evaluate the selectivity of **MP** towards Hg²⁺, changes in fluorescence intensity of **MP** (20 μM) in the presence of different metal ions were investigated. As shown in Fig. 4, the spectrum of isolated **MP** exhibited non-fluorescence emission. Only Hg²⁺ (5 equiv.) resulted in a significant fluorescence enhancement at 470 nm, whereas other metal ions (5 equiv.) did not induce any fluorescence changes. It shows that **MP** has a good selectivity towards Hg²⁺. Then the competition experiments were carried out and the results were shown in Fig. S3 and Fig. S4. The fluorescence emission intensity of **MP** (20 μM) in the presence of Hg²⁺ (20 μM) was almost unaffected by the other metal ions (1 mM of K⁺, Na⁺, Mg²⁺, Al³⁺, Ba²⁺, Ca²⁺, Mn²⁺ and 20 μM of other various metals ions) and anions (100 μM). Obviously, the results reveal **MP** has a specific selectivity and strong anti-interference ability towards Hg²⁺ over the other metal ions and different anions.

Fig. 4. Fluorescence spectra and intensities of **MP** (20 μM) in the absence and presence of various metal ions (100 μM). Inset: the photographs of **MP** in the absence and presence of Hg^{2+} under UV light (365 nm)

3.4. Selective detection of F^- ions by using **FP**

Similarly, the performance of **FP** for the detection of F^- was investigated using fluorescence spectroscopy under the same experimental condition as above. As shown in Fig. S5, in a wide pH range of 4.0-9.0, **FP** (20 μM) exhibited no fluorescence, but displayed an obvious fluorescence enhancement in the presence of F^- (200 μM). It shows that the maximum degree of fluorescence enhancement occurred in the pH range of 6.0-9.0. Therefore, considering consistency of experimental conditions, the neutral pH of 7.4 was chosen for F^- detection in HEPES solution. As shown in Fig. 5, upon the treatment with increasing concentration of F^- (5-1000 μM), the emission intensity of **FP** at 470 nm gradually enhanced. Additionally, there is a good linearity ($R^2 = 0.9812$) between the fluorescence intensity (470 nm) and the concentration of F^- in the range of 0-100 μM with the limit of detection of 1.14×10^{-6} M ($3\delta/\text{slope}$), which was comparable to that of the reported Hg^{2+} probes [50-58] (Table 2). Therefore, it indicates that LOD of **FP** is in the comparable range and also **FP** can determine F^- quantitatively in the linear range.

Fig. 5. Fluorescent titration of **FP** (20 μM) with 0 to 50 equiv. of F^- .

Table 2. The comparison of LOD with some reported F^- probes.

Refs.	LOD	Solution system	Biological application
[50]	0.86 μM and 4.25 μM	DMSO	No
[51]	1.0 μM	CH_3CN	No
[52]	1.9 μM	Bis-Tris solution (10 mM, pH 7.0)	No
[53]	18 μM	THF-HEPES (10 mM, pH 7.2, 1:9, v/v)	Yes
[54]	0.07 mM	EtOH-PBS (5 mM, pH 7.4, 7:3, v/v)	Yes
[55]	0.125 μM	CH_3CN -HEPES (8:2, v/v)	No
[56]	0.656 μM	DMSO	No
[57]	0.216 μM	THF	No
[58]	9.2 μM	CH_3CN -DMSO	No
This work	1.14 μM	EtOH-HEPES (20 mM, pH = 7.4, 1:1, v/v)	Yes

Then the selectivity of **FP** (20 μM) towards F^- (1 mM) was evaluated. **Fig. 6** shows the fluorescence spectra and intensities of **FP** in the absence and presence of various anions. **FP** displayed a remarkable fluorescence enhancement at 470 nm in the presence of F^- but showed nearly negligible fluorescence changes in the presence of the other competitive anions (1 mM). In addition, anti-interference performance of **FP** was also investigated. As shown in **Fig. S6**,

the F^- -triggered enhancement of fluorescence intensity was not influenced by the addition of competitive anions. Both results indicate that **FP** has a good selectivity towards F^- over other competitive anions.

Fig. 6. Fluorescence spectra and intensities of **FP** (20 μ M) in the absence and presence of various anions (1 mM). Inset: the photographs of **FP** in the absence and presence of F^- under UV light (365 nm)

3.5. “AND” logic gate-based detection of Hg^{2+} and F^- ions by using **FMP**

As described above, **MP** and **FP** respectively exhibit high selectivity and sensitivity towards Hg^{2+} and F^- . It means that two recognition moieties can work separately. Whether both recognition moiety in **FMP** can work on Hg^{2+} and F^- simultaneously is very important to perform “AND” logic gate-based detection. Therefore, the relevant experiments were carried out in subsequent studies. As shown in Fig. 8a, 8b), the isolated **FMP** displayed no fluorescence. Upon addition of Hg^{2+} or F^- individually, the fluorescence did not give any “turn-on” responses. However, upon addition of both Hg^{2+} and F^- simultaneously, the significant “turn-on” fluorescence response at 470 nm was observed. We also investigated the pH effect on the fluorescence behavior of **FMP** towards the coexistence of Hg^{2+} and F^- . As shown in Fig. S7, in the presences of Hg^{2+} and F^- , **FMP** exhibited the remarkable fluorescence enhancement in the pH range of 6.0-8.0. It is also demonstrated that the neutral system is suitable choice for monitoring the coexistence of F^- and Hg^{2+} .

Fig. 7. Fluorescent titration of **FMP** (20 μM) with 0 to 10 equiv. of both Hg^{2+} and F^- .

To further evaluate the sensitivity of **FMP** towards the coexistence of Hg^{2+} and F^- , the fluorescence titration experiments were also carried out in the buffer solution. As shown in Fig. 7, fluorescence intensity at 470 nm of **FMP** showed a gradual increase with the same concentration of Hg^{2+} and F^- increasing from 0 μM to 100 μM . And the fluorescence intensity exhibits a good linear relationship ($R^2 = 0.9909$) with the concentration of Hg^{2+} and F^- within the range of 0-100 μM (Fig. S8).

As described above, this sensing system satisfies the requirements of “AND” logic gate, where Hg^{2+} (input 1) and F^- (input 2) are served as two inputs and the fluorescence intensities at 470 nm as one output. The presence and absence of the input signals are defined as 1 (ON state) and 0 (OFF state), respectively. The fluorescence intensities higher and lower than 50 are defined as 1 (ON state) and 0 (OFF state), respectively. As a result, when the input signals are “0-0”, “1-0”, “0-1” and “1-1”, the output signals showed the response of “0”, “0”, “0” and “1”, respectively, which can represent using truth table (Fig. 8c). The results demonstrated that **FMP** could potentially be applied in the development of molecular devices in order to facilitate the detection of the coexistence of Hg^{2+} and F^- .

Fig. 8. a) Fluorescence spectra and intensities of the isolated **FMP** and **FMP** in the presence of Hg^{2+} , F^- and the coexistence of both ions; b) the photographs of the isolated **FMP** and **FMP** in the presence of Hg^{2+} , F^- and the coexistence of both ions under UV light (365 nm); c) truth table; d) general representation of AND logic gate.

3.6. Cell imaging

We next sought to evaluate the ability of three probes to monitor Hg^{2+} and F^- within living HeLa cells. To obtain the optimal concentration of probes for further imaging studies, MTT assay to investigate cytotoxicity of the three probes is necessary (Fig. S9). The HeLa cells treated with **FP** up to 80 μM for 24 hours displayed higher than 85% cell viability. Notably, the cell viability could sustain at 92% upon the treatment of 10 μM **FP** for 24 hours, which indicates that 10 μM **FP** shows no cytotoxic effect on HeLa cells in the imaging experiment. However, **MP** and **FMP** show obvious stronger cell cytotoxicity (Fig. S9). The cell viability sustains at 73% and 70% upon the treatment of **MP** and **FMP** (80 μM), respectively. This is because **MP** and **FMP** both include one more ethyl mercaptoacetate group, promoting the organic dyes go through the cell membrane more easily. Though **MP** and **FMP** have higher cell cytotoxicity, the cell viability all sustains almost at 92% upon the treatment of three probes at the concentration of 10 μM for 24 hours. It indicates 10 μM three probes can be applied for imaging Hg^{2+} and F^- in living cells with almost no cytotoxicity.

Then the imaging experiments were carried out and the results are shown in Fig. S10, S11 and 9. HeLa cells incubated with **MP** or **FP** (10 μM) for 30 min individually showed no intracellular fluorescence (Fig. S10b and S11b). However, **MP**-stained cells exposed to Hg^{2+} (25 μM) for another 30 min and **FP**-stained cells exposed to F^- (25 μM) for another 30 min

1 both display intensive fluorescence signals (Fig. S10e and S11e). The results indicate **MP** and
2 **FP** can serve as turn-on probes to detect intracellular Hg^{2+} and F^- and study the biological
3 processes involving Hg^{2+} and F^- within living cells.

4 As mentioned above, **FMP** could serve as “AND” logic gate-based fluorogenic probe for
5 monitoring the coexistence of Hg^{2+} and F^- in buffer solution. To check up whether the same
6 monitoring performance of **FMP** for the coexistence of Hg^{2+} and F^- in living cells as that in the
7 buffer solution, the cell imaging experiments were carried out. HeLa cells were incubated with
8 **FMP** (10 μM) for 30 min and washed three times with PBS. As shown in Fig. 9b, no obvious
9 fluorescence signal was observed under the confocal microscope. when the **FMP**-stained cells
10 exposed to Hg^{2+} (25 μM) or F^- (25 μM) for another 30 min and then washed three time with
11 PBS, the change of fluorescence signal was almost negligible (Fig. 9e and 9h). However, when
12 the cells treated with **FMP** were incubated with Hg^{2+} (25 μM) and F^- (25 μM) simultaneously
13 under the same incubation time, a remarkable fluorescence signal could be collected at $480 \pm$
14 10 nm by the confocal microscope (Fig. 9k). The imaging results were consistent with
15 fluorescence results in the buffer solution. It indicates **FMP** can act as “AND” logic gate-based
16 probe to detect the coexistence of Hg^{2+} and F^- in both environmental and biological system.

Fig. 9. Confocal microscopy images of **FMP**-loaded living HeLa cells before and after addition of Hg^{2+} and F^- . a, b, c) cells incubated with **FMP** (10 μM) for 30 min; d, e, f) cells incubated with **FMP** (10 μM) for 30 min and then treated with F^- (25 μM) for another 30 min; g, h, i) cells incubated with **FMP** (10 μM) for 30 min and then treated with Hg^{2+} (25 μM) for another 30 min; j, k, l) cells incubated with **FMP** (10 μM) for 30 min and then treated with Hg^{2+} (25 μM) and F^- (25 μM) for another 30 min. From left to right: Bright field, Cyan-channel at $480 \pm 10 \text{ nm}$ ($\lambda_{\text{ex}} = 405 \text{ nm}$) and overlap-channel.

4. Conclusion

In summary, we synthesized three reactive fluorogenic probes (**MP**, **FP** and **FMP**) based on an (imino)coumarin-precursor for the detections of Hg^{2+} and F^- and their coexistence. **FP** and **MP** display good selectivity and sensitivity in the fluorogenic detection of Hg^{2+} and F^- ,

respectively. Moreover, they are biocompatible and can indeed visualize the existence of F^- and Hg^{2+} in living HeLa cells, respectively. **FMP** with two recognition groups exhibits fluorescence response towards the coexistence of Hg^{2+} and F^- as expected. As a result, a “AND” logic gate was easily made by employing Hg^{2+} and F^- as two inputs and a “turn-on” fluorescence as output. The resulted “AND” logic gate can work in both buffer media and living cells. In general, the work demonstrated that the rational use of distinct analytes-mediated *in-situ* formation of fluorescent scaffolds based on multistep reaction cascades is applicable for developing a reactive “AND” logic gate-based fluorescent probes.

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