在纯水体系和活细胞中高选择检测 Fe³⁺的香豆素荧光探针

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摘要：设计、合成了一种香豆素类荧光探针 **CF470**,该探针通过引入高水溶性且选择性与 Fe^{3+} 配位的多羟基基团实现了纯水体系中 Fe^{3+} 的检测。当在探针体系中加入 Fe^{3+} 后,其强烈的蓝色荧光在 2 min 内就可被完全猝灭。探针不仅对 Fe^{3+} 快速、高选择响应,而且由于引入了亲水的多羟基基团具有良好的水溶性。更重要的是,探针对活细胞低毒、生物相容性好,被成功应用于活细胞中 Fe^{3+} 的成像。

关键词：铁离子；香豆素；纯水体系；荧光成像；活细胞

A Highly Selective Coumarin-based Fluorescent Probe for Detecting Fe³⁺ in Pure Water Systems and Living Cells

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Abstract: A novel fluorescent probe **CF470** was designed and synthesized, for detecting Fe^{3+} in pure water systems, composed of coumarin derivative as a fluorophore, and the trihydroxyl group as both the Fe^{3+}-sensing site and hydrophilic group. Adding Fe^{3+} into the solution of **CF470** rendered intense blue fluorescence quenching within 2 min. The probe showed excellent specificity, outstanding water-solubility in virtue of presence of the exceedingly hydrophilic trihydroxyl moiety, and fast response time. More importantly, the low cytotoxic and good biocompatible probe was also successfully utilized to image Fe^{3+} in living cells.

Keywords: iron ions; coumarin; pure water systems; fluorescent imaging; living cells

Many heavy metal ions have attracted great attention owing to their broader influence on the environment and human health[1-2]. In particular, iron (Fe^{3+}) is one of the most plentiful transition elements, and it performs crucial roles in many physiologic processes including cell metabolism, hemoglobin formation and so on[3-7]. However, abnormal Fe^{3+} content may cause many serious diseases to people, for instance, abdominal pain, hyperferremic disorders, Parkinson's disease and Alzheimer's disease[8-10]. Thus, it is of considerable importance to develop an efficient method for highly selective detection of Fe^{3+}.

Compared with conventional analytical methods like colorimetry and voltammetry[11-14], fluorescent probes have been regarded as one of the most valid tools to detect Fe^{3+} due to the advantages of simple

manipulation, high sensitivity and low cost[15-21]. Hence, an increasing number of Fe^{3+} fluorescent probes based on the different mechanisms have been developed[22-31]. Unfortunately, among these developed Fe^{3+} fluorescent probes, many fluorescent probes are easily interfered with Al^{3+}, Cu^{2+} and Cr^{3+}, and few of probes possess ideal water-solubility. To the best of our knowledge, ideal water-solubility is conducive to the biological application of probes. Therefore, it is highly desired to develop a high selective fluorescent probe for detecting Fe^{3+} in 100% aqueous medium. We herein designed and synthesized a novel fluorescent probe **CF470** for sensing Fe^{3+} in deionized water. **CF470** was designed by using coumarin derivative as a fluorophore, and the trihydroxyl group as both the response site and hydrophilic group, as shown in Scheme 1. The intense blue fluorescence of **CF470** was quenched within 2 min after the addition of Fe^{3+} (100 equiv.), which was attributed to the paramagnetic quenching effect. As expected, the probe **CF470** not only possessed good selectivity, ideal water-solubility owing to existence of the exceedingly hydrophilic trihydroxyl group, but also had a rapid response to Fe^{3+}. In especial, the probe, with low toxicity and good biocompatibility, was successfully employed to image Fe^{3+} in A549 cells.

Scheme 1　　　Sensing mechanism of **CF470** for Fe^{3+}
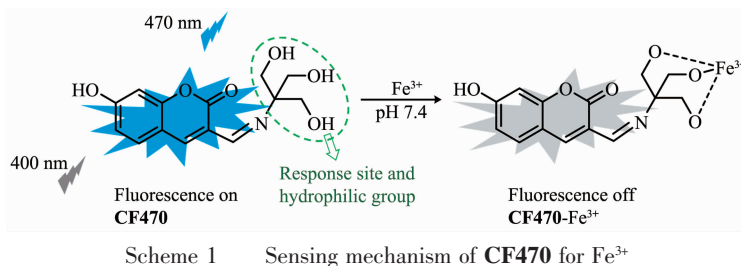
1　Experimental

1.1　Materials and instruments

All materials, unless special stated, were obtained from commercial sources and used without further purification. Fluorescence spectra and absorption spectra were performed on a Hitachi F-2700 fluorescence spectrophotometer and Shimadzu UV-2550 spectrometer, respectively. For all spectral measurements, the excitation wavelength was set at 400 nm, and the slit widths of excitation and emission were 2.5 nm and 5 nm, respectively. NMR spectra were obtained on a Varian Unity INOVA-400 spectrometer with tetramethylsilane (TMS) as an internal standard. A Mettler Toledo pH meter was used for the all pH measurements. High-resolution mass spectra were measured on a Bruker micrOTOF-Q II mass spectrometer. Infrared spectra were acquired with a Bruker Vertex 70 FI-IR spectrometer. The cell experiments were carried out with an Olympus FV1000 confocal microscope.

1.2　Preparation of sample solutions

For all spectrometric studies, **CF470** was prepared in analytical grade DMSO to obtain the stock solution of the probe (1 mmol·L^{-1}). Stock solutions of various metal ions, such as Fe^{3+}, Fe^{2+}, K^+, Mn^{2+}, Ni^{2+}, Cd^{2+}, Al^{3+}, Co^{2+}, Cr^{3+}, Mg^{2+}, Hg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Pb^{2+}, Na^+, Sn^{2+}, and Ag^+ were prepared in doubly distilled water (10 mmol·L^{-1}, respectively).

1.3　Synthesis of probe CF470

Synthesis of **CF470** was presented in Scheme 2. The characterization details were displayed in the supporting information (Fig.S3~S10).

1.3.1　Synthesis of compound **1**

A mixture of 2,4-dihydroxybenzaldehyde (0.69 g, 5.0 mmol), three drops of triethylamine and anhydrous sodium acetate (1.05 g, 11.0 mmol) in propionic anhydride (15 mL) was stirred at 140 ℃ for 6 h. After 6 h, 100 mL ice-water was poured onto the mixture solution. Then the solution was removed by filtration, and the residue was recrystallized from methanol to obtain white solid. Yield: 56%. ^{1}H NMR (400 MHz, CDCl$_3$) δ: 7.54 (s, 1H),7.45(d, J=8.0 Hz, 1H), 7.12 (s, 1H), 7.05 (d, J=8.0 Hz, 1H), 2.70~2.64 (m, 2H), 2.24 (s, 3H), 1.31 (t, J=6.0 Hz, 3H). HRMS: m/z Calcd. for $C_{13}H_{13}O_4^+$ [M+H]$^+$: 233.079 5, Found: 233.080 8.
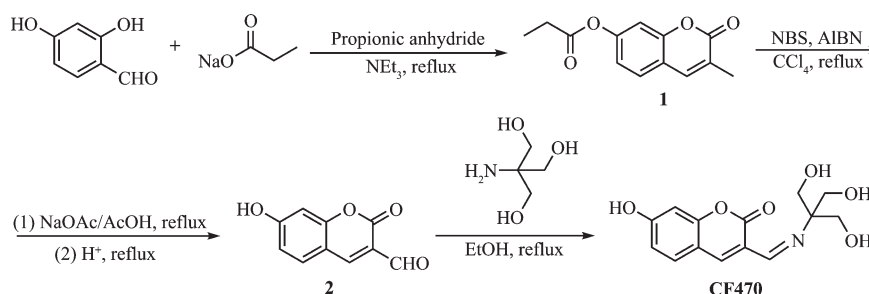
Scheme 2　Synthetic route of **CF470**

1.3.2　Synthesis of compound **2**

A mixture of compound **1** (1.16 g, 5.0 mmol), NBS (2.23 g, 12.5 mmol) and a trace amount of AIBN was dissolved in CCl_4 (150 mL). After the mixture was stirred at 80 C for 12 h, the solvent was evaporated under reduced pressure to give the resulting residue. The mixture of the resulting residue, sodium acetate (2.42 g, 29.5 mmol) and acetic acid (80 mL) was unceasingly stirred at 100 ℃ for 12 h. HCl (2 $mol \cdot L^{-1}$, 80 mL) was poured in the mixture solution and unceasingly stirred for 30 min. The solution then was cooled down to room temperature and was discarded by filtration. The resulting crude solid was washed with water (10 mL) and methanol (10 mL) to afford brown powder. (870 mg, 87%). ^1H NMR (400 MHz, DMSO-d_6) δ: 11.30 (s, 1H), 9.96 (s, 1H), 8.58 (s, 1H), 7.82(d, J=12.0 Hz, 1H), 6.87 (d, J=8.0 Hz, 1H), 6.77 (s, 1H). HRMS: m/z Calcd. for $C_{10}H_7O_4^+$ [M+H]$^+$: 191.019 8, Found: 191.018 6.

1.3.3　Synthesis of probe **CF470**

A mixture of compound **2** (190 mg, 1.0 mmol) and tris (hydroxymethyl) aminomethane (145.2 mg, 1.2 mmol) in ethanol (5 mL) was refluxed at 80 ℃ under the argon atmosphere. After the reaction completed, the solution was cooled to room temperature, the precipitate was acquired through filtration, washed with anhydrous ethanol to obtain orange product (100 mg, 75%). ^1H NMR (400 MHz, DMSO-d_6) δ: 10.60 (s, 1H), 8.05 (s, 1H), 7.60 (d, J=8.0 Hz, 1H), 6.79 (d, J= 8.0 Hz, 1H), 6.72 (s, 1H), 5.35 (s, 1H), 4.75 (s, 1H), 3.70 (d, J=8.0 Hz, 1H), 3.62 (s, J=8.0 Hz, 1H), 3.41 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.03, 160.27, 155.43, 140.69, 130.58, 122.38, 113.83, 111.40, 102.31, 87.70, 69.27, 67.48, 63.11, 62.69. HRMS: m/z Calcd. for $C_{14}H_{16}NO_6^+$ [M+H]$^+$: 294.095 9, Found: 294.097 2. IR (KBr, cm^{-1}) ν: 3 296, 2 801, 1 698, 1 616,1 361, 1 250, 1 191, 1 114, 864, 764, 655, 505.

1.4　Detection limit

The detection limit of **CF470** for detecting Fe^{3+} was calculated by means of following equation:

Detection limit=$3\sigma/k$

σ is the standard deviation of eleven blank measurements and k is the slope of standard curve.

1.5　Cytotoxicity assay

Cytotoxicity studies of **CF470** to A549 cells were evaluated using standard MTT assay. Cells were incubated in 96-well microplates at a density of $1×10^5$ cells per well in Dulbecco′s modified Eagle′s medium (DMEM) containing 10% fetal calf serum for 24 h. After that, different concentrations of **CF470** (0, 5, 10, 15, 20 and 25 $\mu mol \cdot L^{-1}$) were added the culture dish and treated for 24 h. MTT reagent (10 μL) was added and sequentially incubated for 4 h. Then, the culture medium was washed with PBS three times and DMSO (200 μL) was cultured with cells to dissolve formazan. The absorbance at 490 nm was recorded using the microplate reader.

2　Results and discussion

2.1　Spectral properties of probe CF470

In consideration of the effect of pH on the properties of the fluorescent probe, we initially examined the fluorescence intensities of the probe and reaction product within various pH values (3.0~11.0). Fig.1 showed that the probe **CF470** exhibited a maximum fluorescence emission intensity at 470 nm and the fluorescence intensity was almost stable with pH ranging from 7.0 to 8.0. After adding hundred

equivalences of Fe^{3+}, fluorescence intensity at 470 nm of **CF470** was drastically weakened and kept fine stability within a wide pH region of 3.0~11.0. Given the cell imaging and spectral response of **CF470** to Fe^{3+}, all experiments selected pH 7.4.
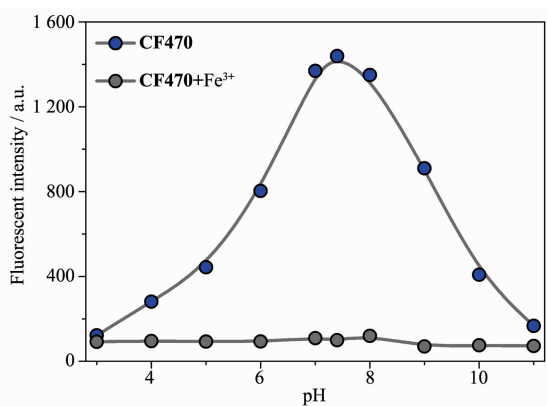
λ_{ex}=400 nm, λ_{em}=470 nm, slit: 2.5 nm/5 nm

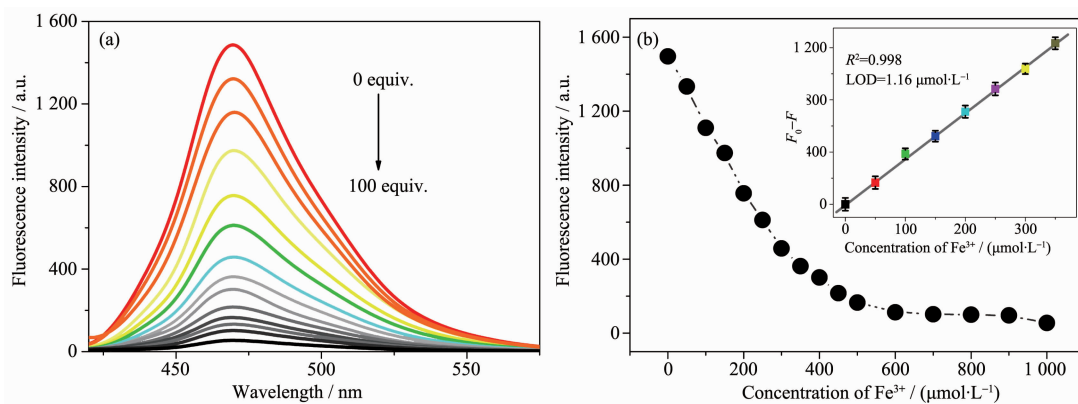Fig.1　Fluorescence intensity at 470 nm of **CF470** (10 μmol·L^{-1}) before and after adding Fe^{3+} (100 equiv.) at different pH values

UV-Vis absorption spectra of **CF470** toward Fe^{3+} was firstly studied in phosphate buffer (pH=7.4) solution (Fig.S1). The free **CF470** exhibited a main absorption peak at 410 nm. With successive addition of Fe^{3+} (0~100 equiv.) into the buffer solution of **CF470**, the absorption peak at 410 nm gradually enhanced. We also estimated the fluorescence spectra of **CF470** to Fe^{3+} (Fig.2a). The probe **CF470** displayed a strong blue fluorescence. However, upon the addition of Fe^{3+}

(0~100 equiv.) to **CF470** solution, a significant fluorescence attenuation was observed, which was attributed to the paramagnetic quenching effect. After the reaction between probe and Fe^{3+} (100 equiv.), the strong fluorescence almost entirely disappeared (Fig.2b). And the fluorescence intensity at 470 nm demonstrated good linear relationship with the concentrations of Fe^{3+} (0~35 equiv.) with a detection limit (1.16 μmol·L^{-1}) (*S/N*=3) (Fig.2b inset). The results showed that **CF470** could realize quantitative detection of Fe^{3+}.

To study the selectivity of **CF470** toward Fe^{3+}, different metal ions were examined under same conditions (Fig.3a). **CF470** demonstrated obvious fluorescence quenching in the presence of Fe^{3+} in phosphate buffer (pH=7.4) solution, while other metal ions did not cause significant fluorescence attenuation and color changes on **CF470** than Fe^{3+} (Fig.3a inset). Simultaneously, competition experiments were also conducted (Fig.3b). When Fe^{3+} (100 equiv.) was added to the mixture solution of **CF470** and other metal ions, a clear fluorescence attenuation was discerned, indicating that the presence of other metal ions could not interfere **CF470** for detecting Fe^{3+}. Thus, the above experimental results demonstrated that **CF470** had high specificity for Fe^{3+}.

To assess the response ability of **CF470** for Fe^{3+}, the kinetic studies were carried out (Fig.4a). The addition of Fe^{3+} (100 equiv.) to the solution of **CF470**

Inset: the linear correlation between fluorescence intensity at 470 nm of CF470 (10 μmol·L^{-1}) and Fe^{3+} concentrations (0~35 equiv.) in phosphate buffer (pH=7.4) solution (λ_{ex}=400 nm, λ_{em}=470 nm, slit: 2.5 nm/5 nm)

Fig.2　(a) Fluorescence spectra of **CF470** (10 μmol·L^{-1}) after adding different amounts of Fe^{3+} (0~100 equiv.); (b) Fluorescence intensity changes of **CF470** (10 μmol·L^{-1}) toward different concentrations of Fe^{3+} (0~100 equiv.) in phosphate buffer (pH=7.4) solution
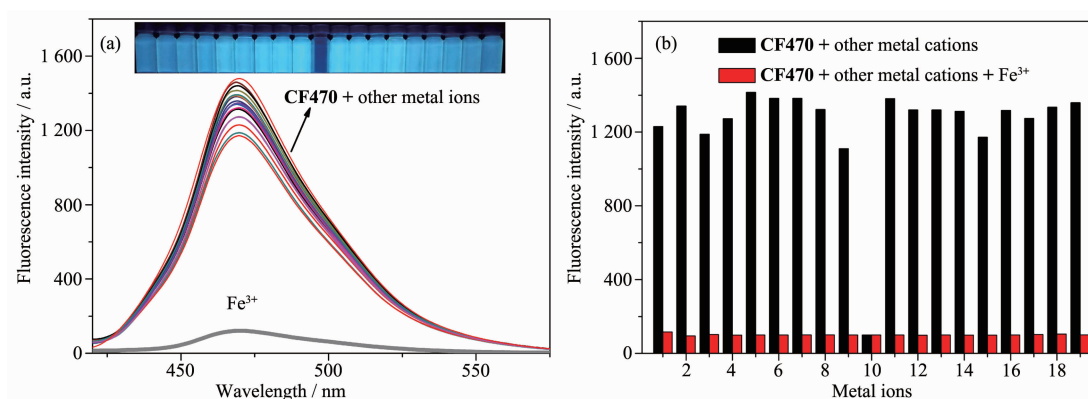
1. Ca^{2+}, 2. Ni^{2+}, 3. Zn^{2+}, 4. Pb^{2+}, 5. Cd^{2+}, 6. Cu^{2+}, 7. Sn^{2+}, 8. Ag^+, 9. Mg^{2+}, 10. Fe^{3+}, 11. K^+, 12. Na^+, 13. Fe^{2+}, 14. Cr^{3+}, 15. Ba^{2+}, 16. Mn^{2+}, 17. Co^{2+}, 18. Hg^{2+}, 19. Al^{3+}; $\lambda_{ex}=400$ nm, $\lambda_{em}=470$ nm, slit: 2.5 nm/5 nm

Fig.3　(a) Fluorescence responses of **CF470** (10 μmol·L⁻¹) to different metal ions (100 equiv.); Inset: photos of **CF470** (10 μmol·L⁻¹) after the addition of different metal ions (100 equiv.) under a handheld 365 nm UV lamp; (b) Fluorescence responses of **CF470** (10 μmol·L⁻¹) to Fe^{3+} (100 equiv.) in presence of different metal ions in phosphate buffer (pH=7.4) solution
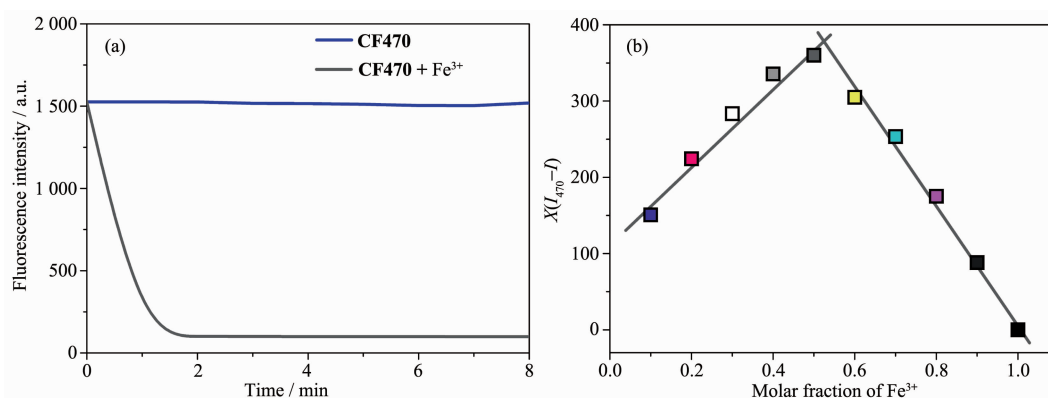
Total concentration of **CF470** and Fe^{3+} was 10 μmol·L⁻¹, and X represented molar fraction of Fe^{3+} ($\lambda_{ex}=400$ nm, $\lambda_{em}=470$ nm, slit: 2.5 nm/5 nm)

Fig.4　(a) Plot of fluorescence intensity at 470 nm versus time for the reaction between **CF470** (10 μmol·L⁻¹) and Fe^{3+} (100 equiv.); (b) Job′s plot of **CF470** in phosphate buffer (pH=7.4) solution

led to a remarkable attenuation in the fluorescence intensity at 470 nm, and the fluorescence intensity attained stable within 2 min, with a 25-fold decrease.

2.2　Proposed sensing mechanism

　　The possible sensing mechanism of **CF470** to Fe^{3+} was depicted in Scheme 1. The complexation of **CF470** with Fe^{3+} resulted in the fluorescence quenching, which was possibly ascribed to the paramagnetic property of Fe^{3+}. HRMS analysis and Job′s plot were carried out to verify this mechanism. As depicted in Fig.4b, a strong fluorescence intensity at mole fraction 0.5 suggested 1:1 stoichiometry for the **CF470**-Fe^{3+} complex. Similarly, the binding mode of **CF470** with Fe^{3+} was also testified by HRMS. After the probe

reacted with Fe^{3+}, a new peak at $m/z=345.998\ 7$ corresponding to [**CF470**-Fe^{3+}] appeared (Fig.S2). As a result, the experimental results fully confirmed the response mechanism of **CF470** toward Fe^{3+}.

2.3　Computational sudies

　　To further prove the sensing mechanism of **CF470** toward Fe^{3+}, theoretical calculation was carried out by the Gaussian 09 program. The optimum structure and electron distributions of **CF470**-Fe^{3+} were obtained using B3LYP/6-31G(d,p) (Fig.5). The electrons on the HOMO of **CF470**-Fe^{3+} were mostly situated on coumarin moiety, whereas the electrons on the LUMO were mainly located around Fe^{3+}, suggesting that the fluorescence quenching was due to the coordination of
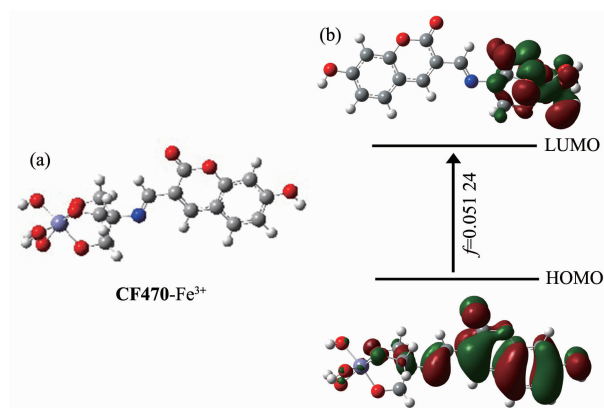
Fig.5　(a) Optimum structure and (b) HOMO-LUMO energy level of **CF470**-Fe^{3+}

Values: mean±standard deviation

Fig.6　Cytotoxicity of **CF470** in A549 cells estimated by the MTT method

CF470 with Fe^{3+}.

2.4　Cellular imaging

To assess the toxicity of **CF470** (0, 5, 10, 15, 20 and 25 μmol·L^{-1}) towards A549 cells, MTT assay was carried out. As shown in Fig.6, even after cells were cultured with high concentration of **CF470** (25 μmol·L^{-1}) for 24 h, cell survival rate was still more than 80%, showing the low cytotoxicity and good biocompatibility of **CF470** to living cells.

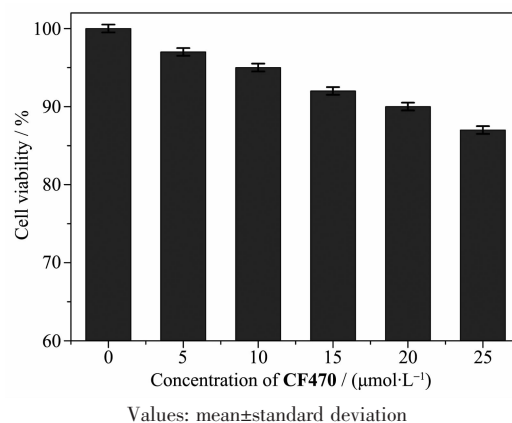Then, the cellular imaging experiments of **CF470** toward Fe^{3+} were conducted on A549 cells. The A549

cells were cultured with **CF470** (10 μmol·L^{-1}) in DMEM for 20 min. After that, cells were washed repeatedly with PBS for three times, and finally cultured with Fe^{3+} (100 equiv.) for 10 min. As seen in Fig.7, strong blue fluorescence was observed after A549 cells was pretreated with **CF470**. In contrast, cells displayed slight fluorescence in the presence of Fe^{3+}. These experimental results manifested that **CF470** possessed good cell membrane permeable as well as could be used for the detection of Fe^{3+} in living cells.
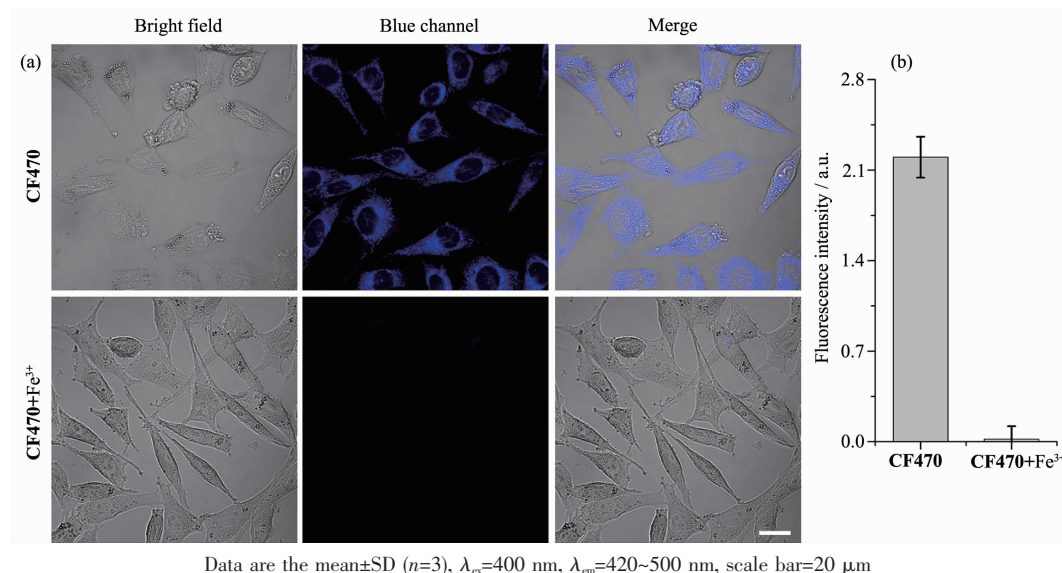

Data are the mean±SD (*n*=3), λ_{ex}=400 nm, λ_{em}=420~500 nm, scale bar=20 μm

Fig.7　Cellular imaging of Fe^{3+} in A549 cells cultured with **CF470** (10 μmol·L^{-1}, 20 min) and further incubated with Fe^{3+} (1 000 μmol·L^{-1}, 10 min); (b) Fluorescence intensity per one blue channel

3　Conclusions

In summary, we have developed a novel fluorescent probe **CF470**, for detecting Fe^{3+} in 100%

aqueous solution through complexation reaction on the basis of fluorescence quenching, comprised of coumarin derivative as a fluorophore, and the trihydroxyl group

as both the Fe^{3+}-sensing site and hydrophilic group. Remarkably, the sensing mechanism of **CF470** to Fe^{3+} was proved by HRMS analysis and theoretical calculation. A palpable fluorescence quenching was observed after the addition of Fe^{3+} into the **CF470** solution. The probe **CF470**, with ideal water-solubility on account of existence of the exceedingly hydrophilic trihydroxyl group, can realize a highly selective and rapid detection of Fe^{3+}. Furthermore, cell imaging results demonstrated that **CF470** with low cytotoxicity had admirable membrane permeability and could detect Fe^{3+} in living cells.

Supporting information is available at http://www.wjhxxb.cn

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