A Ratiometric Selective Fluorescent Probe Derived from Pyrene for Cu\(^{2+}\) Detection

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Abstract: A novel ratiometric Cu\(^{2+}\)-selective probe was rationally constructed based on pyrene derivative. Compared to other tested metal ions, the probe presented the selective recognition for Cu\(^{2+}\) which could be detected by a significant turn-on fluorescent response at 393 nm and 415 nm. Under the optimized conditions, a detection limit of 0.16 \(\mu\)M Cu\(^{2+}\) in aqueous media was found. Besides this, a 1:1 metal–ligand complex was confirmed by MS spectra and Job’s plot experiment, and the binding mode was also studied by \(^1\)H NMR experiment. Meanwhile, the fluorescence imaging in living cells was performed to detect Cu\(^{2+}\) with satisfactory results.

Keywords: Cu\(^{2+}\); probe; pyrene; fluorescence imaging

1. Introduction

Because of wide use of heavy metal ions, analysis and detection of them has been an important means of disease prevention and control in public health and environmental safety [1,2]. Cu\(^{2+}\), as an important heavy metal ion, is confirmed to be harmful to human health in that excessive concentration of Cu\(^{2+}\) is probably involved in the forming of reactive oxygen species to bring out neurodegenerative disorders, such as Wilson’s diseases [3]. To prevent Cu\(^{2+}\)-related pollution and health problems, design of effective tools capable of detection of trace amounts of Cu\(^{2+}\) has inspired considerable interest. Due to these advantages, including rapid analysis, high sensitivity, real-time in situ monitoring, and high spatial resolution, fluorescence probe method is expected to be an ideal choice for sensing and bioimaging of analytes in a variety of samples [4–6]. To date, researchers have developed many Cu\(^{2+}\) fluorescent probes [7–9]. One of the most important approaches is that a specific group is grafted to a fluorophore by organic methods [10–12]. Zhao’s group developed a Cu\(^{2+}\) fluorescent probe by incorporating benzoyl hydrazone group as a receptor into the fluorophore hemicyanine, which could rapidly recognize Cu\(^{2+}\) and was also used for cell image [13]. Li et al. synthesized a Cu\(^{2+}\) fluorescent probe by combining a specific recognition unit phenol derivative with a cyanine dye [14]. However, because Cu\(^{2+}\) is a fluorescence quencher due to its paramagnetic through energy or electron transfer processes, these probes showed an “on-off” fluorescence response [15–17]. Based on sensitivity and selectivity considerations for practical applications, fluorescence quenching probes upon Cu\(^{2+}\) combination are not as popular as fluorescence enhancement probes under Cu\(^{2+}\) binding. In contrast, “turn-on” fluorescent probes by Cu\(^{2+}\)-triggered have...
attracted special interest. A number of these fluorescent probes have been synthesized and characterized [18–20]. For example, You’s group reported a highly fluorescent probe derived from a quinoline moiety for Cu\(^{2+}\) in DMSO-Tris buffer and living cells [21]. In addition, some probes based on rhodamine derivatives could recognize Cu\(^{2+}\) with the aid of structural changes between spirolactam and ring-opened amide to give rise to a stronger fluorescence emission and color change [22]. However, these limitations, including organic cosolvent dependence, poor photostability, poor cellular compatibility and pH dependence [23,24], to some extent, lowered the sensitivity and also restricted the practical applications because the recognition groups had drawbacks in selectivity and sensitivity. Therefore, development of highly selective and sensitive fluorescent probes for Cu\(^{2+}\) sensing with “off-on” response has been still of great challenge.

With this in mind, in this study, probe P was reasonably constructed by connecting an important fluorophore pyrene with 2-aminobenzhydrazide via –C=N– bond (Scheme 1). The responses of P toward Cu\(^{2+}\) were explored in detail by UV and fluorescence analysis. P was also demonstrated that it could react with Cu\(^{2+}\) and effectively turn on fluorescence in living cells. The design concept was explained as follows: (1) Pyrene was used as the fluorophore in view of its excellent photophysical properties and easy modification on the molecular structure [25,26]. In particular, its fine structures of excited state were the ideal signal units for construction of ratio-type fluorescent probes, which measure the ratio of fluorescence intensity at two different wavelengths as a signal parameter. This mode is not affected by the intensity of light source and instrument; (2) Acylhydrazone Schiff base was one of most selective \(\pi\)-conjugated reagents for Cu\(^{2+}\). In addition, this type of compound was characterized by ketone–enol tautomerism, and O atom of the carbonyl group (–C=O), N atom of the amino group (–NH\(_2\)), and the imine group (–N=CH) could participate in coordination with Cu\(^{2+}\) and consequently produced stable coordination complexes according to HSAB principle [27]; (3) C=N isomerization and photoinduced electron-transfer (PET) phenomenon were introduced as common signaling mechanisms to play roles in photophysical processes upon binding of P with Cu\(^{2+}\), which was based on the following considerations. Firstly, the main advantage was that it was easy to prepare and design “off-on” probes based on the two mechanisms; furthermore, the affinity of the chelating group C=N for heavy metal ions was stronger than that for alkali metals and alkaline earth metals [28,29].

![Scheme 1. Design and synthesis of compounds.](image-url)
2. Materials and Methods

2.1. Materials and Instruments

All reagents used in our study were all analytically pure and used directly. Fluorescence experiments were carried out by a Hitachi 4600 spectrofluorimeter (Japan). Absorption spectra were measured by a Hitachi U-2910 spectrophotometer (Japan). Confocal microscopy imaging was determined in an Olympus FluoView Fv3000 laser scanning microscope (Japan). Electrospray ionization (ESI) analyses were performed on a Thermo TSQ Quantum Mass Spectrometer (America), method parameters: Sheath Gas Flow Rate (arb): 30; Aux Gas Flow Rate (arb): 10; Capillary Temp. (°C): 275; Capillary Voltage (V): −45.00 (ES−)/10.00 (ES+); SpraVoltage (kV): 5.00. ¹H NMR and ¹³C NMR spectra were performed by a Bruker AV 400 instrument with tetramethylsilane (TMS) as an internal standard and DMSO-­d₆ as a deuterium generation reagent (Switzerland).

2.2. Synthesis of P and P₁

0.23 g (1 mmol) compound 1 and 0.158 g (1 mmol) compound 2 were stirred in ethanol (40 mL) and refluxed for 4 h, and then cooled slowly to 25°C, followed by filtering precipitate so produced. The obtained yellow solid P was dried and used directly. Yields: 83.4%. MS (ESI+) m/z: 364.1 [M + H⁺], 386.6 [M + Na⁺]. ¹H NMR (δ ppm, d₆-DMSO): 11.80 (s, 1H), 9.46 (s, 1H), 8.81 (d, 1H, J = 7.25), 8.58 (d, 1H, J = 7.72), 8.37 (d, 1H, J = 7.88), 8.27 (d, 1H, J = 8.88), 8.23 (d, 1H, J = 8.92), 8.13 (t, 1H, J = 7.62), 7.72 (d, 1H, J = 7.72), 7.28 (t, 1H, J = 7.60), 6.74 (d, 1H, J = 8.24), 6.67 (t, 1H, J = 7.42), 6.47 (b, 2H). ¹³C NMR (δ ppm, d₆-DMSO): 151.11, 146.48, 133.27, 132.67, 131.78, 131.08, 129.57, 129.42, 129.19, 128.31, 128.12, 127.47, 126.94, 126.61, 126.13, 125.88, 125.09, 124.72, 123.41, 117.41, 115.59 (Figures S1–S3).

Compound P₁ was synthesized similar to P. Yields: 87.2%. MS (ESI+) m/z: 349.3 [M + H⁺], 371.2 [M + Na⁺] (Figure S4).

2.3. Optical Experimental Method

Stock solutions of metal ions and compound P/P₁ were prepared by dissolving appropriate amount in deionized water and DMSO, respectively. Slit widths of excitation and emission were 5 nm and 5 nm, respectively. Excitation wavelength was 350 nm.

2.4. Cell Culture

Hela cells were washed 3 times by PBS (phosphate-buffered saline), and incubated with 1 µM of Cu²⁺ (in PBS) for 30 min at 37 °C, followed by washing another 3 times with PBS. Then, 10 µM of probe P (in DMSO) was added. After treatment for 30 min in the same growth media, it was washed 3 times with PBS again.

Other cell culture experiments were completed according to this method mentioned above.

2.5. Cytotoxicity of P

The cytotoxicity experiment of P in Hela cells was measured using MTT assay. Cells were seeded into 96-well cell culture plate at a rate of 4000/well in 5% CO₂ at 37 °C for 24 h, followed by adding probe P (0, 0.1, 1, 10 µM), and then allowed to incubate for 24 h, respectively. Subsequently, each well was injected with 20 µL MTT (5 mg/mL) and cultured for another 4 h under the same conditions. After that, cells were lysed in the solution composed of 10% SDS, 0.012 M HCl, and 5% isopropanol. A₅₇₀nm of each well was obtained on a microplate reader to calculate the amount of formazan.

3. Results

3.1. Effects of pH

Effect of pH was firstly investigated to evaluate the actual sensing for Cu²⁺. The results were displayed in Figure 1, when Cu²⁺ was not added to the P solution, the fluorescence intensity of P itself remained fairly static in the range of pH 4–10. However, after mixing P with Cu²⁺, the emission intensity gradually increased in the range of pH 4–7, which reached a maximum at pH 6.3. While pH > 7, the fluorescence intensity began to decrease, which tended to the formation of Cu(OH)₂ precipitation. Thereby, the pH-control measurements...
revealed that P was stable in a wide range of pH, which also showed P had the best sensing response to Cu$^{2+}$ at pH 6.3. All measurements were carried out in alcohol–aqueous media (v/v, 8:2, pH 6.3, 0.02 M HEPES).

Figure 1. pH-dependent fluorescence response of P (10 µM) and P (10 µM) toward Cu$^{2+}$ (100 µM) under different HEPES buffers.

3.2. UIV-Vis Response of P

Absorption spectra of P to various common metal ions were illustrated in Figure 2. P exhibited three absorption bands with peaks at 400 nm, 380 nm and 280 nm, which displayed an obvious ratiometric absorbance change (Figure 2b). These results indicated that P possessed the enhancement at 275 nm was also obvious (Figure 2a). These results were enough to account for the binding between P and Cu$^{2+}$.

Figure 2. Absorption spectra. (a) absorption spectra of P (10 µM) toward metal ions (100 µM) in alcohol–aqueous media (v/v, 8:2, pH 6.3, 0.02 M HEPES); (b) absorption titration of P (10 µM) upon addition of increasing amounts of Cu$^{2+}$ (0–10 µM) under the same conditions.

The absorption titration experiment showed a significant decrease of absorbance at 400 nm, 380 nm, and an absorbance increase at 275 nm was observed simultaneously with three isosbestic points at 290 nm, 310 nm and 340 nm, which displayed an obvious ratiometric absorbance change (Figure 2b). These results indicated that P possessed the characteristic of recognizing Cu$^{2+}$ by ratio absorption spectrum, and it also be concluded that Cu$^{2+}$ binding with P blocked conjugation of the double bonds and resulted in the appearance of some shorter absorption wavelengths.
3.3. Characterization of Fluorescent Response of P

The selective properties of P for respective metal ions were further evaluated by fluorescence spectroscopy. P itself showed a very weak fluorescence response with the excitation wavelength 350 nm as seen in Figure 3a, the reason was explained by the transfer of the lone pair electrons from –C=N– group to benzoyl hydrazone unit. In addition, it was likely due to non-radiative decay of the C=N bond in excited state. When comparing Cu²⁺ with other examined metal ions, a remarkable “turn-on” fluorescence response generated at 393 nm and 415 nm by the addition of Cu²⁺, which was the typical monomer emission of pyrene. In this process, we surmised that the N and O atoms in Schiff base maybe participate in forming the complex P-Cu²⁺, and the isomerization of C=N bond and PET process were restricted, so the enhanced fluorescence intensity at 393 nm and 415 nm were induced significantly. These results suggested that P could act as an ideal fluorescent probe for Cu²⁺ over other metal ions. Competitive experiments were also employed to prove the practical application of P as a Cu²⁺-sensitive fluorescent probe. As shown in Figure S5, after Cu²⁺ was added to the mixture of P with different interfering substances, including Na⁺, K⁺, Mg²⁺, Ca²⁺, Pb²⁺, Zn²⁺, Cd²⁺, Co²⁺, Hg²⁺, Cr³⁺, Ag⁺ and Ni²⁺, an obvious enhancement of the fluorescence intensity ratio (I_{393nm}/I_{415nm}) was caused. These results displayed that the fluorescence signal of P toward Cu²⁺ was not susceptible remarkably to the coexistent metal ions, which further confirmed that P was a promising probe to sense Cu²⁺. Furthermore, to evaluate the role of amino group in P, the fluorescence signal of control compound P1 toward the above mentioned metal ions were also measured as seen in Figure 3b. No remarkable emission of P1 at 440 nm was observed, and an emission band at 393 nm and 415 nm was not shown upon addition of Cu²⁺ as found in P, which indicated the amino group played a crucial role in the formed chelating complex P-Cu²⁺.

![Figure 3](image-url)

**Figure 3.** Fluorescence spectra. (a) fluorescence response of P (10 μM) to different metal ions (100 μM) in alcohol-aqueous media (v/v, 8:2, pH 6.3, 0.02 M HEPES); (b) fluorescence emission of P1 (10 μM) for above-mentioned metal ions (100 μM) in alcohol-aqueous media (v/v, 8:2, pH 6.3, 0.02 M HEPES); (c) fluorescence titration of P (10 μM) with various [Cu²⁺], inset: the ratios of fluorescent emission intensity (I_{393nm}/I_{415nm}) of P (10 μM) as a function of [Cu²⁺] within 0.5–8 μM.

The sensing ability of Cu²⁺ with P was also checked by the titration experiment with fluorescence spectroscopy. Along with the increasing concentration of Cu²⁺, the fluorescence intensity at 393 nm and 415 nm enhanced gradually (Figure 3c), which indicated
a ratiometric fluorescence change. The linear fitting curve showed that a perfect linear relationship between the ratios of fluorescent emission intensity (I_{393nm}/I_{415nm}) and [Cu^{2+}] within 0.5–8 μM, and LOD (a detection limit) of 0.16 μM was obtained, respectively. It exhibited that probe P could be used for qualitative and quantitative sensing of Cu^{2+}, and the sensitivity reached 30 μM Cu^{2+} in drinking water in the standard of the WHO (World Health Organization) [30].

3.4. Reaction Mechanism Research

To explore the probable complexation between P and Cu^{2+}, we conducted Job’s plot experiment as displayed in Figure 4 and Figure S6. A maximum emission intensity at 480 nm was found with the proportion of [P]/[Cu^{2+}] of 0.5. As expected, a 1:1 stoichiometry for the complex p-Cu^{2+} was indicated. It was also proved using the Benesi–Hildebrand method [31], the association constant (K_a) was approximately equal to 6.5 × 10^5 M^{-1} from fluorescence titration experiments (Figure S7), compared with reported Cu^{2+}-probe (2.18 × 10^4 M^{-1}) [32], and it indicated that a stable complex was formed between P with Cu^{2+}. Meanwhile, this 1:1 binding stoichiometric mode was also observed on MS spectra of P-Cu^{2+}, a peak at m/z 425.06 (ESI+) belonged to ([P + Cu^{2+}-H^+]^+) (Figure S8).

![Figure 4. Stoichiometry for P and Cu^{2+} by Job’s plot in alcohol–aqueous media (v/v, 8:2, pH 6.3, 0.02 M HEPES). The total concentration of ([P] + [Cu^{2+}]) was 10 μM.](image)

To explore the binding mode of complex P-Cu^{2+} and also to fully strengthen the sensing mechanism of P with Cu^{2+}, ^1H NMR experiment was carried out as shown in Scheme 2. Free P showed the NMR signal of H_b, H_a, H_c at 11.803, 9.485, 6.486 ppm. Upon the addition of 1.0 equiv Cu^{2+}, the NMR signal of H_b (O=C–NH–) disappeared, while that of H_a (–CH=N), H_c (–NH_2) downshifted to 9.639 and 7.912, respectively. Meanwhile, the addition of Cu^{2+} promoted the NMR peaks in the range of 6.5–9.0 ppm, which assigned to protons of pyrene and phenyl to widen and combine together. It suggested that the structure of P became more rigid after coordination with Cu^{2+}.

Keeping these results in mind, a sensing mechanism for the complex P-Cu^{2+} was surmised as depicted as Scheme 2. According to the MS spectra of P-Cu^{2+} complex, 425.06 (ESI+) belonged to [P + Cu^{2+}-H^+]^+, which proved the result that the formation of –OH enhanced the coordination ability of O, and a stable five-membered ring formed in the P-Cu^{2+} complex. Thus, –C=O, –NH_2 and –C=N groups participated in the coordination of P-Cu^{2+} complex. Moreover, the reaction of Cu^{2+} with P blocked the PET and C=N isomerization and greatly increased the emission intensity of pyrene moiety.
3.5. Cell Application

According to the excellent fluorescence properties of P, the application of P in living cells was explored. Firstly, cytotoxicity of P on Hela cells using a standard MTT assay was performed. As was shown in Figure S8, after the cells were incubated for 24 h with P concentrations within 0–10 µM, high cellular viability was still found to be about 95% (Figure S9), which demonstrated the low cytotoxicity and favorable biocompatibility of P to cultured Hela cells.

After that, the biological capability experiment of P to detect Cu$^{2+}$ in Hela cells was conducted. When the Hela cells were supplemented with 10 µM of P at 37 °C for 30 min, a very faint fluorescence intensity was detected (Figure S5), suggesting that background signal in the cells was very feeble. In contrast, when cells treated with Cu$^{2+}$ (1 µM) for 30 min, and then cultured upon addition of P (10 µM) for 30 min, a dramatic fluorescence change was triggered (Figure 5d), demonstrating that P had a good cell-membrane penetration, which also clearly displayed that the enhanced intracellular fluorescence change resulted from interaction of Cu$^{2+}$ with P inside cells. We could deduce that P had low toxicity and good cytocompatibility in view of the morphology of cells. Hence, P may act as a prospective probe to image Cu$^{2+}$ for bioanalysis.

In addition, fluorescence imaging of P (10 µM) with various [Cu$^{2+}$] (0, 2, 4, 6, 8 µM) in Hela cells was measured. It could be seen that the fluorescence intensity was enhanced regularly with increasing concentration of Cu$^{2+}$ as shown in Figure 6a–e, which also determined based on the average fluorescence intensity by confocal laser scanning microscopy as displayed in Figure 6f. These data also established that P could respond to intracellular Cu$^{2+}$ level changes in living cells.
Figure 5. Hela cells fluorescence images. (a) cells cultured with 10 μM of P for 30 min in the growth medium; (b) image of bright field cells in panel (a); (c) images of overlay in panels (a,b); (d) after supplementing with 1 μM of Cu^{2+} for 30 min, HeLa cells incubated with P (10 μM) for additional 30 min; (e) image of bright field cells in panel (d); (f) images of overlay in panels (d,e).

Figure 6. Confocal fluorescence images of Hela cells. (a–e): cells supplemented with 0, 2, 4, 6, 8 μM Cu^{2+} in the growth media for 30 min at 37 °C, and then incubated with 10 μM P for 30 min respectively; (f) fluorescence intensity of P with various [Cu^{2+}] ([a–e]: 0, 2, 4, 6, 8 μM).

The subcellular localization of P in Hela cells was also conducted, Green 488 was selected as a nuclear dye. Afterwards, Hela cells were co-cultured with Cu^{2+} (10 μM), P (10 μM) and Green 488 in the growth medium as shown in Figure 5. The fluorescence imaging was recorded as represented in Figure 7, P (blue panel, Figure 7a) located in the periphery of Green 488 (green panel, Figure 7b); we concluded that P was mainly located in the cytoplasm of living cells as shown in Figure 7c.
Figure 7. Subcellular localization of P in Hela cells. (a) cells supplemented with Cu\textsuperscript{2+} (10 μM) for 30 min, followed by the incubation with P (10 μM) for 30 min; (b) cells treated upon addition of Cu\textsuperscript{2+} (10 μM) for 30 min, P (10 μM) for another 30 min, and then cultured with Green 488 for 30 min; (c) images of overlay in panel (a,b).

Furthermore, the cytoplasm location of P in living Hela cells was also explored by conducting co-localization experiment (Figure 8). Mito Tracker Red and Green 488 were utilized as a mitochondria-specific dye and a nuclear-specific dye, respectively. The cell image of probe P (red panel, Figure 8a) and dye Mito Tracker Red (blue panel, Figure 8b) could not overlay completely in mitochondria (Figure 8d), so it could be concluded that probe P was not locate in mitochondria of Hela cells.

Figure 8. Cell localization experiment. (a) Cells were incubated with P for 30 min, and then Cu\textsuperscript{2+} (10 μM) for 30 min; (b) cells were incubated with P for 30 min, Cu\textsuperscript{2+} (10 μM) for 30 min, and Mito Tracker Red for 20 min at 37 °C, subsequently; (c) cells were incubated with P for 30 min, Cu\textsuperscript{2+} (10 μM) for 30 min, and Mito Tracker Red for 20 min at 37 °C, Green-488 for 10 min at 37 °C subsequently; (d) overlay field image of cells in panels (a–c).
3.6. Method Performance Comparison

The photophysical properties of reported probes and our proposed P toward Cu\(^{2+}\) were summarized as displayed in Table 1. Probes derived from different fluorophores, including spiropyran, pyridine, naphthalene, rhodamine and pyrene, exhibited fluorescence enhancement or quench which possessed good selectivity and high sensitivity, low LOD [24,32–35], and meanwhile most Cu\(^{2+}\)-probes can adopt dual signal changes towards Cu\(^{2+}\) except some probes [15,33,35,36]. Especially, some Cu\(^{2+}\) probes could display a ratiometric fluorescence change [32,35,37] as well as our proposed probe. Though the availability of fluorescence is increasing, there are still numerous challenges in developing new fluorescent probes for practical applications, such as unfavorable signal mode [15,34] rigorous testing media [15,36,37], no applicability [15,24,33–37] and narrow linear range [35]. Our newly designed pyrene-based probe possessed several advantages such as good selectivity and high sensitivity, a ratiometric dual fluorescence and wide linear range, encouraging applicability.

**Table 1. Performances comparison of various fluorescent probes for Cu\(^{2+}\).**

| Modes | Reagents | Selectivity | Linear Range (µM) | LOD (µM) | Testing Media | Applications | Remarks | Ref. |
|-------|----------|-------------|-------------------|----------|---------------|--------------|---------|------|
| Quenching \(\lambda_{ex}/\lambda_{em} = 556/603\) nm | Spiropyran derivative | Good | 0.75–3.6 | 0.15 | Ethanol or pH 6.98 (0.1 M Tris-HCl) | NA | Fluorescence changes | [15] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 530/580\) nm | Rhodamine derivative | Good | 0.05–0.9 | 0.03 | Water/methanol (1:4, v/v, pH 6.0, 20 mM HEPES) | NA | Dual chromo- and fluorogenic changes | [24] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 342/375–460\) nm | Pyridine derivative | Good | 0.1–20 | 0.02 | Tris-HNO\(_3\) (9:1 v/v, pH 7.0) | Water samples and living cells | Dual chromo- and fluorogenic changes, ratiometric. | [32] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 370/410–415\) nm | Pyrene derivative | Good | 0.1–1.9 | 0.000234 | Water/DMF solution (1:1, v/v) | NA | Fluorescent changes, ratiometric | [33] |
| Quenching \(\lambda_{ex}/\lambda_{em} = 355/565\) nm | Coumarin derivative | Good | 0.5–4.5 | 0.0634 | HEPES aqueous buffer (pH 7.0) | NA | Dual chromo- and fluorogenic changes | [34] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 451/473–525\) nm | Naphthalimide derivative | Good | 0–0.1 | 0.01 | Water-ethanol (6:4, v/v, pH 72, 50 mM HEPES) | NA | Fluorescent change, ratiometric | [35] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 350/495\) nm | Pyrene derivative | Good | NA | 0.15 | CH\(_3\)CN | NA | Fluorescent changes | [36] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 360/430–489\) nm | Naphthalimide derivative | Good | NA | NA | CH\(_3\)CN | NA | Dual chromo- and fluorogenic changes, ratiometric | [37] |
| Enhancement \(\lambda_{ex}/\lambda_{em} = 350/393–415\) nm | Pyrene derivative | Good | 0.5–8 | 0.16 | Alcohol–water media (v/v, 8.2, pH 6.3, 20 mM HEPES) | Cell imaging | Dual chromo- and fluorogenic changes, ratiometric | This work |

4. Conclusions

In summary, we took advantage of C=N isomerization and PET mechanism to design and synthesize facilely a newly pyrene-based probe for Cu\(^{2+}\). The formation of P-Cu\(^{2+}\) complex effectively led to the enhanced fluorescence response of pyrene moiety, which realized ratiometric detection of Cu\(^{2+}\). The probe exhibited an excellent feature of a dual-responsive colorimetric and fluorescent response to Cu\(^{2+}\), which showed a high selectivity for Cu\(^{2+}\) compared with other metal ions in alcohol–aqueous media (v/v, 8.2, pH 6.3, 0.02 M HEPES). The proposed probe can be applied to the quantification of Cu\(^{2+}\) with a linear range
of 0.5–8 μM and the detection limit of 0.16 μM, and it was also applied for the imaging of Cu²⁺ in living cells, which showed P had low toxicity and good cytocompatibility. Further investigations on its applications in life science are still underway. We believe that the design concept should provide reference to develop new “off-on” probes for transition metal ions.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/chemosensors10060207/s1](https://www.mdpi.com/article/10.3390/chemosensors10060207/s1). Figure S1: MS spectra of P; Figure S2: ¹H NMR spectrum of P; Figure S3: ¹³C NMR spectrum of P; Figure S4: MS spectra of P1; Figure S5: Fluorescence interfering experiment; Figure S6: Job’s plot for P and Cu²⁺; Figure S7: Benesi–Hildebrand plot between P and Cu²⁺. Figure S8: MS spectra of P-Cu²⁺; Figure S9: MTT experiment.

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