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A polypyridyl-pyrene based off-on Cd^{2+} fluorescent sensor for aqueous phase analysis and living cell imaging

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ABSTRACT

By retaining the quadrapyridyl receptor of polypyridylhexaazatriphenylene (a Cd^{2+} sensor reported by us) and extending its chromophoric group with pyrene, a chemical sensor (**1**) was designed and synthesized in this work. This sensor exhibit selective off-on fluorescence response to Cd^{2+} over other metal ions, and the detection limit is as low as 0.02 μM. The Cd^{2+} sensing of **1** has high water toleration and can be carried out in the media with the water content up to 70%. Additionally, **1** was successfully applied to the *in vivo* imaging of intracellular Cd^{2+} in living HaLa cells, and showed low cytotoxicity and cell membrane permeability in these experiments. These results suggest that **1** has potential application in the Cd^{2+} analysis of environmental and biological samples.

1. Introduction

In recent decades, contamination of the environment with heavy metal has been an important concern around the world. This kind of pollutants is undegradable, and could be accumulated along the food chain to the levels much higher than that in the environment [1]. Especially, cadmium (Cd), which is one of the most toxic heavy metals and widely used in agriculture and industry [2,3], has induced a serious of environmental and public health problems. The harmful effects of Cd on human body include a number of lesions in many organs and tissues such as cardiovascular diseases [4], emphysema and itai-itai disease [5], so the World Health Organization (WHO) stipulated a very strict contact standard for this pollutant (provisional tolerable weekly intake is 7 μg/kg of body weight) [6]. Therefore, it is in great need for the development of the methods for the qualitative and quantitative detection of Cd [7–10].

Compared with other analytical methods [11,12], fluorescent sensor shows great potential for the detection of ion species because of its operability and simplicity. Due to its *in vivo* detection feature, fluorescence sensor could also be used to visualize the intracellular distribution of metal ions [13–16]. The most important indexes to describe the detection ability of a chemical sensor are its selectivity and sensitivity [17,18]. Although a large number of Cd^{2+} fluorescence sensors have been reported in recent years, most of them suffer from the interference of other metal ions, especially Zn^{2+} [19–22], which is the second most abundant transition metal ion in living systems [21,23,24]. To improve the sensitivity of chemical sensor, besides reinforcing its binding ability with the target, enhancing the fluorescence emission of the complex formed between sensor and analyte is also a common strategy [25,26]. Additionally, water toleration is another requirement for a chemical sensor related to its practical application in environmental and biological detection, because Cd^{2+} such metal ions usually distribute in the aqueous phase. However, most of the reported Cd^{2+} sensors, including some developed by our group, can only work with the maximum water content of 5 – 10% in the media [27].

In our previous works [28], a hexaazatriphenylene (HAT) based sensor, hexa-2-pyridyl hexaazatriphenylene (HPDQ) has been developed. The study about this sensor showed that the quadridentate quadrapyridyl structure has high binding selectivity to Cd^{2+}. Based on these results, we expect that retaining the receptor structure of HPDQ and extending its chromophoric group would give a more sensitive response to Cd^{2+}. Therefore, in this work, by fusing the pyridyl substituted HAT and pyrene moieties, we

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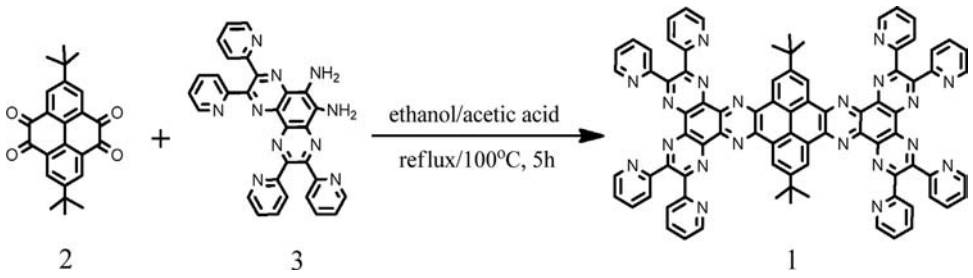


Scheme 1. Synthetic route of **1**.

designed and synthesized a new compound **1** (structure shown in Scheme 1), which has excellent fluorescence sensing ability for Cd^{2+} in aqueous media. Furthermore, **1** was also found to be able to monitor Cd^{2+} in living cells.

2. Experimental

2.1. Materials and general methods

All the starting reagents and chemicals for the synthesis of **1** were purchased from commercial sources and used as received. The solvents used for fluorescence measurements were purified by standard procedures. ^{1}H NMR spectra were recorded in CF_3COOD on a Bruker 300 MHz NMR spectrometer. High-resolution mass spectra were measured on an IonSpec 7.0T FT-ICR mass spectrometer. IR spectra were recorded on a TENSOR 27 OPUS Fourier transform infrared (FT-IR) spectrometer (Bruker) using KBr disks dispersed with sample powders in the 4000–400 cm^{-1} range. Elemental analyses (C, H, and N) were tested using a Perkin-Elmer 240 C analyzer. UV–vis absorption spectra were recorded by a Shimadzu UV-2450 UV–vis spectrophotometer. Fluorescence spectra were measured at room temperature on a Varian Cary Eclipse fluorescence spectrometer.

2.2. UV–vis absorption and fluorescence study

The solution of **1** (0.01 mM) was prepared with the mixed solvent of H_2O and EtOH (v/v=7/3). Stock solutions of the perchlorate salts of K^+, Na^+, Li^+, Mg^{2+}, Al^{3+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ag^+, Ni^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+} and Cd^{2+} (0.03 M) were prepared with deionized water. During titration tests, an appropriate aliquot of metal ion solution (0 – 50 μL) was added to the solution of **1** (3 mL), and the UV–vis and fluorescence spectra of the mixed solution were measured. For fluorescence measurements, the emission spectra were recorded at the maximum excitation wavelength of 350 nm, and the excitation and emission slit widths were both 5 nm.

2.3. Synthesis of the receptor 1

The synthesis route of **1** is shown in Scheme 1. Intermediates 2,7-di-tert-butylpyrene-4,5,9,10-tetraone(**2**) [29] and 2,3,8,9-tetra (pyridine-2-yl)pyrazino-[2,3-f]quinoxaline-5,6-diamine (**3**) [30,31] were prepared by reported procedures.

A solution of **2** (0.16 g, 0.43 mmol) dissolved in a mixture of ethanol (50 mL) and acetic acid (2 mL) were heated at 100 °C under the protection of N_2, and then **3** (0.5 g, 0.96 mmol) was added slowly. The mixture was refluxed for 5 h. After cooling to room temperature, the precipitate formed was collected by filtration and washed with ethanol, acetone and diethyl ether sequently to give the crude product as light yellow solid. This crude product was purified by column chromatography (silica gel) using CH_2Cl_2/CH_3OH (v/v=50/1) as eluent to give receptor **1** as light yellow solid in about 32% yield. ^{1}H NMR (300 MHz, CF_3COOD): δ (ppm): 1.58 (s, 18H), 8.18 (d, J=6 Hz, 8H), 8.44 (s, 8H), 8.58 (s, 4H), 9.09 (d, J=9 Hz, 8H), 10.01 (s, 8H). MS-ESI m/z: 1344.1 $[M+H]^+$. HRMS m/z: 683.24 $[M+H+Na]^{2+}/2$, 694.23, $[M+2Na]^{2+}/2$. IR (KBr, cm^{-1}): 3055, 2917, 2849, 1586, 1472, 1364, 1253, 768, 695. Anal. Calcd. for $C_{84}H_{54}N_{20}\cdot 3H_2O$: C, 72.19%; H, 4.33%; N, 20.05%; Found: C, 72.42%; H, 4.70%; N,19.64%.

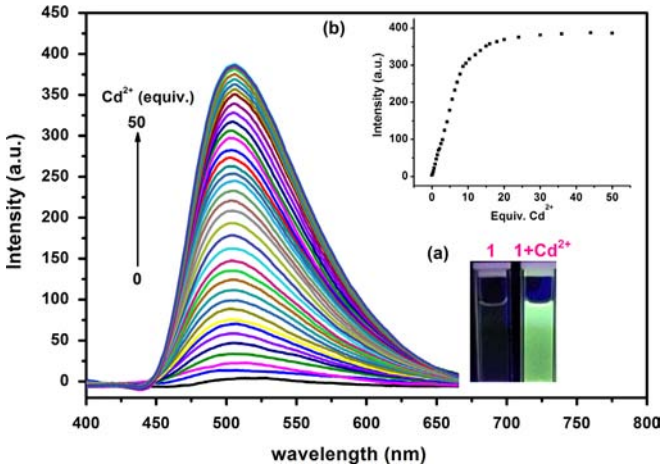


Fig. 1. Fluorescence titration of **1** (10 μM) with Cd^{2+} (from 0 to 500 μM) in H_2O–EtOH (v/v=7/3) solution. The excitation wavelength was 350 nm, and the excitation and emission slit widths were both 5 nm. Inset: (a) fluorescence change of **1** upon the addition of Cd^{2+} (500 μM) under the irradiation of a handheld UV lamp (centered at 365 nm); (b) change of the fluorescence intensity monitored at 506 nm.

2.4. Cell imaging

Experiments were performed on 16 mm × 16 mm glass slides. HeLa cells were seeded in a 6-well plate at a density of 4 × 104 cells per well with Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% fetal bovine plasma (FBS) in the atmosphere of 5% CO_2 at 37 °C overnight to get a suitable density. Certain amounts of the stock solution of Cd^{2+} (0.1 mL, 3 μM) were added to four wells, and then the cells were further incubated in the presence of Cd^{2+} for 12 h to prepare Cd^{2+}-uptaken HeLa cells. The cells in the other two wells in the absence of **1** or Cd^{2+} were also incubated for 12 h for comparison.

3. Results and discussion

3.1. Fluorescence response

We first investigated the fluorescence response of compound **1** to Cd^{2+} with the excitation at 350 nm. As shown in Fig. 1, compound **1** shows a very weak fluorescence emission with the

maximum at 500 nm. After the addition of 500 μM of Cd^{2+}, a remarkable fluorescence peak appeared at 506 nm. Relative to free **1**, the emission intensity increased by 128-fold ($I_{506}^{1-Cd}/I_{500}^{1}$), and the fluorescence quantum yield (Φ_f, determined with quinine sulfate in 0.5 M H_2SO_4 as the standard [32–34]) exhibits an approximate 70-fold enhancement (from 0.002 to 0.14). In accordance with the change in fluorescence spectra, under the irradiation of UV lamp, the solution of **1** changed from emission-less to cyan luminous upon the addition of Cd^{2+} (inset a in Fig. 1), which could be distinguished easily by naked eyes.

Since the water toleration is essential to a sensor for its practical application, we study the effects of water content on the fluorescence response of **1** to Cd^{2+} ion to optimize its detection conditions for environmental and biological samples. These experiments were carried out using a serious of mixed solvents of ethanol and water with different proportions. As shown in Fig. 2, in the vol% (H_2O) range of 0–70, the fluorescence intensity after the addition of Cd^{2+} decreased slowly with the increase of water content in the solution. In the 7:3 H_2O–EtOH solution, **1**-Cd^{2+} can still remain 44% of the emission intensity of that in pure ethanol. These results show that the water toleration of **1** is extremely high compared with other HAT based sensors, which can only operation in pure organic phase [28] or the solvent with minor content of water (< 10%) [31]. However, when the vol% (H_2O) was above 80, further increase of water content would cause a drastic decrease of the fluorescence emission of **1**-Cd^{2+} to nearly zero. Based on these results, 7:3 H_2O–EtOH solution is considered to be the optimum for the practical detection of compound **1**, and in the following experiments, its fluorescence response was mainly characterized in this media.

The effect of pH on the fluorescence response of **1** was investigated in the range of pH 1–11. As shown in Fig. S1, the fluorescence emission of **1**-Cd^{2+} enhances with pH value in the range of pH 1–7 and gives a plateau in the range of pH 7–10. Further enhancement of the pH value would decrease the fluorescence intensity, probably due to the formation of $Cd(OH)_2$ precipitate. These results show that the optimal performance of **1** is reached in the pH range of 7–10, which is suitable for the analysis of normal environmental and biological samples.

The reproducibility of this Cd^{2+} sensing method was estimated by performing ten independent experiments of the measurement of the fluorescence change upon the addition of Cd^{2+} to the solution of **1** (Fig. S2). The fluorescence enhancements observed in these repetitive experiments are highly consistent with each other, and the fluorescence intensity changes give a small standard deviation of 1.6%. The good reproducibility of the fluorescence response ensures the accuracy of Cd^{2+} analysis.

A temporal scan of the fluorescence intensity after the addition of Cd^{2+} was carried out to examine the response time of **1**. As shown in Fig. S3, the fluorescence emission gave a sharp enhancement immediately after Cd^{2+} addition and leveled off in half a minute, showing the fast response rate of this sensing method.

To evaluate the sensitivity of compound **1** toward Cd^{2+}, fluorescence titration experiments were carried out (also shown in Fig. 1). It was observed that the fluorescence emission intensity enhanced sharply with the addition of Cd^{2+} in the low concentration range but leveled off at high concentration to saturation (inset b). Compound **1** gives a good linear response to Cd^{2+} in the range of 0 – 100 μM (R^2=0.9985, Fig. S4). From the slope of the fitting line (k) and the normal deviation of fluorescence measurement (σ), the detection limit ($3\sigma/k$) of **1** towards Cd^{2+} was calculated to be 0.02 μM, which is sufficient for the detection of sub-micromolar concentrations of Cd^{2+} in many biological and environmental systems [35]. This detection limit is remarkably lower than those of other HAT based sensors [27,28], probably due to the extending of the chromophoric group with pyrene in **1** exaggerates the fluorescence response.

Fluorescence titration results also provide information about the binding mode and stability of **1**-Cd^{2+} [36,37]. As shown in Fig. 3, the plot of $\log[(F-F_0)/(F_\infty-F)]$ *vs.* $\log[Cd^{2+}]$ exhibited a slope of 2.26, which illustrates an approximately 1:2 stoichiometric ratio between **1** and Cd^{2+}. This 1:2 stoichiometry of **1**-Cd^{2+} is supported by the Job's plot analysis (Fig. 4). From the fitting of fluorescence titration results, the logarithm of the cumulative stability constant ($\log\beta$) of **1**-Cd^{2+} is determined to be 9.68 ± 0.14, which suggests a strong binding affinity of **1** towards Cd^{2+}. Considering the structure of compound **1** and that the ligands with quadridentate quadrapyridyl structure usually have high affinity for Cd^{2+}, we expect that **1**-Cd^{2+} has the binding mode shown in Scheme 2, and the fluorescence response of **1** to Cd^{2+} is based on a chelation enhancement of fluorescence (CHEF) mechanism [38].

Additionally, with the alternative addition of Cd^{2+} and EDTA (effective removal of Cd^{2+}), the reversibility of the fluorescence response of **1** was tested (Fig. 5). After the fluorescence enhancement caused by the addition of Cd^{2+}, the following addition of EDTA would make the fluorescence intensity recover to the level before Cd^{2+} addition. The second cycle of the sequential addition

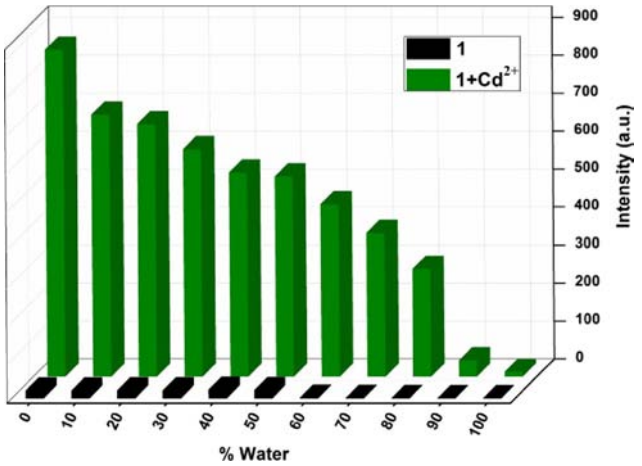


Fig. 2. Fluorescence response of **1** (10 μM) to Cd^{2+} (500 μM) in H_2O–EtOH solutions with different proportions. The columns represent the fluorescence intensity at 506 nm with the excitation at 350 nm.

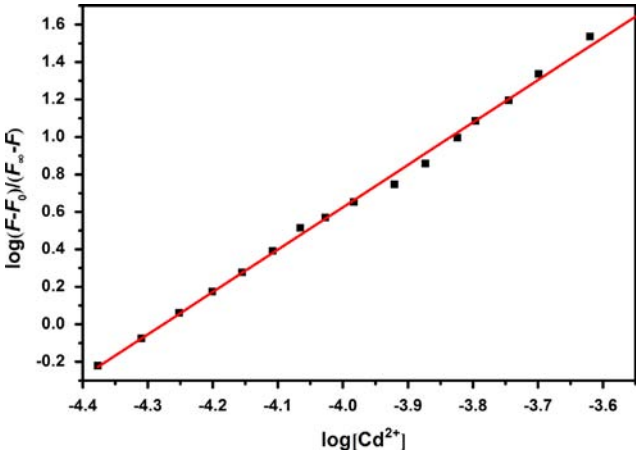
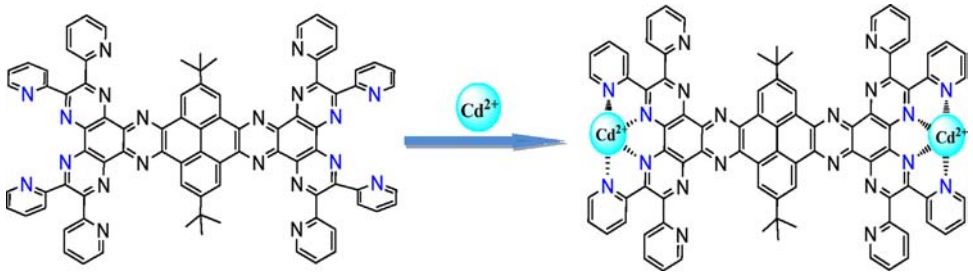


Fig. 3. The linear fitting of fluorescence intensity at 487 nm with $\log[(F-F_0)/(F_\infty-F)]=n\log[Cd^{2+}]+\log\beta$. n is the complex-ratio of **1** to Cd^{2+}; β is the stability constant of **1**-Cd^{2+}; F_0 is the fluorescence intensity of the solution of **1** without any cations; F is the fluorescence value obtained after adding a given amount of Cd^{2+} to the **1** solution; F_∞ is the fluorescence value of **1** in the presence of excess amount of Cd^{2+}.



Scheme 2. Proposed binding mode for complex **1**-Cd^{2+}.

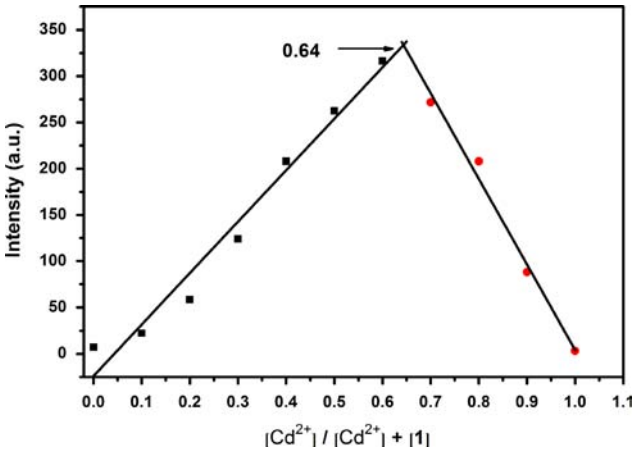


Fig. 4. Job's plot for **1**-Cd^{2+} complex ([1]+[Cd^{2+}] =100 μM).

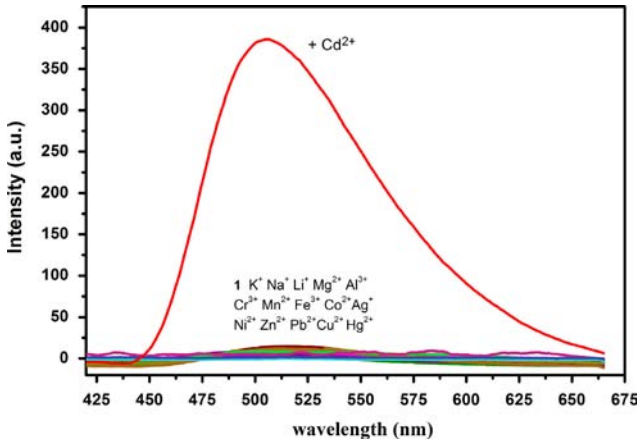


Fig. 6. Fluorescence spectra of **1** (10 μM) in H_2O–EtOH (v/v=7/3) solution after the addition of different metal ions (500 μM), λ_{ex}=350 nm.

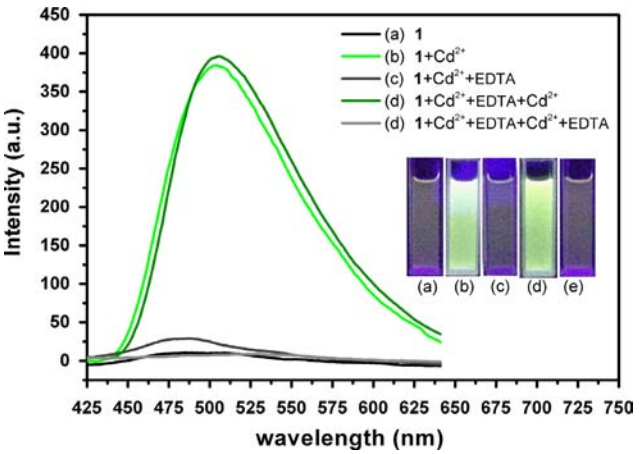


Fig. 5. The reversibility of the fluorescence response of **1** in the sensing of Cd^{2+}. Cd^{2+} (500 μM) and EDTA (500 μM) were added alternatively to the solution of **1** (10 μM). Inset: visual fluorescence change under the irradiation of a handheld UV lamp (centered at 365 nm).

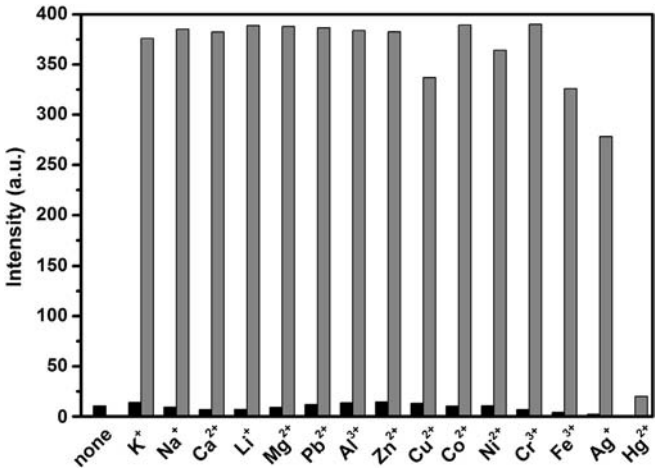


Fig. 7. Competitive selectivity of 1 towards Cd^{2+} in the presence of different metal ions. Black and gray bars represent the fluorescence intensity at 506 nm of the solution containing 1 (10 μM) and different metal ions (500 μM) before and after the addition of Cd^{2+} (500 μM), respectively.

of Cd^{2+} and EDTA gives almost the same fluorescence change with that of the first one. These results prove that the fluorescence response of **1** to Cd^{2+} is completely reversible, and this sensor could be reused to the detection systems.

The UV–vis titration of Cd^{2+} was also carried out with the solution of **1** (10 μM) in H_2O–EtOH (v/v=7/3) media. As shown in Fig. S5, the absorbance of **1** enhanced gradually with the addition amount of Cd^{2+} in the whole tested spectrum range, and gave an absorption band with the maximum at 370 nm. This absorption band extends to the visible region (> 400 nm), which makes the solution change from colorless to light yellow upon the addition of Cd^{2+} (inset of Fig. S5). The linear fitting of the absorbance change at 370 nm with Cd^{2+} amount gives the binding stoichiometry of 1:2.33 and logβ of 9.04 (Fig. S6), which are close to the values obtained from fluorescence titration experiments (1:2 and 9.68, respectively, discussed above). These results corroborate that **1** forms stable coordination complex with Cd^{2+} in the sensing process.

3.2. Selection and competition experiments

The selectivity of sensor **1** was tested with the addition of excess amount of other metal ions, such as K^+, Na^+, Li^+, Mg^{2+}, Al^{3+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ag^+, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+} to its solution. In contrast to Cd^{2+}, the addition of these ions

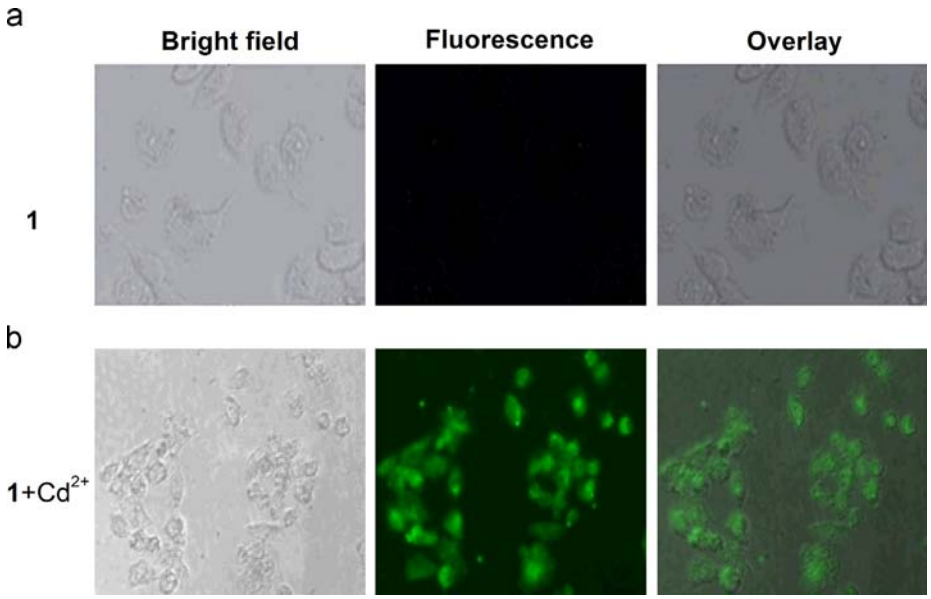


Fig. 8. Fluorescence images of HaLa cells treated with **1** and Cd^{2+}. (Left) bright field image; (Middle) fluorescence image; (right) merged image.

only cause negligible change in the fluorescence spectra (λ_{ex}=350 nm, Fig. 6). Even Zn^{2+}, which has similar electronic structure and chemical properties to those of Cd^{2+}, only gives a 0.8-fold enhancement on the emission intensity. These results show that **1** has specific response to Cd^{2+} over other metal ions.

A critical characteristic for a selective ion sensor is its ability to distinguish the target ion under the interference of other competing species. To further explore the anti-interference ability of **1** for Cd^{2+} sensing, we conduct competition experiments by observing its fluorescence response to Cd^{2+} in the presence of other metal ions. As shown in Fig. 7, all the tested metal ions, except for Hg^{2+}, exerted no or little influence on the fluorescence detection to Cd^{2+}, which further confirm the high binding affinity of **1** with Cd^{2+}. Hg^{2+} shows a remarkable quenching effect on the emission of **1**-Cd^{2+}, which may be due to its heavy atom effect [39–41].

3.3. Living cell imaging

After studying the fluorescence response of **1** in aqueous media, we turned our attention to its performance in living cells. These experiments were carried out with HeLa cells, a commonly used human cell line derived from cervical cancer cells, and the images were taken with confocal fluorescence microscopy. HeLa cells were first incubated for 12 h in the presence of **1** to prepare sensor loaded cells, which showed ignorable background fluorescence (Fig. 8a). Most of the tested cells (> 90%) remained alive during the incubation process, indicating the low cytotoxicity of sensor **1**. Treatment of the loaded cells with Cd^{2+} (as a harmful exotic for the cell) gave an obvious cyan fluorescence (Fig. 8b). The bright-field transmission and fluorescence images overlaid perfectly, which proves that the fluorescence signal comes from the intracellular region and sensor **1** containing the hydrophobic tertiary butyl pyrene core is cell membrane permeable. All these results indicate that sensor **1** is suitable for the *in vivo* imaging of Cd^{2+} in living cells.

4. Conclusions

In summary, an off-on fluorescence sensor, compound **1**, has been designed and synthesized. Having the receptor with quadrapyridyl structure, this compound shows selective fluorescence response to Cd^{2+} over other metal ions, and the detection limit could be as low as 0.02 μM. Additionally, the water toleration, low cytotoxicity and cell membrane permeability of this sensor make it can be used to the *in vivo* monitoring of intracellular Cd^{2+} levels. Based on above experimental observations, we believe that, as a metal ion sensor, **1** has potential application in environmental and biochemical events. The results of this work also illustrates that the strategy of remaining the receptor structure but altering the reporter component is a reliable and convenient strategy for the design of the chemical sensors for a given species.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.046.

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