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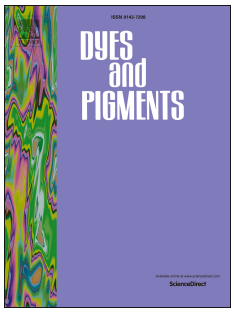

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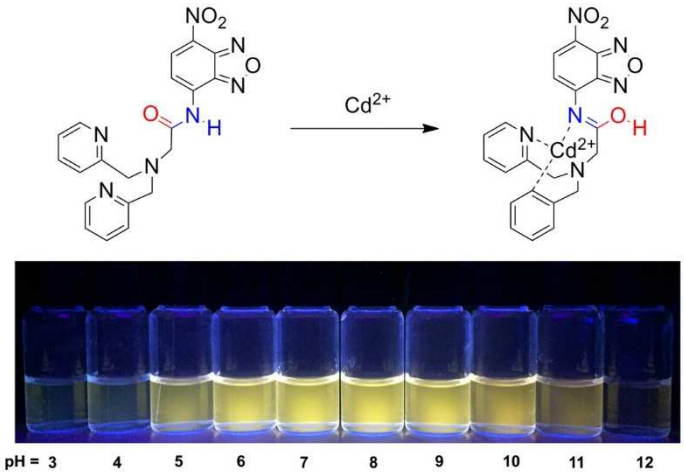

CdTS can fluoresently recognize Cd^{2+} across a wide pH range.

Cd^{2+}–Triggered Amide Tautomerization Produces a Highly Cd^{2+}–Selective Fluorescent Sensor across a Wide pH Range

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Abstract: An NBD-derived fluorescent sensor termed **CdTS** was reported to sense Cd^{2+} with very high binding selectivity and significant fluorescence turn-on signal selectivity (65 fold enhancement). The amide/di-2-picolylamine receptor binds Cd^{2+} in an imidic acid tautomeric form, but binds most of other metal ions in an amide tautomeric form. The transformable ability makes **CdTS** have the specific selectivity for Cd^{2+}. Additionally, **CdTS** can fluoresently and colorimetricly recognize Cd^{2+} across a wide pH range from 4.5 to 11.5. Finally, we applied **CdTS** to detect Cd^{2+} in living cells.

Keywords: Amide tautomerization; Fluorescent sensor; Cadmium ions; Wide pH range; Cells imaging

1 Introduction

Cadmium is extremely toxic metals and can cause renal dysfunction, calcium metabolism disorders and an increased incidence of cancers of the lung, prostate, pancreas, and kidney[1-3]. Its wide use in industry and agriculture lead to a high level of absorption and accumulation in plants and other organisms, thus causing cadmium contamination. Although it has been demonstrated that the uptake of Cd^{2+} can affect cellular functions, the molecular mechanisms of

Cd^{2+}-causing diseases remains unclear[4]. Fluorescent sensors are powerful tools to monitor in vitro and/or in vivo biologically relevant species such as metal ions due to the simplicity and high sensitivity of fluorescence[5-8]. Until now, a number of fluorescent sensors for Cd^{2+} have been reported with some successful applications to image Cd^{2+} in living cells[9-33]. However, specific binding selectivity for Cd^{2+} over Zn^{2+} and Hg^{2+} in the same family as well as biologically abundant transition metal ions like Fe^{2+}/Fe^{3+} and Cu^{2+} is still a challenge for fluorescent sensor design. Cadmium speciation, adsorption and distribution in soils depends strongly on pH, with its mobility decreasing with increasing alkalinity[34-35]. pH is also one of the most important environmental factors determining cadmium bioavailability to organisms[34,36]. For example, Bervoets *et al*. reported that cadmium uptake by the midge larvae Chironomus riparius increased with increasing pH of exposure in the range of 5.5–9.0 but decreased between pH 9.0 and 10.0[37]. It has been widely reported that lowering environmental pH reduces cadmium toxicity in bacteria[38-39]. For example, Worden *et al*. confirmed that cadmium was less toxic to *Escherichia coli* at pH 5 than at pH 7 in M9 minimal salts medium[40]. Understanding mechanisms by which pH mediates cadmium toxicity would be useful for minimizing cadmium toxicity in the environment and for gaining insight into the interactions between organic and inorganic components of life. Taking into account the complexity and uncertainty of environmental pH, a fluorescent probe that is able to identify cadmium ions over a wide range of pH, covering acidic to basic, will be very helpful to understand the bioavailability and toxicity of cadmium ions. Unfortunately, these reported fluorescent sensors have the ability to identify Cd^{2+} only in a narrow pH range around neutral.

Most receptors for metal ions have a confined binding ‘cavity’. The high selectivity to an

analyte is extremely difficult to achieve due to a single binding pattern. If the receptor is transformable to bind the analyte of choice, that is the binding pattern of the receptor with analyte is different from that with other competitors, the receptor may bind the analyst more specific and favorable. In our previous work, we found an amide-containing DPA receptor shows extreme selectivity for Zn^{2+} attributing to their transformable ability[41-42]. The receptor binds Zn^{2+} in an imidic acid tautomeric form with highest affinity but most other HTM ions in an amide tautomeric form. We conjugated this receptor to two different fluorophores naphthalimide and coumarin to get zinc probes termed **ZTRS**[38] and **CTS**[42], respectively. In this paper, we introduced the amide-DPA receptor to an NBD fluorophore. The newly synthesized compound **CdTS** displayed unexpected high binding selectivity for Cd^{2+} rather than Zn^{2+} along with a unique dramatic fluorescence enhancement. The ^{1}H-NMR and fluorescence studies reveal that the receptor binds Cd^{2+} in an imidic acid form. It is worth to notice that **CdTS** can fluoresently and colorimetricly recognize Cd^{2+} across a wide pH range from 4.5 to 11.5. **CdTS** was easily synthesized from 4-Chloro-7-nitrobenzofurazan in three steps as shown in Scheme 1.

Scheme 1

2 Experimental

2.1 Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification.^{1}H-NMR and ^{13}C-NMR spectra were recorded on a VARIAN INOVA-500 spectrometer, using TMS as an internal standard. Mass spectrometry data were obtained with a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. UV-visible spectra were collected on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. Fluorescence measurements

were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018).

2.2 Synthesis

*2.2.1 Synthesis of **2**.*

To a solution of **1** (400 mg, 1 mmol) in MeOH (20 mL) was added ammonium hydroxide (4 mL, 28% in water) at room temperature. The reaction solution was stirred at room temperature for 24 h under a nitrogen atmosphere. The solvent was evaporated in vacuo, then the crude product was purified by silica gel chromatography with PE:EA = 1:1 to afford desired product as a brown solid (194 mg, 54% yield).

*2.2.2 Synthesis of **3**.*

A solution of 2-chloroacetyl chloride (146 mg, 1.30 mmol, 1.2 eq.) in 5 mL of dry CH_2Cl_2 was added dropwise to a solution of **2** (194 mg, 1.08 mmol) and 4-dimethylaminopyridine (DMAP) (171 mg, 1.40 mmol, 1.3 eq.) in 20 mL of dry CH_2Cl_2 stirred in an ice bath. After stirred 2 h at room temperature, the mixture was removed under reduced pressure to obtain a pale solid, which was purified by silica gel column chromatography with PE:EA = 5:1 to afford desired product as a yellow solid (119 mg, 43% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.52 (s, 1H, N*H*), 8.58 (d, J = 8.0 Hz, 1H), 8.48 (d, J = 8.0 Hz, 1H), 4.35 (s, 1H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.5, 145.9, 143.8, 136.1, 134.3, 130.8, 114.6, 43.9. HRMS (ESI): Calcd for $C_8H_6ClN_4O_4$ $[M+H]^+$ 257.0078; found 257.0080.

*2.2.3 Synthesis of **CdTS**.*

3 (100 mg, 0.39 mmol), di-(2-picolyl)amine (DPA) (42 mg, 0.39 mmol), K_2CO_3 (107 mg, 0.78 mmol), and potassium iodide (50 mg) were added to CH_3CN (50 mL). After stirring at 60 ^{o}C for

10 h under nitrogen atmosphere, the mixture was cooled to room temperature, and the mixture was removed under reduced pressure ,then the residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH =100:1) to afford **CdTS** as a pale yellow solid (101 mg, 62 % yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.37 (s, 1H, N*H*), 8.58 (d, *J* = 4.5 Hz, 2H), 8.53 (d, *J* = 8.0 Hz, 1H), 8.45 (d, *J* = 8.0 Hz, 1H), 7.62 (t, *J* = 7.5 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 7.17 (t, *J* = 6.0 Hz, 2H), 4.05 (s, 4H), 3.62 (s, 2H) ; ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.3, 157.6, 149.6, 145.4, 143.3, 136.8, 134.4, 134.3, 130.5, 123.2, 122.7, 112.8, 60.6, 58.9. HRMS (ESI): Calcd for $C_{20}H_{18}N_7O_4$ $[M+H]^+$ 420.1420; found 420.1436.

2.3 Culture of CHO cells and fluorescent imaging

CHO cells was hatched in an atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) at 37 ^{o}C. The cells were seeded in 24-well flat-bottomed plates and then incubated for 72 h at 37 ^{o}C under 5% CO_2. 5 μM **CdTS** in the culture media containing 0.1% (v/v) DMSO was added to the cells and the cells were incubated for 1 h at 37 ^{o}C. After washing twice to remove the remaining sensor, the cells were treated with 10 μM $Cd(ClO_4)_2$ for 30 min. Fluorescence imaging was observed under a confocal microscopy (Olympus FV1000) with a 60×objective lens.

3 Results and discussion

3.1 Cd^{2+} selectivity

The selectivity of the fluorescent response of **CdTS** to metal ions was first examined. **CdTS** has a good water solubility, and in HEPES buffer at pH 7.4 (0.5% DMSO) displays very weak emission centered at 555 nm (Φ = 0.005) upon excitation at 462 nm. Addition of 1 equiv of Cd^{2+} induces a bathochromic shift of the dominant emission band to 567 nm with a significant

fluorescence increase (65-fold, Φ = 0.32) (Fig. 1a). The **CdTS** /Zn^{2+}, **CdTS** /Hg^{2+} and **CdTS** /Pb^{2+} complex showed slight enhanced emissions (Fig. 1a inset). The addition of other metal ions, such as Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Ag^{+} and Pd^{2+}, produced a negligible change in the fluorescence spectra of **CdTS** (Fig. 1a). Thus, **CdTS** has a very high fluorescence selectivity for Cd^{2+}.

Fig. 1

It's worth noting, even the changes were very small, that the binding of Zn^{2+} and Hg^{2+} blue-shifted the emission to 548 nm and 552 nm, respectively, and the binding of Pb^{2+}, similar to Cd^{2+}, red-shifted the emission to 563 nm (Fig. 1a inset). Inspired by the transformable sensing mechanism of **ZTRS** and **CTS** which show blue-shifted emission in an amide tautomeric binding form, while red-shifted emission in an imidic acid tautomeric form, we propose that **CdTS** binds Cd^{2+} and Pb^{2+} in an imidic acid tautomeric form, but Zn^{2+} and Hg^{2+} in an amide tautomeric binding form. Subsequently, we performed competition experiments in the presence of 30 equiv of Li^{+}, Na^{+}, K^{+}, Mg^{2+}, or Ca^{2+}, and 3 equiv of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Ag^{+}, Hg^{2+}, Pb^{2+} or Pd^{2+}, with the subsequent addition of 1 equiv of Cd^{2+}. As shown in Fig. 1b, the emission profile of the **CdTS** /Cd^{2+} complex is unperturbed in the presence of alkali and alkaline earth cations. Of transition metal ions we tested, only Zn^{2+} and Cu^{2+} limit the turn-on response of **CdTS**, indicating the strongest affinity and selectivity for Cd^{2+} (Fig. 1b) over these metal ions. We believe the specificity for Cd^{2+} and unique fluorescence responses result from the transformable ability of **CdTS** that is the displacement of other metal ions by Cd^{2+} induces transformation of chelation

from an amide to an imidic acid tautomeric form. Accordingly, the addition of Cd^{2+} induced a much more significant red-shift in absorption than other metal ions (Fig. 1c). Further studies indicated the detection limit of **CdTS** for Cd^{2+} is down to 10 nM.

3.2 Binding mechanism

To confirm the imidic acid tautomeric binding form with Cd^{2+}, we conducted ^{1}H-NMR titration experiments in DMSO-*d6*. The chemical shift of the amide NH can be used to distinguish whether Cd^{2+} is bound to carbonyl oxygen or imidic acid nitrogen. The complexation of the carbonyl oxygen with metal ions blocks the amide resonance and then shifts the NH resonance upfield. Correspondingly, the binding of the amide nitrogen with metal ions acts as an electron-withdrawing group to shift the OH resonance downfield. As shown in Fig. 2a, the resonance of the H4-9 protons undergo down-field shifts in DMSO with the addition of 1 equiv of Cd^{2+}, which demonstrate the coordination of Cd^{2+} with two pyridyl nitrogens and one aliphatic amine nitrogen. With the addition of Cd^{2+}, the chemical shift of the amide NH changed, which indicated the coordination of Cd^{2+} with the amide group. As aforementioned, the clear down-field of H3 from 11.98 to 12.13 suggested that **CdTS** binds Cd^{2+} in an imidic acid tautomeric form in DMSO.

Fig. 2

Fig. 3 showed the fluorescence and absorption titration experiments of **CdTS** with Cd^{2+} in HEPES. When Cd^{2+} was added to the solution of **CdTS**, a red-shifted emission with a maximum at 567 nm was increased subsequently (Fig. 3a). The inset job plots in Fig. 3a indicated the **CdTS**/Cd^{2+} complex had 1:1 stoichiometry. On addition of 1 equiv of Cd^{2+} to the solution of

CdTS, the absorbance at 400 nm decreased sharply to its limiting value, while the one at 462 nm increases prominently with an isosbestic point at 424 nm, which induces a color change from colourless to yellow (Figure 3b).

Fig. 3

3.3 Effect of pH on Cd^{2+} detection

The influence of pH on the detection properties of **CdTS** for Cd^{2+} was then examined by fluorescence titration in HEPES solution (Figure 4). From pH 4 to 12, the fluorescence intensities of **CdTS** were all increased by the addition of Cd^{2+}. Particularly between pH 4.5 and 11.5, the fluorescence increase responses were significant, indicating the excellent fluorescent sensing properties of **CdTS** for Cd^{2+} in this pH range (Fig 4a). More importantly, the obvious colour change to yellow and yellow fluorescence of **CdTS** in the prescence of Cd^{2+} from pH 4.5 to 11.5 facilitate the Cd^{2+} detection and expand the detection scope (Fig. 4b). Particularly, the probe **CdTS** has a great potential to investigate the pH-dependent distribution and toxicity of Cd^{2+}. **CdTS** is also anticipated to help the understanding of mechanisms by which pH mediates cadmium toxicity.

Fig. 4

3.4 Cell imaging of Cd^{2+}

We then sought to examine the Cd^{2+} sensing properties of **CdTS** in living cells. CHO cells treated with 5 μM **CdTS** alone exhibited very weak background fluorescence (Fig. 5a). The cells incubated with 10 μM $Cd(ClO_4)_2$ and **CdTS** displayed enhanced fluorescence (Fig. 5b). These experiments indicate **CdTS** can recognize intracellular Cd^{2+} fluorescently. The cytotoxicity of

CdTS was examined toward CHO cells by a MTT assay (Fig S7). The results showed that > 90% CHO cells survived after 24 h (5.0 μM **CdTS** incubation), demonstrating that **CdTS** was of low toxicity toward cultured cell lines.

Fig. 5

3.5 **Conclusion**

In summary, we have developed an NBD-based fluorescent sensor **CdTS** for Cd^{2+} recognition which contains a transformable amide-DPA receptor. **CdTS** has the strongest affinity with Cd^{2+} among competitive metal ions and displays an excellent fluorescent selectivity for Cd^{2+} with an enhanced emission resulting from the Cd^{2+}–triggered amide tautomerization. More importantly, **CdTS** can fluoresently and colorimetricly recognize Cd^{2+} across a wide pH range from 4.5 to 11.5, which makes **CdTS** a candidate to investigate the pH-dependent distribution and toxicity of Cd^{2+}. However, Cd^{2+}–triggered amide tautomerization in **CdTS** is beyond our expectation. In conjunction with Zn^{2+}–triggered amide tautomerization in ZTRS and CTS, we propose that various metal ions may trigger the amide tautomerization of amide-DPA receptor in different systems.

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Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.dyepig.XXXX.XX.XXX .

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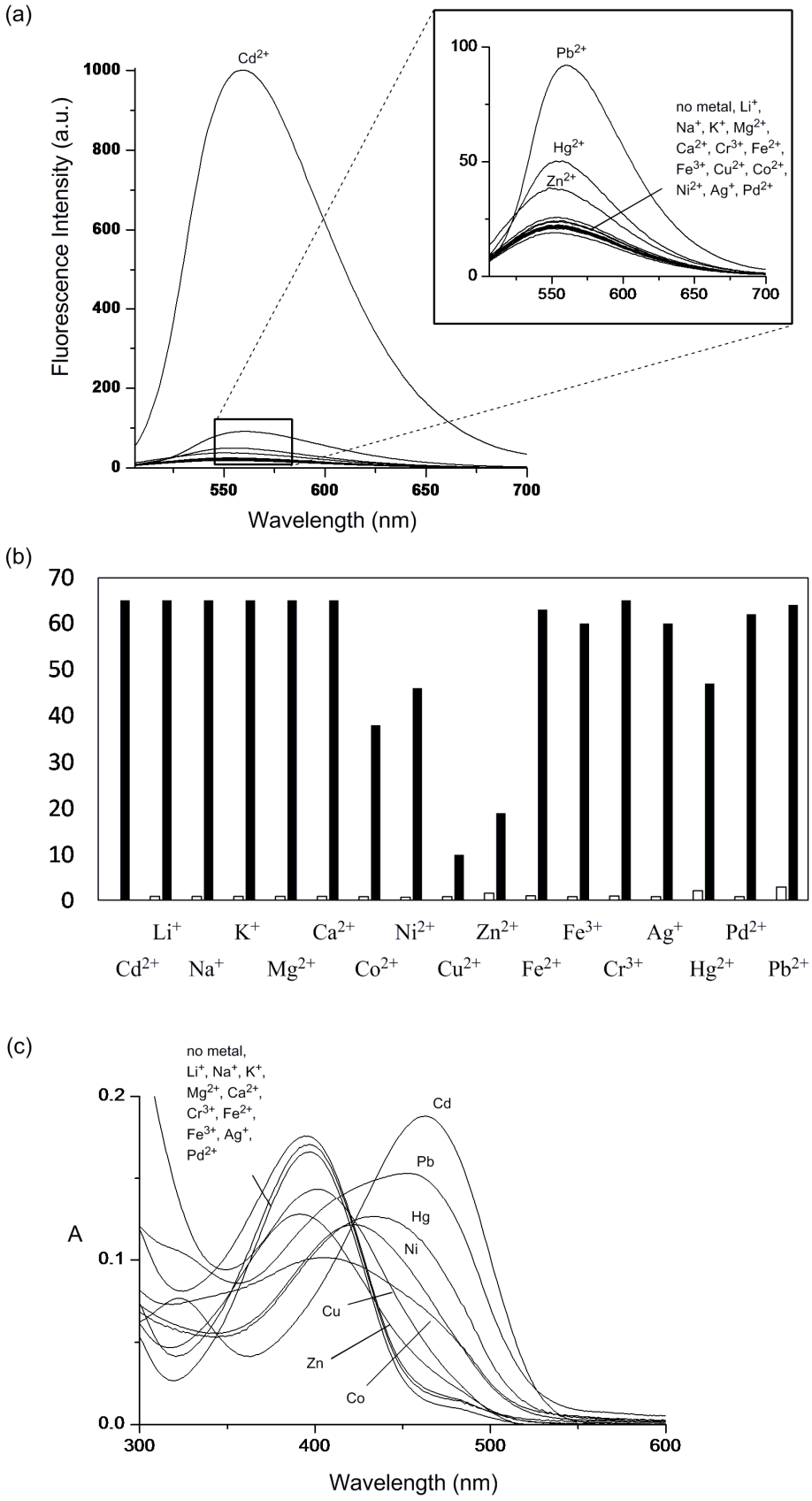

(a)
Fluorescence Intensity (a.u.)
Wavelength (nm)
Cd2+
Pb2+
Hg2+
Zn2+
no metal, Li+, Na+, K+, Mg2+, Ca2+, Cr3+, Fe2+, Fe3+, Cu2+, Co2+, Ni2+, Ag+, Pd2+
(b)
Li+ K+ Ca2+ Ni2+ Zn2+ Fe3+ Ag+ Pd2+
Cd2+ Na+ Mg2+ Co2+ Cu2+ Fe2+ Cr3+ Hg2+ Pb2+
(c)
no metal, Li+, Na+, K+, Mg2+, Ca2+, Cr3+, Fe2+, Fe3+, Ag+, Pd2+
Cd
Pb
Hg
Ni
Cu
Zn
Co
A
Wavelength (nm)

Fig. 1

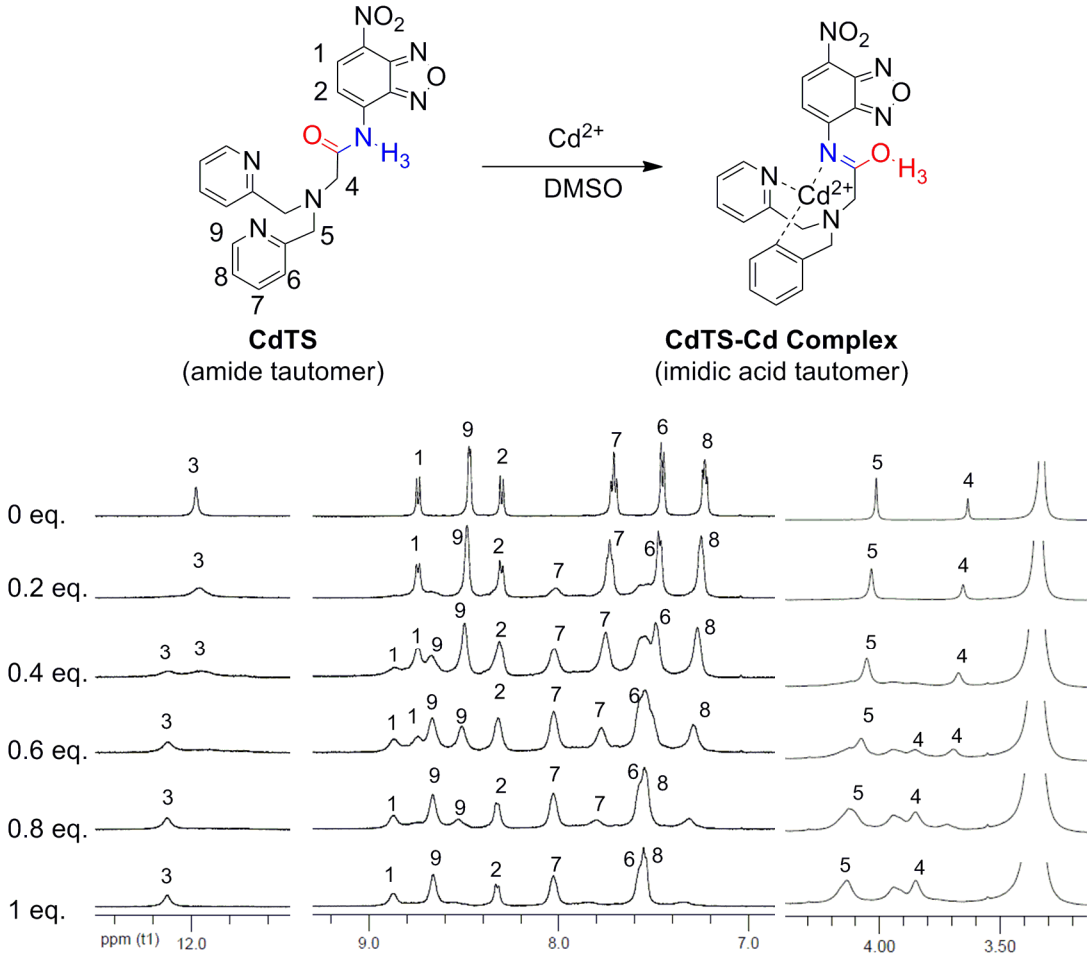


Fig. 2

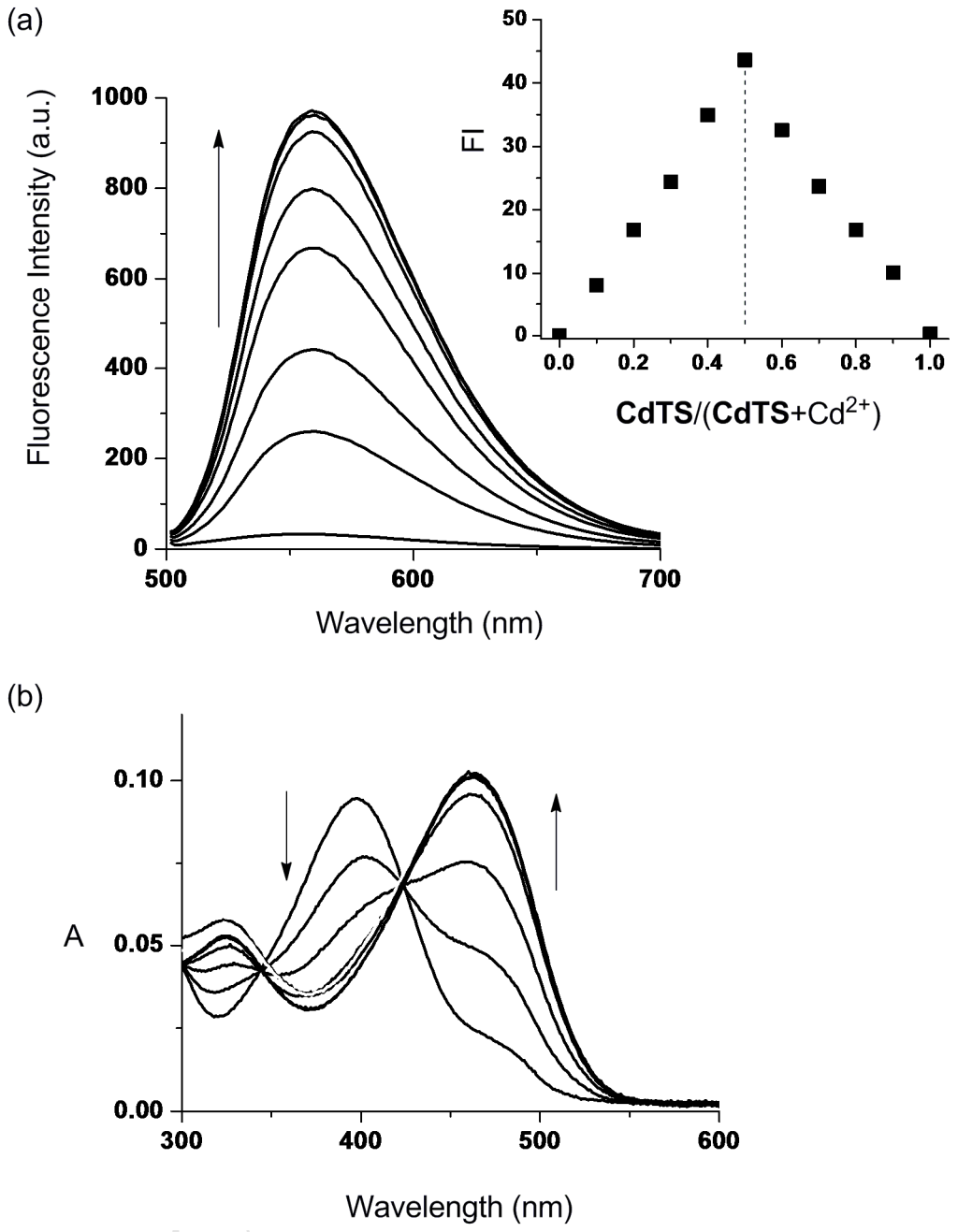

(a)
Fluorescence Intensity (a.u.)
1000
800
600
400
200
0
500
600
700
Wavelength (nm)
FI
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
CdTS/(CdTS+Cd2+)
(b)
A
0.10
0.05
0.00
300
400
500
600
Wavelength (nm)

Fig. 3

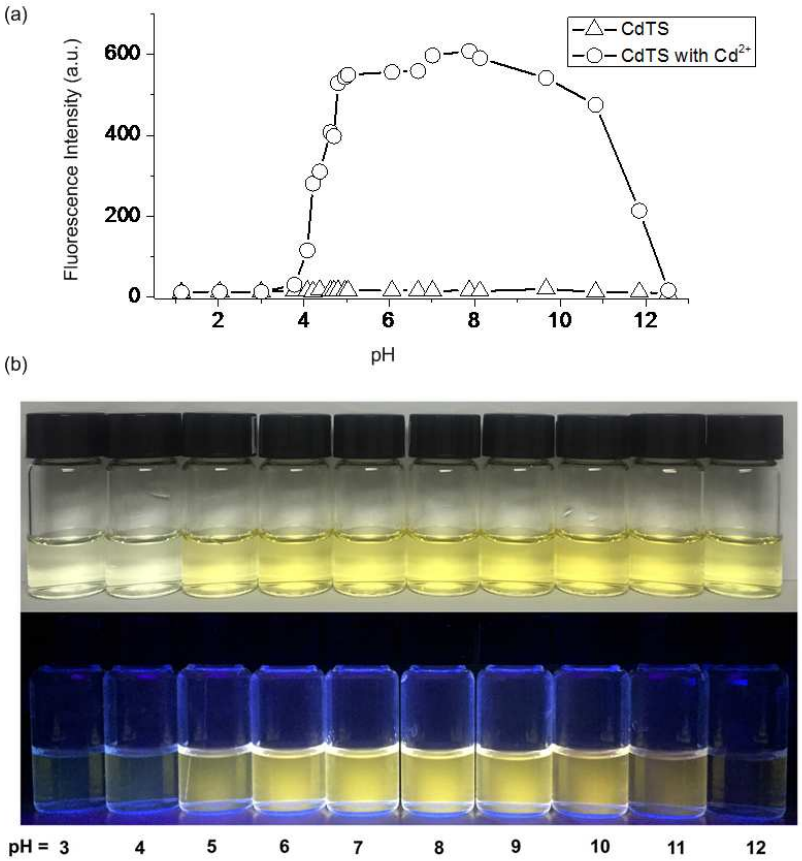

(a)
Fluorescence Intensity (a.u.)
600
400
200
0
2
4
6
8
10
12
pH
CdTS
CdTS with Cd2+
(b)
pH = 3
4
5
6
7
8
9
10
11
12

Fig. 4

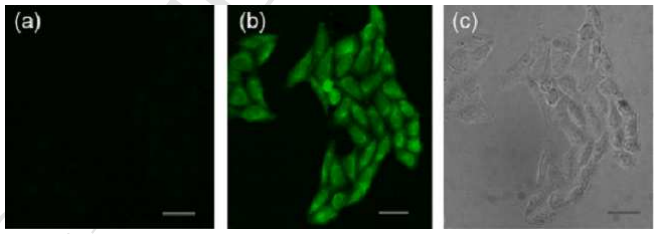
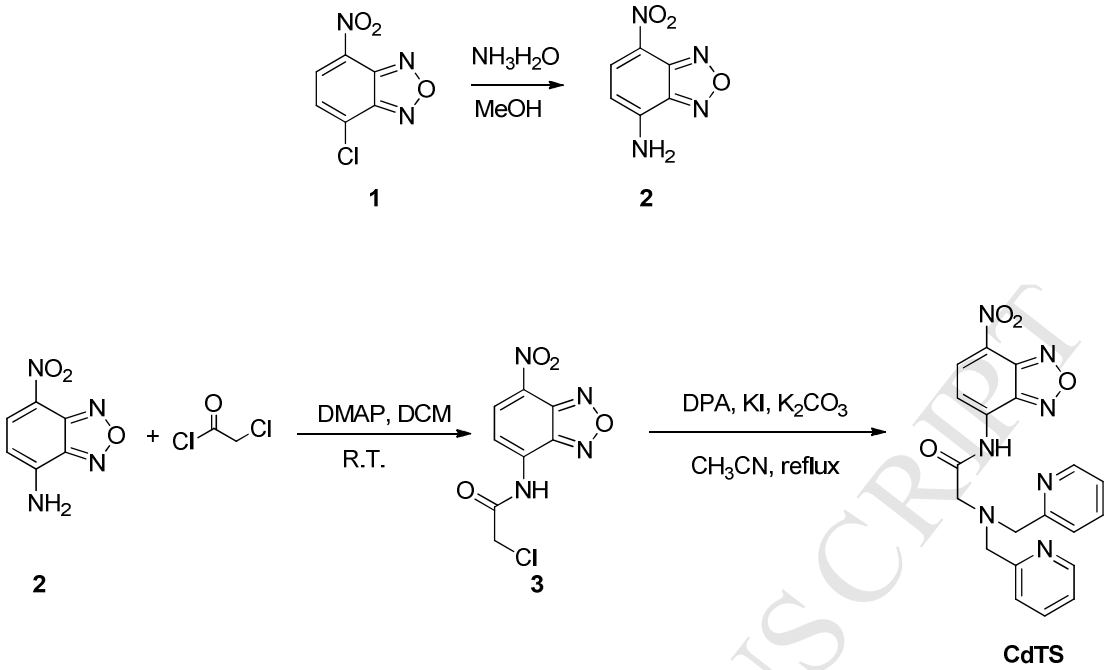

(a)
(b)
(c)

Fig. 5



Scheme 1

Scheme 1 Synthesis of the fluorescent sensor **CdTS**.

Fig. 1 (a) Fluorescence spectra of 10 μM **CdTS** in the presence of various metal ions in aqueous solution. (b) Fluorescence responses of **CdTS** to various metal ions in aqueous solution. Bars represent the final fluorescence intensity at 567 nm over the original emission. White bars represent the addition of 3 equiv of metal ions (for Na^+, K^+, Mg^{2+} and Ca^{2+}, 30 equiv) to a 10 μM solution of **CdTS**. Black bars represent the subsequent addition of 1 equiv of Cd^{2+} to the solution. (c) Absorption spectra of 10 μM **CdTS** in the presence of various metal ions in aqueous solution.

Fig. 2 ^{1}H-NMR spectra of **CdTS** in the presence of Cd^{2+} in DMSO-*d6*.

Fig. 3 The fluorescence (a) and absorption (b) titration experiments of **CdTS** with Cd^{2+} in HEPES. The inset shows the Job plot evaluated from the fluorescence with a total concentration of 10 μM.

Fig. 4 (a) Influence of pH on the fluorescence sensing of **CdTS** for Cd^{2+}. (b) Colour changes and visible emission observed from samples of **CdTS**/Cd^{2+} in different pH solutions.

Fig.5 Fluorescence images of CHO cells incubated with 5 μM **CdTS** and Cd^{2+}. Cells treated with **CdTS** a) in the absence and b) presence of 10 μM of $Cd(ClO_4)_2$. (c) bright field image.

- Cd^{2+} can trigger amide tautomerization in the amide-DPA receptor.
- The probe is extremely sensitive to Cd^{2+} with 65-fold emission enhancement.
- The probe has an excellent selectivity for Cd^{2+}.
- The probe can recognize Cd^{2+} across a wide pH range from 4.5 to 11.5.
- The probe can image Cd^{2+} in living cells.