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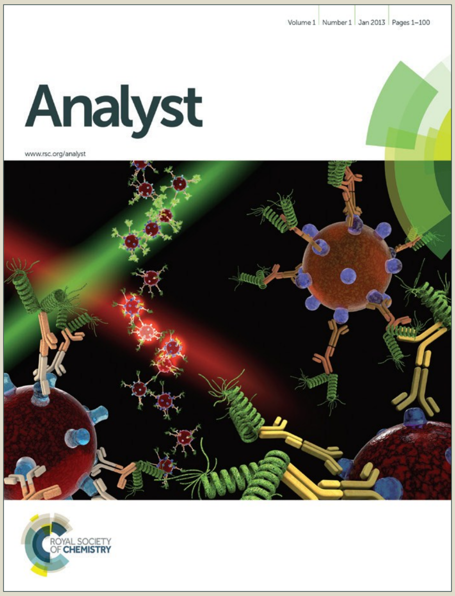

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Turn-On fluorescence chemosensor based on a tripodal amine [tris(pyrrolyl-α-methyl)amine]-rhodamine conjugate for the selective detection of zinc ions

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A novel tetradendate ligand derived from a tris(pyrrolyl-α-methyl)amine (H_3tpa) and rhodamine-based conjugate (**PR**) has been designed for use as a sensor, synthesized and characterized spectroscopically. **PR** {(tris(5-rhodamineiminopyrrol-2-ylmethyl)amine} serves as a selective colorimetric as well as a fluorescent chemosensor for Zn^{2+} in acetonitrile/water (1:1, v/v). In the presence of Zn^{2+}, **PR** exhibited obvious absorption (558 nm) and emission (577 nm) peaks whose intensity increased along with increasing Zn^{2+} concentrations. Titration experiments revealed that a large excess of Zn^{2+} was required to saturate the absorption (λ_{max}) and emission intensities. Upon the addition of 1000 equivalents of Zn^{2+}, the fluorescence intensity of the **PR** underwent a ~500-fold increase (Φ_f=0.34) with the emission maximum at 580 nm. These kinetics studies demonstrated that the absorption and emission changes were proportional to the Zn^{2+} concentration. The color of the solution changed from colorless to a dark pink color. The fluorescence of the **PR-Zn^{2+}** complex can be reversibly restored by using ammonium water or by heating. Competitive ion tests revealed that the intensity of the **PR-**Zn^{2+} was not suppressed by excess amounts of other metal ions. The counter anions did not exert obvious influences on the absorption and emission profiles. ^{1}H-NMR and FT-IR spectroscopic investigations of **PR** and **PR-Zn^{2+}** revealed that the pyrrole motifs, -C=N- groups and spirolactam of rhodamine B are capable of coordinating cation guest species. Because each arm of the tripodal ligand tautomerizes independently, only moderate fluorescence enhancement could be seen until all three –C=N- groups were coordinated by zinc, which may be due to the spirolactam ring opening mechanism of the rhodamine unit. Once all three –C=N- groups were locked by coordinating with the excess of Zn^{2+}, the isomerization was arrested, and **PR** exhibited highly enhanced fluorescence. In addition, energy optimized structures of **PR** were found to be cage-like by Gaussian 09, further supporting that it can access large excesses of Zn^{2+}. Intriguingly, imaging of HeLa cells by confocal microscope revealed that this **PR** probe could be used for biological applications.

Introduction

The fluorescent detection and imaging of target biological molecules in living systems has become an active topic in fields ranging from biological research to medical diagnosis because of its high selectivity and ability to offer high spatial resolution via fluorescence microscopy[1,2]. Fluorescence offers significant advantages over other methods for metal ion detection and measurement because of its generally nondestructive character, high sensitivity, and instantaneous response[3]. Among heavy metal ions, Zn^{2+} has attracted great interest from chemists in recent years because of its special chemical properties and the important roles it plays in various fundamental biological processes, such as gene transcription, immune function and mammalian reproduction, brain function and pathology, regulation of metalloenzymes, neural signal transmission and apoptosis[4-6], as well as in certain pathological processes such as Alzheimer's disease, epilepsy, ischemic stroke, and infantile diarrhea[7].

However, elevated levels of Zn^{2+} in humans have been implicated in neurodegenerative and other disorders[8]. These issues indicate the urgent need to develop effective methods for detecting zinc species. Zinc is spectroscopically silent due to the inert electronic configuration of Zn^{2+} ($3d^{10}4s^0$), so colorimetric and fluorometric methods are particularly useful for the detection of Zn^{2+}. In recent years, progress in the area of chemosensors has contributed significantly to the development of a variety of Zn^{2+} probes based on quinoline, anthracene, BODIPY, porphyrin and fluorescein as fluorophores[9-11].

However, designing chemosensors that can effectively differentiate Zn^{2+} from Cd^{2+} has been a great challenge[9]. Because these two elements belong to the same group in the periodic table, they possess similar chemical properties and exhibit very similar spectral properties with various reported ligands. Hence, most of the

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† Electronic Supplementary Information (ESI) available: Experimental details, ^{1}H-NMR spectra data of intermediates, PR and complexes and sensing response of PR with Zn^{2+} by UV-vis and fluorescence spectral data. See DOI: 10.1039/b000000x/.

developed sensors have exhibited poor selectivity between Cd^{2+} and Zn^{2+} ions. Recently, certain chemosensors have been developed for the selective detection of Zn^{2+} ions[12]. We also previously developed rhodamine-based sensors for the detection of Cu^{2+} and Pd^{2+} [13]. Continuing our research on fluorescent chemosensors, we report herein the development of a new sensor for the selective detection of Zn^{2+} based on the Schiff base derived from a tris(pyrrolyl-α-methyl)amine (H_3tpa) and rhodamine-based conjugate (probe PR). H_3tpa has previously been utilized for its size selective anion binding properties and was obtained in a single step by a triple Mannich reaction involving 3 equivalents of pyrrole, 3 equivalents of formaldehyde, and ammonium chloride[14]. The synthesized H_3tpa was further subjected to Vilsmeier–Haack formylation followed by condensation with rhodamine-based amine derivatives to yield the probe PR. This newly designed sensor possesses three rhodamine units connected through –C=N- bonds and was found to selectively sense Zn^{2+} ions at room temperature in $CH_3CN:H_2O$ (1:1, v/v) at pH 7.0. This probe exhibited highly enhanced fluorescence "turn-on" sensing of Zn^{2+} ions. Spectroscopic evidence indicated that the –C=N- isomerization locking and spirolactam ring opening mechanisms worked cooperatively to bind Zn^{2+} and engender this behaviour.

Experimental

Reagents and Instruments

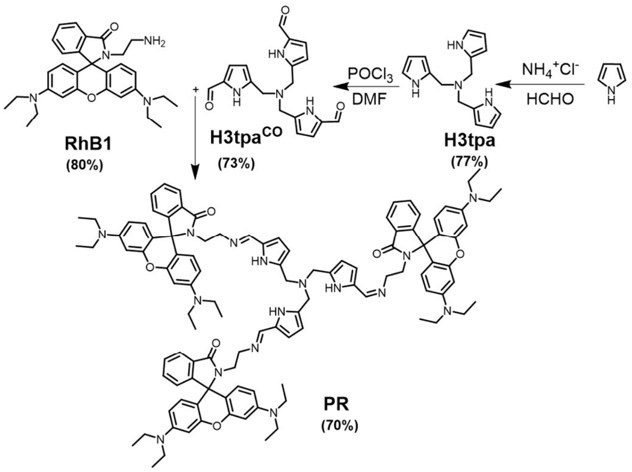
All chemicals, including rhodamine B and pyrrole, were purchased from Acros Chemicals and used without further purification. All solvents were purified before use. Fourier transform infrared spectra (FTIR) were recorded on a Jasco VALOR III Fourier transform infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AMX-400 high-resolution NMR spectrometer in deuterated solvents. UV/Vis absorption spectra were measured with a Jasco V-550 spectrophotometer in $CH_3CN:H_2O$ (1:1, v/v). All the solvents used for the syntheses were spectroscopic grade. The fluorescence spectra were measured with a Hitachi F-4500 fluorescence spectrophotometer at λ_{ex}=510 nm. All of the fluorescent images were taken with an Eclipse E 600 (Nikon Instech Co., Ltd., Kawasaki, Japan) with 10x magnification. Unless otherwise mentioned, all of the absorption and emission spectra were recorded in $CH_3CN:H_2O$ (1:1, v/v) at 25°C and pH 7.0 in HEPES buffer solution. For all of the optical responses of **PR** with metal ions, the samples were incubated for 30 min after each addition of metal solution before the spectra were recorded.

Synthesis of intermediates (RhB1, H_3tpa, H_3tpa^{CO}) and the probe (PR)

All the intermediates **(RhB1, H_3tpa, H_3tpa^{CO})** were synthesized according to reported procedures[13a,b, 14b, 15], and the detailed experimental procedures and characterization of the products are given in the supporting information (ESI†). The solubility of **PR** was tested in an aqueous medium, and it was revealed that **PR** is soluble in $CH_3CN:H_2O$ (1:1, v/v), therefore, all the absorption and emission experiments were carried out using a 1:1 mixture of $CH_3CN:H_2O$ unless otherwise mentioned.

Results and Discussion

The probe **PR** {(tris(5-rhodamineiminopyrrol-2-ylmethyl)amine} was accessed through a simple synthetic route starting with pyrrole reacting with formaldehyde in the presence of ammonium chloride, yielding the tripodal amine (H_3tpa). The H_3tpa then underwent Vilsmeier–Haack formylation to yield tris(5-formylpyrrol-2-ylmethyl)amine (H_3tpa^{CO}). Finally, the desired imine probe was synthesized by simple condensation of the H_3tpe^{CO} with excess rhodamine-based amine derivative, yielding the probe as an apricot colored solid (**Scheme 1**). Both the intermediate and receptor molecules were characterized spectroscopically (ESI).



Scheme 1 Synthesis of Probe **PR**

The ^{1}H-NMR spectrum of H_3tpa displayed a new peak at 3.6 ppm corresponding to an alkyl group attached to the pyrrole unit (–CH_2-Pyrrole), indicating the formation of H_3tpa from pyrrole (**Fig. S2**). Similarly the appearance of a new peak at 9.4 ppm corresponded to the protons of the aldehyde group (H-C=O), revealing the successful formylation of H_3tpa to H_3tpa^{CO} (**Fig. S3**). The ^{1}H-NMR spectrum of the final compound **PR** exhibited peaks at approximately 7–8 ppm, corresponding to aromatic protons of the rhodamine unit, and a peak at 11 ppm, corresponding to pyrrole –NH- protons, revealing the successful formation of the Schiff base, which was further confirmed by mass spectra (**Fig. S4** and **S5**). All the spectroscopic data are consistent with the structure of the molecules.

Binding properties of PR

The cation binding properties of **PR** were studied by employing the perchlorate salts of Cu^{2+}, Pd^{2+}, Ni^{2+}, Na^+, Mn^{2+}, Li^+, Hg^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Al^{3+} and Ag^+ in $CH_3CN:H_2O$ (1:1, v/v at room temperature) at pH 7.0. The samples were incubated for 30 min after each addition of metal solution before recording the spectra of complexes. The time course study revealed that **PR** metal recognition (λ_{max}) is completed nearly 180 min after the addition of the metal ion, however, obvious enhancement were observed up to 30 min (**Fig. S8** and **S9**). The **PR** receptor solution in $CH_3CN:H_2O$ (1:1, v/v) was colorless and non-fluorescent, indicating that **PR** exists in the closed spirocyclic form, as expected.

Upon the addition of metal solutions to the **PR**, only Zn^{2+} caused a strong absorption band (558 nm) and emission band (577 nm) along with a color change from colorless to dark pink, which indicates that **PR** can coordinate only with Zn^{2+}. No significant color changes and no obvious absorption or emission enhancement were promoted by the other metal ions (**Fig. 1** and **Fig. 3**).

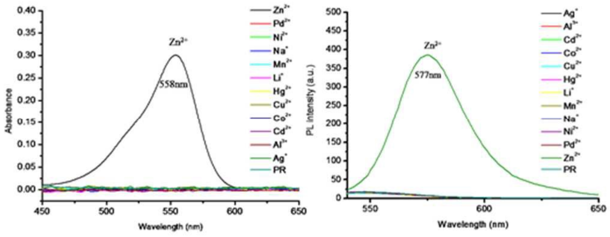


Fig. 1 UV (a) and fluorescence (b) spectral changes of **PR** (1μM) upon addition of 10 equivalents of metal ions (Ag^{2+}, Al^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Li^{+}, Mn^{2+}, Na^{+}, Ni^{2+}, Pd^{2+} and Zn^{2+}).

Therefore, absorption and fluorescence titrations were carried out for the binding of **PR** with Zn^{2+}. The titrations revealed that the intensity of the absorption (**Fig**. **2**) and emission increased at regular intervals as $\mathbf{Zn^{2+}}$ was progressively added (up to 100 equivalents), resulting in a brilliant pink coloration of the solution, which accompanied the spirolactam ring opening of the rhodamine unit. An emission band centered at 580 nm in the fluorescence spectra appeared upon Zn^{2+}addition, and the emission intensity increased linearly with $[Zn^{2+}]$(**Fig. 2**).

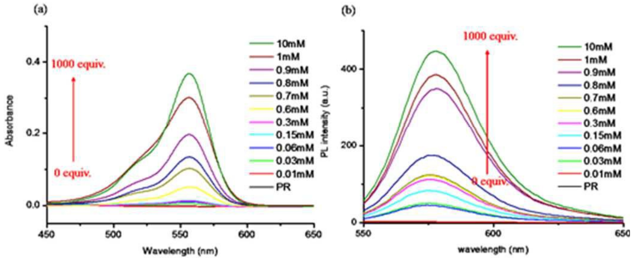


Fig. 2 Changes in the absorption and emission spectra of **PR** (1μM) at pH 7.0 upon titration with Zn^{2+}

The kinetics study demonstrated that the absorption and emission changes were proportional to the Zn^{2+} concentration. The titrations were continued up to 1000 equivalents of metal ions and revealed that the absorption and emission intensities were saturated only at higher equivalents of metal ions (1000 equivalents). This suggested that the probe could access large excesses of metal ions, which could be due to the molecule undergoing metal induced aggregation in the presence of Zn^{2+}. This phenomenon was further supported by the disappearance of the highly pink color of the complexes during heating. During heating, the secondary forces responsible for aggregation are cancelled, causing disappearance of the color. Additionally, molecular simulation of **PR** using Gaussian 09 software revealed that the probe **PR** exists as a "cage-like" structure (**Fig. S13**), further supporting the theory that it can access a large excess of zinc ions.

From the titration profiles, the association constant (K_a) for PR-Zn^{2+} was determined as 4.47 x 10^{-5} M by a Hill plot (**Fig. S14**). Based on 3δ/k (where δ is the standard deviation of the blank solution and k is the slope of the plot), the limit of detection for Zn^{2+} was found to be up to 0.2 micro molar solution. The less sensitivity compared to other reported Zn^{2+} sensors may be due to the sterically hindered bulky structure of **PR** as well as –C=N- isomerization phenomena.

During the addition of the large excess of Zn^{2+}, the fluorescence intensity increased more than 500-fold (PL intensity of PR=0.89, PR+Zn^{2+}=448.5 from **Fig. 2,** Φ_f raised to 0.34 for the addition of 100 equivalents of Zn^{2+}), which was the most significant change in fluorescence signals compared with the other metal ions.

Moreover, the acid-base titration experiments demonstrated that **PR** likely exists in closed spiro form in a basic medium, while in a strongly acidic medium (pH<4), the spirolactam ring of the rhodamine unit is opened, which enhances the intensity of the absorption (**Fig. S6**). This indicates that **PR** did not emit any notable fluorescence between pH 8.0–14.0.

Interference from other metal ions should be taken into account when considering physiological applications. Therefore, the absorption changes of **PR** in the presence of other competitive metal ions were investigated. We performed competitive experiments by titrating all the metal ions into a solution of the **PR-**$\mathbf{Zn^{2+}}$ complex. The results from the competitive ion tests reveal that the **PR-**$\mathbf{Zn^{2+}}$ intensity was not suppressed by excess amounts of other metal ions such as Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mn^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, Pd^{2+} and Sb^{2+} in $CH_3CN:H_2O$ (1:1, v/v at room temperature) at pH 7. To examine the interference of other background cations, we conducted the titrations using a dual metal system, for example (PR+Zn^{2+})+ Ag^{+}, (PR+Zn^{2+})+ Al^{3+}, (PR+Zn^{2+})+ Cd^{2+} etc. as shown in **Fig. 4** (red bars).

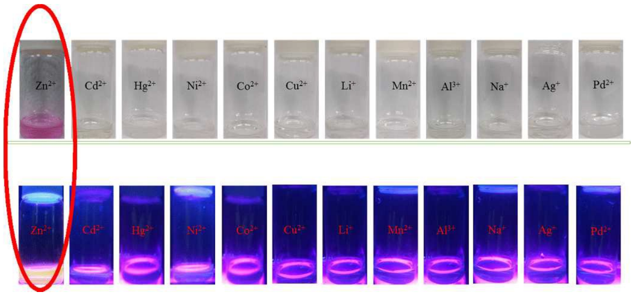


Fig. 3 Images of PR with different metal ions (a) under visible light and (b) under UV light.

The enhanced fluorescence of **PR** in the presence of Zn^{2+} was not substantially perturbed by the background cations. Notably, even Cd^{2+}, which exhibits the most similar characteristics to Zn^{2+}, did not affect the sensing selectivity (**Fig. 4**). The results show that Zn^{2+} can be detected in the presence of other metal ions using the synthesized **PR** probe.

Moreover, counter anions (perchlorate, chloride and nitrates) exhibited similar optical responses (even though the solubility of zinc nitrate is not excellent in the tested solvent), which indicates that counter anions have a negligible effect on this probe.

The absorption and emission spectra of titration kinetics of **PR** with Zn^{2+} reveal that the binding assay of Zn^{2+} with **PR** cannot be determined using the method of continuous variations (Job's plots) because **PR** can access large excesses of metal ions. This raises a question about the coordination mechanism of Zn^{2+} with **PR**. To gain further mechanistic insights, **PR**-Zn^{2+} was subjected to FTIR and NMR analyses, as crystals suitable for X-ray studies could not be obtained.

FTIR analysis (**Fig. S7)** revealed that the amide carbonyl absorption of **PR** at 1631 cm^{-1} shifts to a lower frequency (1612 cm^{-1}) upon the addition of **Zn^{2+}**. This result confirms that the amide carbonyl group is involved in the coordination of the **Zn^{2+}** cation[15b]. The peaks at approximately 2900 cm^{-1} for **PR** revealed the presence of an imine group (–C=N-), which disappeared after complexation with **Zn^{2+}.** This indicates that –C=N- is involved in the coordination of Zn^{2+}.

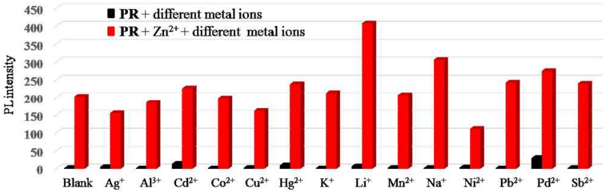


Fig. 4 Fluorescence intensity of **PR** at 577 nm *in the presence of one single* metal ion (black) and mixture of Zn^{2+} and various metal ions (red).

^{1}H-NMR titrations of **PR** with excess Zn^{2+} were performed in CD_3CN/D_2O, seen in **Fig. 5**, which shows that the aromatic protons at approximately 7.43 ppm shifted downfield to 7.55 ppm, indicating that Zn^{2+} underwent a spirolactam ring opening. Other peaks also shifted significantly downfield, such as the multiplet corresponding to the protons of the pyridyl group (from 6.3 to 6.6 ppm) and C=NH (from 7.82 to 7.97 ppm), indicating that these groups (pyridyl and C=NH-) were also involved in the coordination with Zn^{2+}. Therefore, based on both FTIR and NMR, it is clear that C=NH-, aromatic groups and pyridyl groups are involved in the coordination of Zn^{2+}.

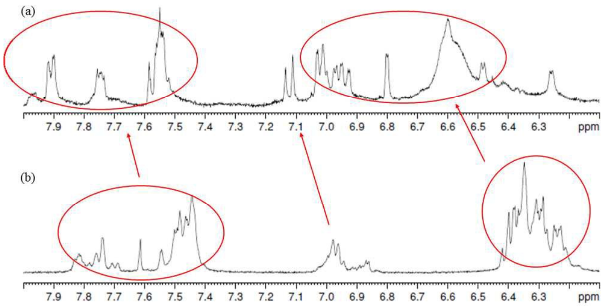
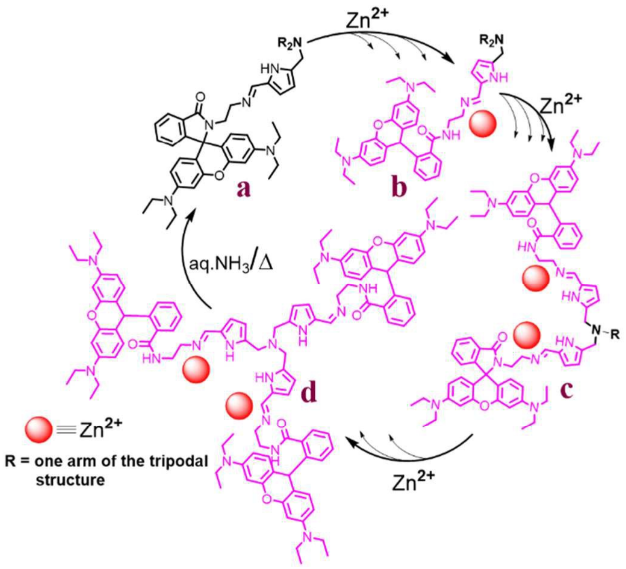


Fig. 5 ^{1}H-NMR spectra of **PR** with Zn^{2+} (a) and PR (b) in CD_3CN/D_2O (1:1 v/v)

It was noted that at lower concentrations of Zn^{2+} ions, PR shows slow responsiveness, while at higher concentrations of Zn^{2+}, PR responds immediately. This suggested that we focus on the isomerization of –C=N- before and after the addition of metal ions. The isomerization of the imine -C=N- bond is known to be the main decay process of excited states in compounds with an unbridged C=N structure, so such compounds are often nonemissive. The rapid isomerization of the imine C=N bond in the excited state may be why **PR** is almost nonfluorescent. However, a solution of **PR** shows an almost 500-fold fluorescence enhancement when a large excess of zinc ions is added. The initial addition of metal ions to the **PR** probe exhibited moderate fluorescence enhancement due to the spirolactam ring opening of the rhodamine units, as observed for many rhodamine-based sensors. Further successive addition of metal ions may lead to occupation of the pyridyl and –C=N- groups, thereby locking –C=N- rotation, so clear fluorescence enhancement was expected. However, high levels of fluorescence enhancement were not observed. This may be because all three –C=N- units in the PR molecule might not arrest, therefore the possibility of isomerization leads to lower than anticipated enhancement of emissions. Therefore, successive addition of more metal ions leads to the anticipated strong fluorescence enhancement by locking all of the –C=N- units in PR. Our results indicated that all three –C=N- units of the tripodal structure of **PR** did not undergo simultaneous tautomerization. Each arm of **PR** underwent tautomerization independently. Because **PR** possesses three units of -C=N-, the high level emission enhancement cannot be observed until all three -C=N- units were arrested by metal ions. This theory of independent tautomerization is further supported by a report from Alison R. Fout, using iron complexes of a tripodal based Schiff base[14c]. Based on this data, a plausible mechanism for the sensing ability of PR towards Zn^{2+} has been proposed[9c-f] and is depicted in **Scheme 2**.



Scheme 2. The potential mechanism of **PR** binding with Zn^{2+}

For practical applications, reversibility, the ability to regenerate the free sensors from the complexed system, is a significant parameter for fluorescence sensors. To examine the reversibility of **PR**, EDTA or aqueous ammonia (1 mM) was added to the **PR-Zn^{2+}** solution as a suitable counter ligand {**PR** (10 μM) with Zn^{2+} (1 mM)} in CH_3CN/H_2O (1:1 v/v, pH= 7) and the absorption and emission spectra before and after the NH_3 addition (or EDTA) were examined (**Fig. S10**). The absorption/emission bands decreased dramatically after the addition of the aqueous ammonia (as well as EDTA), which is ascribed to the formation of $[Zn(NH_3)_4]^{2+}$ (or znEDTA^{2-}). Moreover, the pink colour of the **PR-Zn^{2+}** complexes disappeared. Complexation of ammonia with Zn^{2+} is much stronger than that of **PR**, which leads to the reversible coordination of Zn^{2+} with ammonia. This result suggested that the coordination mechanism of **PR** with Zn^{2+} is reversible and that the **PR** probe could therefore be recycled. Because $[Zn(NH_3)_4]^{2+}$ is soluble in water but **PR** is not, this suggests an easy recycling path for PR. Similar phenomena was observed for EDTA.

Imaging studies in living cells

To demonstrate the potential application of this zinc probe, we conducted fluorescence imaging experiments[16]. The good performance (high quantum yield and metal selectivity) of **PR** with Zn^{2+} prompted us to examine it using confocal fluorescence microscopy. HeLa cells were incubated with the **PR** probe (5 mM) for 30 min, followed by the addition of Zn^{2+} ions (5 mM) and then another 30 min of incubation. The fluorescence microscopy images revealed a clear enhancement of fluorescence (**Fig. 6**). This suggests that the ability of the probe to penetrate the cell membrane can be used for tracking Zn^{2+} ions in living cells and potentially in vivo.

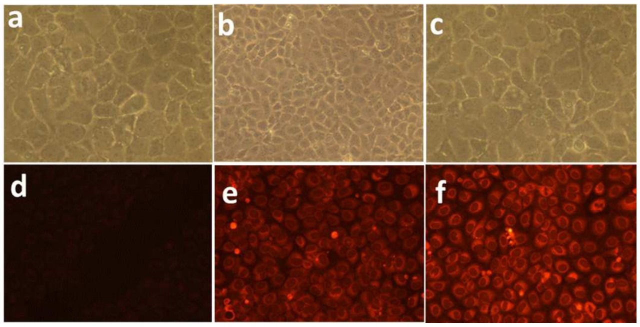


Fig. 6 Fluorescence images of the Zn^{2+} ions in HeLa cells. (a-c) Bright-field transmission image of HeLa cells incubated with the probe (5 mM). (d) Fluorescence image of HeLa cells incubated only with the probe (5 mM). (c-f) Fluorescence image of HeLa cells incubated with the probe for 30 min, washed three times, and then further incubated with 5 mM Zn^{2+} ions for 30 min.

Conclusions

We developed a colorimetric and fluorescent "on-off" probe **PR,** which selectively binds with Zn^{2+}. The emission profile of the **PR-Zn^{2+}** complex was unperturbed in the presence of other metal ions. Moreover, counter anions did not exert obvious influences on the emission profile. A fast and immediately obvious fluorescence enhancement was observed at higher concentrations of Zn^{2+}, which may be due to the presence of three imine units that may separately undergo isomerization. Locking the imine isomerization and opening the rhodamine spirolactam ring caused this probe to be an effective fluorescent sensor towards Zn^{2+} under the given conditions. A plausible mechanism was proposed for the binding of **PR** with Zn^{2+} from the results of FTIR and NMR. This probe can be recycled by treatment with EDTA or aqueous ammonia. Moreover, living cell imaging studies using confocal fluorescent microscopy suggested possible additional value of this probe for practical applications in biological systems.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details, ^{1}H-NMR spectra data of intermediates, PR and complexes and sensing response of PR with Zn^{2+} by UV-vis and fluorescence spectral data. See DOI: 10.1039/b000000x/.

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