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A Single Molecular Probe for Multi-analyte (Cr^{3+} , Al^{3+} and Fe^{3+}) in Aqueous and Its Biological Application

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Abstract

An ESIPT based fluorescent sensor 1 was developed, which could selectively detect and differentiate trivalent metal ions Cr^{3+} , Al^{3+} and Fe^{3+} in aqueous. The cell imaging experiments confirmed that 1 can be used for monitoring intracellular Cr^{3+} and Al^{3+} levels in living cells.

Trivalent cations have important biological properties and are directly involved in the cell function where there is a critical control of M^{3+} levels.¹ For example, Cr^{3+} has direct impacts on the metabolism of carbohydrates, fats, proteins and nucleic acids by either activating certain enzymes or stabilizing proteins and nucleic acids.² Chromium deficiency can increase the risk factors associated with diabetes and cardiovascular diseases.³ Al^{3+} could also have adverse effect on human's health, as excessive amount of Al^{3+} in the brain, is believed to cause neurodementia such as Parkinson's disease, Alzheimer's disease and dialysis encephalopathy.⁴ Fe^{3+} plays an indispensable role in many biochemical processes at the cellular level,⁵ and in the oxygen transport processes in all tissues in the form of hemoglobin.⁶ The deficiencies or excesses of Fe^{3+} can lead to a variety of diseases, such as Alzheimer's, Huntington's, and Parkinson's diseases.⁷ Thus, there is an urgent need to develop chemical sensors that are capable of detecting the presence of Cr^{3+} , Al^{3+} and Fe^{3+} ions in biological samples.

Due to their paramagnetic nature, trivalent chromium (Cr^{3+}) and iron (Fe^{3+}) are among the most effective fluorescent quenchers,⁸ which makes it difficult to develop a fluorescence turn-on sensor. For this reason, very few sensors for $\text{Cr(III)}^{3c,8,9}$ and Fe(III)^{10} have been reported, and far fewer find application in cell imaging.^{3c,9d,10h} In contrast, Al^{3+} is diamagnetic, whose binding to sensors often enhance the fluorescence.¹¹ Due to strong hydration of Al^{3+} in water, however, most reported dyes for Al^{3+} are required to be used in organic solvents or mixed solvents, with very few being suitable for Al^{3+} imaging applications.^{11a,11e} Recently, the study by Costero *et al.*^{9a} reported a fluorescein derivative,

whose fluorescence at 475 nm could be turned on by Cr^{3+} , Fe^{3+} and Al^{3+} in dry CH_3CN . The presence of 4% of water in CH_3CN , however, will quench the fluorescence of fluorescein complex with Cr^{3+} and Fe^{3+} ions. It remains a challenge to design a fluorescent sensor that *not only* can recognize *but also* differentiate the trivalent cations (Al^{3+} , Cr^{3+} and Fe^{3+}), especially in aqueous solution. Herein, we disclose a sensor that can simultaneously detect Cr^{3+} , Al^{3+} and Fe^{3+} ions by naked eyes in aqueous.

The sensor design incorporates 2-(2'-hydroxyphenyl) benzoxazole (HBO) unit as an emitting fluorophore, whose emission has a large Stokes' shift (> 150 nm) arising from the excited-state intramolecular proton transfer (ESIPT). As shown in Figure 1, the cation binding is expected to occur by using two stronger ligands 2-(pyridin-2'-yl)hydrozono groups (**2**). Due to steric hindrance with H_a atom, the $\text{C}=\text{N}$ will be twisted away from the coplanarity (with HBO) upon binding metal cations, resulting in decrease of the conjugation length (absorbance hypsochromic shift). It is assumed that only those metal cations that can bind strongly with the imine bonds will be able to compete, thus separating the trivalent cations from the divalent and monovalent ones. In addition, the cation binding removes the adverse effect of Schiff base on fluorescence, thereby turning on the emission. The proposed metal complex **2** has four-coordination, which should be more stable than **3** (three coordination). The two different binding modes could be easily identified by their ESIPT ON/OFF property (large or smaller Stokes' shift). The design also includes a phenol group, whose participation in the cation binding event could act as a switch to manipulate the ESIPT property. By using the dual channel control (i.e., fluorescence intensity and ESIPT), the study aims to differentiate the trivalent cations, since the ESIPT of HBO unit is quite sensitive to electronic perturbation from the cation binding.¹²

The absorption of **1** revealed a major peak (at 315 nm, attributed to $\pi-\pi^*$) and a minor peak (at 432 nm, attributed to $n-\pi^*$) (ESI Figure S1b). The fluorescence response of **1** was examined in aqueous medium ($\text{H}_2\text{O}:\text{EtOH} = 8:2$) by addition of various metal ions (Figure 1). Sensor **1** showed weak red fluorescence ($\Phi_F = 0.1$) with a large Stokes' shift (180 nm), attributing to emission from its *keto* tautomer associated with its ESIPT property. Sensor **1** was silent to monovalent and divalent metal ions. Surprisingly, fluorescence of **1** showed immediate fluorescence turn-on upon addition of trivalent ions Cr^{3+} , Al^{3+} and Fe^{3+} , although Cr^{3+} and Fe^{3+} are widely known as fluorescent quenchers. The sample with Fe^{3+} showed the initial fluorescence turn-on but quickly decay within 5 minutes (ESI Figure S5b), while the yellow fluorescence with Cr^{3+} complexes was quite stable (ESI Figure S3). The fluorescence with Al^{3+} complexes changed to blue-green (527 nm). The distinctive feature in optical response thus allowed us to distinguish Fe^{3+} (which gives pulse-like fluorescence) from Cr^{3+} (emission $\lambda_{\text{em}} \sim 556$ nm, yellow green) and Al^{3+} ($\lambda_{\text{em}} \sim 527$ nm, blue-green). In summary, different response to trivalent ions enabled the naked eyed detection of Cr^{3+} , Fe^{3+} and Al^{3+} cations. (Cr: $\Phi_F = 0.63$; Al: $\Phi_F = 0.31$). It was noted that the sensor also showed different response between Fe^{2+} and Fe^{3+} , as Fe^{2+} only slightly quenched the fluorescence without showing initial turn-on.

To elucidate the metal binding mode, the UV-vis spectra of **1** were recorded upon addition of different equiv. of Cr^{3+} (ESI Fig. S2). As is known, the deprotonation of the phenol upon metal binding will lead to large spectral bathochromic shift together with fluorescence blue

shift, resulting in a smaller Stokes' shift.^{6a, 6d} Upon addition of Cr^{3+} , the absorption band at 432 nm ($n\text{-}\pi^*$) progressively decreased, indicating that the cation was binding to the Schiff base. The new $\pi\text{-}\pi^*$ absorption band was observed at about 358 nm. It should be noted that the emission of the metal complex exhibited a large Stokes' shift (195 nm for Cr^{3+} and 170 nm for Al^{3+} respectively), which indicates the existence of the free phenol group and ruled out the binding mode **3**. All these facts pointed to formation of the metal complex **2**, where Al^{3+} and Fe^{3+} showed the similar binding mode.

Mass spectral studies revealed that ligand **1** disappeared (TOF-MS-ES⁺ at peaks 472.1815 and 494.1637) upon addition of Cr^{3+} (ESI, Fig. 8d). The newly formed positively charged peak of TOF-MS-ES⁺ at 577.1374, which matched $[\mathbf{1} + \text{Cr}^{3+} + \text{MeO}^- + \text{EtO}^-]^+ = 577.1507$, indicating the formation of **2** (ESI Fig. S8) with 1:1 ratio of ligand to trivalent metal ions. In aqueous solution, TOF-MS-ES⁺ also detected the formation of **2** where $\text{L}=\text{L}'=\text{OH}$ (ESI Fig. S8a) with six-coordination geometry. ¹H NMR spectra revealed consistent evidence to support the formation of **2**. Upon addition of 1.0 equiv. of $\text{Cr}(\text{NO}_3)_3$, the turbid solution of **1** in $\text{CD}_3\text{OD}/\text{DMSO-}d_6$ became transparent, indicating the Cr^{3+} complex formation. The characteristic pyridine signal H_b and imine H_f (at ~8.5 ppm) were shifted downfield to about ~8.7 ppm, supporting the assumption that both pyridine units and imine groups were bound to the Cr^{3+} ion, as shown in the complex **2** (ESI Fig. S19).

To gain a better understanding of M^{3+} binding mode, compound **4** and **5** were prepared (Figure 2). UV-vis absorption of **4** exhibited nearly no change (at ~417 nm, Figure 2a) upon addition of the M^{3+} cations, in comparison with **1**. In sharp contrast, UV-vis absorption of **5** showed clear blue shift upon addition of M^{3+} (Figure 2b). The observation supported the proposed binding mode that the cation was binding to both Schiff base groups as shown in **2**, whose formation caused the twisting of imine bonds. This was also consistent with the hypsochromic shift in UV-vis absorption. Fluorescence of **5** also exhibited a similar blue shift as **1** upon interaction with M^{3+} cation (ESI Fig. S20). The fluorescence of metal complex **5-M}^{3+}** exhibited a small Stokes' shift (~60 nm), which is far smaller than that of **1-M}^{3+}** (~190 nm). The results thus indicated the formation of complex **2**, whose ESIPT remained to be on.

In an effort to seek additional evidence for the binding mode, **4** was further examined with addition of Zn^{2+} cation, as its complex can involve the adjacent phenol as observed from **5**.^{12b} While ligand **4** exhibited weaker interaction with Zn^{2+} in aqueous (ESI Figures S16–S17b), it reacted readily with Zn^{2+} in ethanol to form stable 4-Zn complexes (ESI Figures S9e and S18). As a consequence of removal of phenolic proton, the UV-vis absorption of **4** ($\lambda_{\text{max}} \approx 375$ nm) was notably shifted to $\lambda_{\text{max}} \approx 417$ nm *via* forming **4-Zn** (Scheme 1, and ESI Fig S18a). In addition, the weak emission of **4** at ~582 nm (assigned to ESIPT from *keto* tautomer) disappeared, which was accompanied with a stronger new emission at ~477 nm upon formation of the zinc complex. It was clear that participation of the phenol in the cation binding would result in a large spectral shift with a small Stoke's shift (60 nm). Since no large spectral shift was observed when ligand **1** was binding to trivalent Cr^{3+} cation, it was not likely that the phenol participated binding. Therefore, the proposed binding mode **2** was predominant for Cr^{3+} complex of **1**. For Al^{3+} cation, the resulting complex with **1**

initially formed via binding mode **2**, which gradually changed to binding mode **3** to give blue-green emission (ESI, Fig S15). In the sensing of Fe^{3+} ion, the formation of complex **2** initially turned on the fluorescence. However, the tautomerization of **2** took place quickly within 5 mins leading to the formation of **2'** (ESI, Scheme S1), which was captured by TOF-MS- ES^+ : the positively charged peak of TOF-MS- ES^+ at 503.0999 matched $[\text{1}+\text{Fe}^{3+}-2\text{H}^+]^+ = 503.0762$ (see ESI Figure S21). The formation of **2'** could be responsible for the observed fluorescence quench.

In order to examine the selectivity of **1**, some other cations were added to a solution of **1** under the same conditions. Addition of M^+ and M^{2+} cations (10 equiv.) induced almost no change in the UV-vis spectra of **1** (except Cu^{2+} , see ESI Figure S1). The result indicated the weak binding between dye **1** and the mono- and divalent metal ions. Physiologically important metal ions which exist in living cells, such as Ca^{2+} , Mg^{2+} , Na^+ and K^+ gave negligible fluorescence response. Addition of one of the metal ions with subsequent addition of $\text{Cr}^{3+}/\text{Al}^{3+}$, the green-yellow fluorescence was then turned on (see ESI Figure S10). Therefore, **1** is a highly selective chemosensor for $\text{Cr}^{3+}/\text{Al}^{3+}$ with the detection limits as $0.2\mu\text{M}$ and $0.5\mu\text{M}$ for Cr^{3+} and Al^{3+} respectively.

The potential application of **1** for both Cr^{3+} and Al^{3+} in biological samples was examined by using confocal fluorescence microscopy. In the control experiment, staining human mesenchymal stem cells (hMSCs) with $10\mu\text{M}$ dye **1** for 30 mins led to negligible intracellular fluorescence (Figure 3d). When the cells were first incubated with $30\mu\text{M}$ of metal ions (Cr^{3+} or Al^{3+}) for 30 mins, then further treated with $10\mu\text{M}$ sensor **1** for another 30 minutes, a significant increase in the fluorescence from the intracellular area was observed (Figures 3e and 3f). Bright-field measurements confirmed that the cells, after being treated with $\text{Cr}^{3+}/\text{Al}^{3+}$ and **1**, were viable throughout the imaging experiments. These results demonstrate that the probe is permeable to cells, binds to intracellular Cr^{3+} and Al^{3+} , and emits strong fluorescent light, thus is highly suitable for determining intracellular Cr^{3+} and Al^{3+} ions. Response to Cr^{3+} and Al^{3+} ions with distinctly different colors (yellow-green and cyan, respectively) from the cell samples raised the prospect that the Cr^{3+} and Al^{3+} ions could be simultaneously determined.

In conclusion, we have demonstrated a single fluorescent molecular probe that can *specifically* detect trivalent ions (Cr^{3+} , Al^{3+} and Fe^{3+}) in aqueous medium. In the molecular design, the sensor cleverly utilized two “hydrazone Schiff base” (binding mode **2**) to bind trivalent cations Al^{3+} , Cr^{3+} and Fe^{3+} (M^{3+} cation), while being silent to mono- and divalent metal ions. Simultaneous binding to two “hydrazone Schiff base” by M^{3+} cation removed the fluorescence “quenching effect” associated with Schiff base, thereby leading to great fluorescence turn-on. Large response from both fluorescence intensity and spectral shift provided distinctly different profile for each of three trivalent metal ions, thereby allowing their naked eyed detection. By using the different cation binding modes to switch the ESIPT ON and OFF, the study further illustrates an effective and novel strategy to differentiate Al^{3+} , Cr^{3+} and Fe^{3+} . Cell imaging of confocal fluorescence microscopy further demonstrated that **1** can be used for monitoring intracellular Cr^{3+} and Al^{3+} levels in living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Top: Dye **1** and its Cr^{3+} complex **2** (**3** is possible but not preferred). The cation in **2** is likely to adopt six-coordination geometry with two additional ligands (L & L'). Bottom: fluorescence response of **1** upon addition of 10 equiv of different metal ions in aqueous (8:2 = water : EtOH).

Figure 2. UV-vis response of **4** (a) and **5** (b) upon addition of 10 equiv. of different metal ions in aqueous (8:2 = water : EtOH).

Figure 3.

Confocal fluorescence images of Human mesenchymal stem cells (hMSCs) excited with a Diode laser (405 nm) on an Olympus FV-1000 laser scanning microscope. The images were collected at bright field (a, b and c) and fluorescent channels (d, e, f). a→d: the cells were incubated with dye **1** in PBS for 60 mins; b→e: the cells were first treated with Cr³⁺ (30 μM) for 30 mins and further exposed to dye **1** (10 μM) in PBS for another 60 mins, and image e was collected from 535–565 nm; c→f: the cells were first treated with Al³⁺ (30 μM) for 30 mins and further exposed to dye **1** (10 μM) in PBS for another 60 mins, and image f was collected from 505–525 nm.

Scheme 1.

Structures of **4** and its metal complexes.