An Indole-Based Fluorescent Chemosensor for Detecting Zn$^{2+}$ in Aqueous Media and Zebrafish

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Abstract: An indole-based fluorescent chemosensor IH-Sal was synthesized to detect Zn$^{2+}$. IH-Sal displayed a marked fluorescence increment with Zn$^{2+}$. The detection limit (0.41 µM) of IH-Sal for Zn$^{2+}$ was greatly below that suggested by the World Health Organization. IH-Sal can quantify Zn$^{2+}$ in real water samples. More significantly, IH-Sal could determine and depict the presence of Zn$^{2+}$ in zebrafish. The detecting mechanism of IH-Sal toward Zn$^{2+}$ was illustrated by fluorescence and UV-visible spectroscopy, DFT calculations, $^1$H NMR titration and ESI mass.

Keywords: zinc ion; indole; fluorescent chemosensor; calculations; bio-imaging

1. Introduction

Zinc ion, the second richest in body, has essential roles related to various physiological functions like gene transcription and immune and brain functions [1–8]. However, the imbalance of zinc ions may result in several pathological problems, such as epilepsy, infantile diarrhea, Parkinson’s disease, ischemic stroke and Alzheimer’s disease [9–11]. Thus, effective probing and monitoring of zinc ions in biological systems has become an important issue [12].

Various analytical methods, like electrochemical methods, inductively coupled plasma atomic emission spectroscopy (ICP-AES) and atomic absorption spectrometry (AAS), have been applied for determining zinc ions [13,14]. However, they require complicated sample preparation, expensive instruments and time-consuming procedures [15]. By contrast, fluorescent chemosensors have merits such as high selectivity, simplicity and low cost [16–22]. Moreover, fluorescent chemosensors could be applied to living organisms for bio-imaging [23–27]. Meanwhile, it is a huge obstacle to distinguish zinc ions from cadmium ions, since they show similarity in chemical properties [28–30]. Thus, chemosensors capable of discriminating zinc ions from cadmium ions are especially needed.

Indole derivatives have been widely applied to chemosensors for detecting various ions, such as F$^-$, CN$^-$, I$^-$, Cu$^{2+}$ and Hg$^{2+}$ [31–35], because of their unique fluorescent characters and good water solubility [36,37]. In addition, they are bio-compatible and essential in biological systems [38–41]. As a result, some of the indole-based chemosensors have shown applications in aqueous media, which contributed to bio-imaging [42–45]. Nevertheless, only five indole-based Zn$^{2+}$ chemosensors have been reported to date, and only one of them presented an application in living organisms [46–50].

Herein, we demonstrate an indole-based fluorescent probe IH-Sal for probing Zn$^{2+}$, that was provided by condensation reaction of 2-(1H-indol-3-yl)acetoxydrazide and salicy-laldehyde. IH-Sal showed efficient fluorescence turn-on for Zn$^{2+}$ and could be applied to recognize and quantify Zn$^{2+}$ in real samples and zebrafish.
2. Experiments

2.1. Materials and Equipment

Reagents were provided commercially. Electrospray ionisation mass spectrometry (ESI-MS) and nuclear magnetic resonance spectroscopy (NMR) data were provided with a Thermo Finnigan quadrupole instrument (Thermo Finnigan LLC, San Jose, CA, USA) and a Varian spectrometer (Varian, Palo Alto, CA, USA). Fluorescent and UV–visible spectra were provided by Perkin Elmer spectrometers (Perkin Elmer, Waltham, MA, USA).

2.2. Synthesis of IH-Sal ((E)-N′-(2-hydroxybenzylidene)-2-(1H-indol-3-yl)acetohydrazide)

Following the method for synthesizing IH-Sal reported in the literature [51], salicylaldehyde (61.1 mg, 5 × 10⁻⁴ mol) was added to 2-(1H-indol-3-yl)acetohydrazide (100.3 mg, 5.3 × 10⁻⁴ mol) in methanol (2 mL) with stirring for 2 h at 23 °C (Scheme 1). A white precipitate was filtered, rinsed with methanol and dried (118.1 mg; 80.5%); ¹H NMR in DMSO-­⁶: δ 11.77 (s, 0.67H), 11.27 (s, 0.33H), 11.16 (s, 0.67H), 10.94 (s, 0.67H), 10.88 (s, 0.33H), 10.12 (s, 0.33H), 8.41 (s, 0.67H), 8.28 (s, 0.33H), 7.72–6.86 (m, 9H), 4.01 (s, 0.67H), 3.65 (s, 1.33H). ¹³C NMR in DMSO-­⁶: δ 172.1 (0.33C), 166.9 (0.67C), 157.2 (0.67C), 156.2 (0.33C), 146.7 (0.67C), 136.0 (0.33C), 131.1 (1C), 130.8 (1C), 129.3 (1C), 127.0 (1C), 123.7 (1C), 121.0 (1C), 120.7 (1C), 118.5 (1C), 118.3 (1C), 116.2 (1C), 111.4 (1C), 107.7 (1C), 31.3 (0.67C), 29.2 (0.33C). ESI-MS (m/z): [IH-Sal + H⁺ + DMSO]+: calculated, 372.14, found, 372.25.

Scheme 1. Synthesis of IH-Sal (see the experimental section for details).

2.3. Preparation of Spectroscopic Experiments

Sensor IH-Sal (2.93 mg, 10 µmol) was dissolved in DMSO (1 mL) for a stock solution (10 mM). A Zn²⁺ stock (20 mM) was prepared by dissolving Zn(NO₃)₂ in bis-tris buffer (1 × 10⁻² M, pH 7). We also prepared other metal ion stocks using their nitrate salts or perchlorate salts, such as Ga(NO₃)₃, Co(NO₃)₃, NaNO₃, Cr(NO₃)₃, Fe(CIO₄)₂, Ga(NO₃)₃, Fe(NO₃)₃, Pb(NO₃)₂, Mn(NO₃)₂, Cd(NO₃)₂, Mg(NO₃)₂, In(NO₃)₃, Cu(NO₃)₂, Al(NO₃)₃ and KNO₃. All spectroscopic experiments were conducted immediately after mixing them for a few seconds.

2.4. Imaging in Zebrafish

Zebrafish embryos were reared under previously described conditions [52,53]. The 6-day-old embryos were treated with 2 × 10⁻⁵ M of IH-Sal (containing 0.02% DMSO in E2 media) for 21 min. After washing with E2 media to eliminate the remnant IH-Sal, the embryos were treated with two different amounts of Zn²⁺ solution (2.5 and 5.0 × 10⁻⁵ M) in E2 media for 20 min and washed again. Before observing changes, the embryos were narcotized by adding ethyl-3-aminobenzoate methanesulfonate. An imaging experiment was performed with a fluorescent microscope and the intensity of the images was measured by Icy software (Institut Pasteur, Paris, France).

2.5. Calculations

The results of theoretical calculations were given with the Gaussian 16 program (Gaussian, Inc., Wallingford, CT, USA) [54]. Before calculating electronic states of IH-Sal and IH-Sal-Zn²⁺ complex, their optimal geometries were provided with the density functional theory (DFT) method [55,56]. The hybrid functional was B3LYP, and the 6-31G(d,p) basis set was implemented for all atoms except for Zn²⁺ [57,58]. Additionally, the LANL2DZ basis
set was applied for effective core potentials (ECP) to Zn\(^{2+}\) [59–61]. Imaginary frequency was not shown in optimized forms of IH-Sal and IH-Sal-Zn\(^{2+}\), implying that they meant local minima. With IEFFPCM, the solvent effect of water was considered [62]. Based on energy-optimized forms of IH-Sal and the IH-Sal-Zn\(^{2+}\) complex, the plausible UV–Vis transition states were verified with the DFT method with the twenty lowest singlet states.

3. Results and Discussion

3.1. Structural Characterization of IH-Sal

The \(^1\)H NMR of IH-Sal showed pairs of singlets having a 1:2 ratio of integral value for the protons H\(_1\), H\(_6\), H\(_8\), H\(_9\) and H\(_{14}\), implying that it has two isomeric forms originated from keto-enol tautomerization (Figure S1). The compound IH-Sal was further verified by \(^{13}\)C NMR and ESI-MS.

3.2. Spectroscopic Examination of IH-Sal to Zn\(^{2+}\)

To comprehend the fluorescent characteristic of IH-Sal, the fluorescent variation was checked with varied cations in bis-tris buffer (Figure 1a). IH-Sal itself exhibited no fluorescence emission. Upon the addition of the cations except for Zn\(^{2+}\), IH-Sal displayed either no variation or a trivial increase in the fluorescent emissions. Meanwhile, the addition of Zn\(^{2+}\) displayed a striking fluorescence increment at 465 nm (\(\lambda_{\text{ex}} = 369\) nm) with a large stokes shift. The stokes shift was the largest among indole-based Zn\(^{2+}\) sensors (Table S1). The quantum yields (\(\Phi\)) of IH-Sal and IH-Sal-Zn\(^{2+}\) were calculated to be 0.014 and 0.153, respectively. Therefore, IH-Sal can work as a fluorescence sensor for a clearly selective probing of Zn\(^{2+}\). In the literature, the displacement of the indole moiety in IH-Sal by a benzene ring or tetraphenylethylene showed that the sensors sensed Zn\(^{2+}\) ions only in organic or semi-aqueous solvents [63,64], confirming that the indole moiety might play an important role in increasing water solubility of IH-Sal.

Figure 1. (a) Fluorescence changes in IH-Sal (1 × 10\(^{-5}\) M) with varied cations (8.5 equiv). Photograph: the fluorescence images of IH-Sal and IH-Sal-Zn\(^{2+}\) under UV light (\(\lambda_{\text{ex}}\): 369 nm); (b) fluorescence titration of IH-Sal (1 × 10\(^{-5}\) M) with varied amounts of Zn\(^{2+}\) (0–9 equiv); (c) UV–Visible variations in IH-Sal (1 × 10\(^{-5}\) M) with varied amounts of Zn\(^{2+}\) (0–8 equiv).
To demonstrate the sensing characteristics of IH-Sal to Zn$^{2+}$, a fluorescence titration of IH-Sal and Zn$^{2+}$ was conducted (Figure 1b). The fluorescence intensity of IH-Sal at 465 nm consistently increased up to 8.5 equivalent (equiv) of Zn$^{2+}$. The photophysical characteristics of IH-Sal were also tested with UV–Vis spectrometry (Figure 1c). With the addition of Zn$^{2+}$ to IH-Sal, the absorption of 250 and 360 nm consistently increased, and that of 290 and 320 nm decreased. There were clean isosbestic points at 257 and 340 nm, implying that one species was provided by the complexation of IH-Sal with Zn$^{2+}$. On the other hand, the UV–Vis change in IH-Sal with various metal ions showed that IH-Sal was not selective to Zn$^{2+}$ (Figure S2).

To confirm the stoichiometry of complexation, the Job plot experiment was carried out (Figure S3). The biggest intensity was shown at a mole fraction of 0.5, suggesting that IH-Sal and Zn$^{2+}$ formed a 1:1 binding compound. The 1:1 binding of IH-Sal-Zn$^{2+}$ was verified by ESI-MS analysis (Figure S4). Positive ion mass displayed that the peak of 511.58 ($m/z$) was suggestive of [IH-Sal(-H$^+$) + Zn$^{2+}$ + 2DMSO]$^+$ (calculated, 512.07). Based on the stoichiometry, the Benesi–Hildebrand equation [65,66] was used to calculate $K$ (association constant) for IH-Sal-Zn$^{2+}$ (Figure S5). The $K$ value was given to be $1.6 \times 10^4$ M$^{-1}$, which was within the scope of those ($1$--$1.0 \times 10^{13}$) addressed for Zn$^{2+}$ sensors.

The $^1$H NMR titrations were executed to demonstrate the binding interaction of IH-Sal and Zn$^{2+}$ (Figure 2). With the addition of Zn$^{2+}$ to IH-Sal, the proton H$_{14'}$ disappeared and the proton H$_9$ was slightly moved to upfield. These results implied that the enol form of IH-Sal could interact with Zn$^{2+}$ using the oxygen of the deprotonated phenol and the nitrogen of the imine group (Scheme 2).

Figure 2. $^1$H NMR titration of IH-Sal with Zn$^{2+}$ (0, 0.5, 2.0 and 3.0 equiv).
We used IH-Sal to measure the amount of Zn$^{2+}$ in real water samples, based on a calibration plot of IH-Sal to Zn$^{2+}$ (Figure S6). As real water samples, we chose tap and drinking water (Table 1). Quantification of each sample was repeated twice and showed proper recovery and relative standard deviation (R.S.D.), indicating that IH-Sal could work as an efficient chemosensor for monitoring Zn$^{2+}$ in real samples. From the calibration curve, the detection limit of IH-Sal for zinc ions was calculated to be 0.41 µM based on $3\sigma/k$, which was greatly below that suggested by the WHO (76.0 µM) for Zn$^{2+}$ ions [67]. The value is the lowest among those previously found for indole-based Zn$^{2+}$ chemosensors in a near-perfect aqueous solution (Table S1).

Table 1. Determination of Zn$^{2+}$. *^a^

| Sample       | Zn$^{2+}$ Added (µM) | Zn$^{2+}$ Found (µM) | Recovery (%) | R.S.D. (n = 3) (%) |
|--------------|----------------------|----------------------|--------------|-------------------|
| Drinking water | 0.0                  | 0.0                  | -            | -                 |
|              | 10.0                 | 10.0                 | 100.01       | 1.58              |
| Tap water    | 0.0                  | 0.0                  | -            | -                 |
|              | 10.0                 | 10.1                 | 101.00       | 0.40              |

*a Conditions: [IH-Sal] = 1 × 10⁻⁵ M in buffer.

To prove the practicability of IH-Sal as a practical probe for zinc ions, competitive tests were executed (Figure S7). With the same amount of Zn$^{2+}$ and other cations with IH-Sal, most cations did not inhibit the sensing ability of IH-Sal for zinc ions. However, Cu$^{2+}$, Fe$^{2+}$, Cr$^{3+}$, Fe$^{3+}$ and Co$^{2+}$ interfered with the fluorescence emission of IH-Sal with Zn$^{2+}$.

The pH dependence of IH-Sal-Zn$^{2+}$ for biological application was tested with various pH values (6–9, Figure S8). While there was no fluorescence emission at pH 6, IH-Sal-Zn$^{2+}$ showed a remarkable fluorescence response between pH 7 and 9, indicating that IH-Sal can clearly recognize Zn$^{2+}$ by the fluorescence application within the environmental pH range [68]. Based on the result of the pH dependence, fluorescence imaging of zebrafish was performed to widen the biological application. While the zebrafish cultured with IH-Sal (20 µM) alone did not show any fluorescent signal (Figure 3), blue emission on the zebrafish cultured with IH-Sal gradually increased as the amount of Zn$^{2+}$ increased from 0 to 50 µM. The mean intensity of the images was calculated with Icy software (Figure S9), given the detection limit of 5.07 µM. These results supported the biocompatibility of IH-Sal.
as a useful fluorescent probe for sensing Zn$^{2+}$ in live organisms. Importantly, this is the second indole-based Zn$^{2+}$ chemosensor for application to living organisms (Table S1).

**Figure 3.** Fluorescent images of zebrafish cultured with IH-Sal followed by addition of Zn$^{2+}$. (a$_1$–a$_3$): IH-Sal only; (b$_1$–b$_3$): IH-Sal with $2.5 \times 10^{-5}$ M Zn$^{2+}$; (c$_1$–c$_3$): IH-Sal with $5 \times 10^{-5}$ M Zn$^{2+}$. [IH-Sal] = $2.0 \times 10^{-5}$ M. Scale bar: 0.91 mm.

3.3. Calculations

With the results of the Job plot and ESI mass, the optimal structures of IH-Sal-Zn$^{2+}$ and IH-Sal were provided with DFT calculation (Figure 4). IH-Sal with a dihedral angle of $-179.427^\circ$ (1O, 2N, 3N, 4O) had a moderately distorted structure (Figure 4a). IH-Sal-Zn$^{2+}$ had a structure with a flipped phenol group (Figure 4b), showing a dihedral angle of 2.495°. Based on the energy-optimized forms of IH-Sal and IH-Sal-Zn$^{2+}$, transition energies and molecular orbitals were examined with TD-DFT calculations. For IH-Sal, the main absorption of HOMO-$1$→LUMO transition (314.18 nm) exhibited $\pi \rightarrow \pi^*$ transition (Figure S10). The major absorption of IH-Sal-Zn$^{2+}$ derived from HOMO→LUMO transition (358.71 nm, Figure S11) also displayed $\pi \rightarrow \pi^*$ transition (Figure S12). The red shift (320 to 360 nm) shown in the UV–Vis spectra was greatly matched with the calculated transitions and corresponded to a decreased energy gap. These outcomes implied that the fluorescence turn-on of IH-Sal to Zn$^{2+}$ may be a chelation-enhanced fluorescence (CHEF) effect [69]. With the Job plot, ESI-MS, $^1$H NMR titration and calculations, the appropriate structure of IH-Sal-Zn$^{2+}$ is proposed in Scheme 2.
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Figure 4. Energy-optimized patterns of (a) IH-Sal and (b) IH-Sal-Zn²⁺.

4. Conclusions

We illustrated an indole-based fluorescent probe, IH-Sal, which was produced from the condensation of 2-(1H-indol-3-yl)acetohydrazide and salicylaldehyde. IH-Sal could work as an effective fluorescent probe for monitoring Zn²⁺. The detection limit (0.41 μM) for Zn²⁺ was significantly below that suggested by the WHO (76.0 μM). The value is the lowest among those previously found for indole-based Zn²⁺ chemosensors in a near-perfect aqueous solution. IH-Sal could be reliably applied to real samples and showed its practical applicability to recognize Zn²⁺ in zebrafish. Importantly, this is the second indole-based Zn²⁺ chemosensor for application to living organisms. Thus, we believe that IH-Sal can be an efficient fluorescent chemosensor to determine Zn²⁺ in biological and practical applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/103390/s1, Table S1: Examples of indole-based Zn²⁺ chemosensors found to date; Figure S1: 1H NMR spectrum of IH-Sal; Figure S2: UV–Vis changes in IH-Sal (1 × 10⁻⁵ M) with various metal ions (8 equiv); Figure S3: Job plot for the binding of IH-Sal with Zn²⁺ (50 μM) in bis-tris buffer (10 mM, pH 7.0); Figure S4: Positive-ion ESI mass spectrum of IH-Sal (100 μM) upon the addition of 1 equiv of Zn²⁺; Figure S5: Benesi–Hildebrand equation plot (at 465 nm) of IH-Sal (10 μM) based on fluorescence titration, assuming 1:1 stoichiometry for association between IH-Sal and Zn²⁺; Figure S6: Calibration curve of IH-Sal as a function of Zn²⁺ concentration; Figure S7: Competitive selectivity of IH-Sal (10 μM) toward Zn²⁺ (8.5 equiv) in the presence of other metal ions (8.5 equiv, λex = 369 nm); Figure S8: Fluorescent intensity of IH-Sal (10 μM) and IH-Sal-Zn²⁺ species, respectively, at different pH values (6–9); Figure S9: Quantification of mean fluorescence intensity in Figure S7 (a₁, b₁ and c₁); Figure S10: (a) The theoretical excitation energies and the experimental UV–Vis spectrum of IH-Sal. (b) The major electronic transition energies and molecular orbital contributions of IH-Sal; Figure S11: (a) The theoretical excitation energies and the experimental UV–Vis spectrum of IH-Sal-Zn²⁺. (b) The major electronic transition energies and molecular orbital contributions of IH-Sal-Zn²⁺; Figure S12: The major molecular orbital transitions and excitation energies of IH-Sal and IH-Sal-Zn²⁺.
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