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Hydrolysis-activated Fluorophore System as a Molecular Sensor for Selective Detection of Zn^{2+}

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Abstract: A new selective Zn^{2+} fluorescent sensor, N′-(2-hydroxybenzylidene)-4-(2-hydroxy-benzylidene-amino) benzohydrazide (HHB), was synthesized. With the harbored 2-hydroxybenzylidene group, HHB exhibits an emission band centered at 475 nm with high quantum yield (f = 0.59) upon the addition of zinc ions. It features excellent fluorescence enhancement and provides favorable sensitivity for Zn^{2+} detection under the biological pH window of 6.8 ~ 7.7, and high selectivity for Zn^{2+} over biologically relevant alkali metals, alkaline earth metals and some of the first row transition metal. It works through the metal-assisted hydrolysis mechanism, and the hydrolysis residue sensor HB, salicylaldehyde-4-aminobenzoylhydrazone, was also investigated for comparison.

Key words: fluorescent sensor; Zn^{2+}; hydrolysis; selectivity

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1 Introduction

Recently, fluorescent sensors that can selectively detect zinc in live cells or even tissue slices to provide static and kinetic information with high spatial and temporal resolution have received more attention[1~18]. Initially, aryl sulfonamide-based sensors, including N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ) and its derivatives, were employed to visualize chelatable Zn(Ⅱ) in the mammalian hippocampus and in live cells[19~26]. By rational design, a variety of fluorescent zinc sensors that are bright and highly selective and respond rapidly to Zn(Ⅱ) with tunable emission wavelengths, zinc affinity, cell permeability, and subcellular localization have been documented[27~38]. Nevertheless, the design and implementation of new Zn(Ⅱ) imaging reagents is required to continue advances in this field.

More recently, we presented a strategy for the design of highly selective zinc sensors by introducing a carboxylic acid hydrazone group into quinoline-based fluorescent dyes (QB1 and QB2)[39]. By modulating the N_2O chelator with a suitable binding strength, this approach has been utilized to obtain high selectivity for zinc over the first-row transition metals. With the aim of enhancing the sensitivity of our sensors for zinc detection, herein we reported a hydrolysis-activated fluorophore sensor HHB, N′-(2-hydroxy-benzylidene)-4-(2-hydroxy- benzylideneamino) benzohydrazide for highly selective and sensitive detection of Zn^{2+} in DMSO-aqueous media.

HHB consists of a N-2-hydroxybenzaldehyde carbohydrazide moiety, the chromophore and part of the NO_2 chelator, which is utilized as metal ionphore for the first-row transition metals[40~44]. The sensor is synthetically simple and is envisioned to exhibit high sensitivity for Zn^{2+} based on the photoinduced electron transfer (PET) principles with high quantum yield[45,46], like that of some other N-(2-

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hydroxybenzylidene) benzohydrazide derivatives[47,48]. The harbored 2-hydroxybenzylidene group, which has the potential to hydrolyze in the presence of metal ion, would quench the fluorescence of the metal-free dye, such that the HHB chemosensor should exhibit low background luminescence.

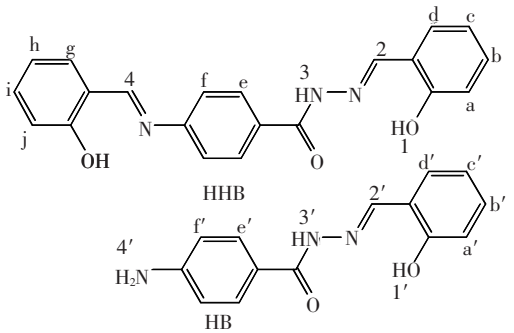

Fig. 1 Chemical structures of the chemosensors HHB and HB

2 Experiments

2.1 Materials and Methods

Unless otherwise stated, materials were obtained from commercial suppliers and used without further purification. Compound HB was synthesised according to the literature procedure[49]. ^{1}H NMR spectra were recorded on a VARIAN INOVA-400 spectrometer with chemical shifts reported as ppm (in d6-DMSO, TMS as internal standard). IR (KBr pellet) spectra were recorded on FI/IR NEXUS spectrophotometer. Mass spectrometric data were obtained on a HP1100LC/MSD mass spectrometry. Element analysis of C, N and H were performed with Vario EL Ⅲ I analyzer. Fluorescence emission spectra were obtained using JASCO FP-6500 luminescence spectrometer. Stock solution (2×10^{-2} mol·L^{-1}) of the aqueous salts of K^+, Na^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Cr^{3+}, Ag^+, Pb^{2+}, and Hg^{2+} were prepared. HB and HHB (2.0×10^{-5} mol·L^{-1}) were prepared in aqueous DMSO solutions (DMSO/H_2O, 80∶20 in *V*/*V*). The quantum efficiency of metal-free or metal-bound ligands was measured using Rhodomine B (ϕ = 0.89)[50] as the reference. The concentration of the reference was adjusted to match the absorbance of the test sample. For all the fluorescent measurements, the excitation wavelength was 370 nm. Both excitation and emission slit widths were 3 nm. Optical absorption spectra were measured on HP8453 UV/vis spectrophotometer in an aqueous DMSO solution (DMSO/H_2O 80∶20) at room temperature. All the spectroscopic measurements were performed at least in triplicate and averaged.

2.2 Preparation of HHB

5 drops of acetic acid were added to the mixture of 4-aminobenzoylhydrazide (0.15 g, 1.0 mmol) and salicylicaldehyde (0.3 g, 2.5 mmol) in methanol (30 mL). After refluxing for 24 h, the yellow product was collected by filtration, washed by methanol and dried in vacuum. Yield: 0.35 g, 96%.

Element analysis for HHB ($C_{21}H_{17}N_3O_3$), Calc.: H 4.77, C 70.18, N 11.69 %; Found: H 4.78, C 70.02, N 11.74.

^{1}H NMR (d_6-DMSO, ppm): 12.81 (s, 1H, H_5), 12.17 (s, ^{1}H, H_3), 11.32 (S, 1H, H_1), 9.04 (S, ^{1}H, H_4), 8.67 (s, 1H, H_2), 8.06 (d, 2H, H_f, J = 8.4 Hz), 7.71 (d, 1H, H_d, J = 7.6 Hz), 7.57 (d, 3H, H_e+H_g), 7.47 (t, 2H, H_i, J = 8.0 Hz), 7.31 (t, 1H, H_b, J = 7.6 Hz), 7.01 (t, 2H, H_c+H_h), 6.94 (t, 2H, H_a+H_j).

API-MS m/z: 359.1 ([M-H^+]), 381.0 ([M-Na^+]). Calc. for $C_{21}H_{17}N_3O_3Cl$: 394.3.

IR (solid KBr pellet, cm^{-1}): 3 251 (s), 3 052 (w), 2 747 (w), 1 667 (s), 1 611 (s), 1 571 (s), 1 537 (s), 1 491 (s), 1 454 (w), 1 372 (m), 1 357 (m), 1 276 (s), 1 191 (m), 1 172 (s), 1 116 (w), 849 (m), 827 (w), 753 (s) and 685 (w).

2.3 Preparation of Zinc Complex 1

HHB (0.03 g, 0.085 mmol) and $Zn(ClO_4)_2\cdot6H_2O$ (0.03 g, 0.10 mmol) were mixed in THF in the presence of triethylamine (0.1 mmol). The yellow precipitate was collected by filtration. Crystal suitable for X-ray structural analysis was obtained by slowly diffusing ethyl acetate to DMF solution.

Element analysis for Zn(HB)(DMF)(THF)-ClO_4, $C_{21}H_{23}ClN_4O_8Zn$, Calc.: H 4.14, C 45.02, N 10.00; Found: H 4.26, C 45.18, N 10.19.

2.4 Crystallography

Crystal data for Zn-complex 1 $C_{21}H_{23}ClN_4O_8Zn$, M = 559.25, Triclinic, space group P-1, yellow

needle, a = 1.061 2(1) nm, b = 1.157 7(1) nm, c = 1.188 3(1) nm, α = 63.961(6)°, β = 72.107(6)°, γ = 84.273(6)°, V = 124.72(2) nm^3, Z = 2, Dc = 1.492 g · cm^{-3}, μ(Mo-Kα) = 1.144 mm^{-1}, T = 293(2) K.

The intensities of the crystal data were collected on a Bruker SMART CCD diffractometer with graphite-monochromated Mo-Kα (λ = 0.071 073 nm) using the SMART and SAINT programs[51]. The structure was solved by direct methods and refined on F^2 by full-matrix least-squares methods with SHELXTL version 5.1[52]. 15 242 reflections were collected of which 4 394 reflections were unique (R_{int} = 0.043 5). The final refinement gave R_1 = 0.060 6, and wR_2 = 0.186 2 for 3 087 reflections with $I > 2\sigma(I)$. All of the non-hydrogen atoms were refined with anisotropic thermal displacement coefficients. Hydrogen atoms were fixed geometrically at calculated distances and allowed to ride on the parent non-hydrogen atoms. The oxygen atoms of the perchlorate anion were refined disordered with the s. o. f of the parts being refined using free variable.

3 Results and Discussions

3.1 Spectroscopic Properties and Optical Responses to Zn^{2+}

The absorption spectrum of HB undergoes a blue shift from 335 nm to 330 nm upon zinc binding, reflecting the phenol oxygen atom to be involved in the Zn(Ⅱ) coordination (Supporting information S1). At the same time, a new peak at 392 nm appears which is possibly assigned to the deprotonation of the phenol proton and a perturbation of the benzocarboxy hydrazide π-system during the zinc coordination[47,48]. Compound HHB exhibits the similar absorbance spectrum at about 300 ~ 600 nm and a similar blue shift of the band at 335 nm during the zinc bonding. The different spectra obtained from titration of HB and HHB show comparable features with the absorption decreasing at about 300 nm and 345 nm, and an increasing at 392 nm occurs on the addition of Zn^{2+} (Fig. 2). At the mean time, compound HHB exhibits a shoulder peak at about 260 nm which becomes stronger when Zn^{2+} is added.

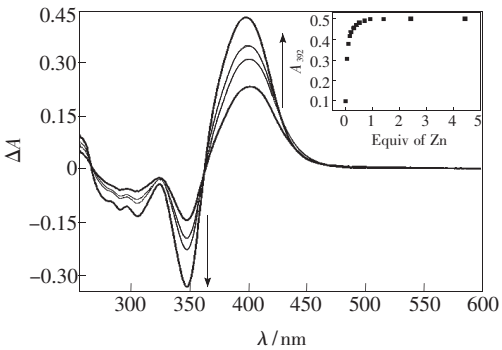

Fig. 2　UV-vis difference spectra resulting from the addition of Zn(Ⅱ) to HHB (20 mmol · L^{-1} in DMSO-H_2O 80∶20). Inset: The Uv-vis titration curve at 392 nm upon the addition of Zn^{2+}.

A simple comparison of the two families of the spectra demonstrates that such a shoulder peak is possibly caused by the second 2-hydroxybenzylidene attached to the amino group.

Like those of N-(2-hydroxybenzylidene)benzohydrazide derivatives, free HB exhibits a weak luminescence band at about 475 nm in DMSO-H_2O (80∶20) aqueous solution (20 mmol · L^{-1}) when excited by 370 nm[47,48]. Addition of zinc nitrate causes a large fold fluorescence enhancement with the quantum yield of 0.63 (λ_{max} = 475 nm). The fluorescence enhancement with the fluorescent wavelength being fixed suggests a simple photo induced electron transfer (PET) responding mechanism. From a mechanistic viewpoint, upon the complexation of Zn(Ⅱ) ion with HB, PET is hindered by use of the lone pair of N of the —C═N— group in a large rigid π-electron system[1,7]. In addition, the coordination of Zn(Ⅱ) to the whole π-electron system may increase the rigidity of the HB molecule, which may alter the relaxation processes from the excited state, radiative decay and interconversion. It is suggested that both the conformation restriction of the rigid conjugate system and the binding induced electron transfer from the N atom in the —C═N— group and the O atom in the phenol to a metal ion are attribute to the fluorescence of HB with the Zn^{2+}[53].

As shown in Fig. 3, compound HHB in DMSO-H_2O (80∶20) aqueous media exhibits a very weak emission band at 475 nm, the addition of Zn(Ⅱ) induces a significant luminescence enhancement

(excited by 370 nm). And the almost same emissions in HHB to that of HB with the high quantum yield (0.59) of the zinc-HHB complexation species indicate the same luminescent origin of the two chemosensors. Compared with only one nitrogen lone pair in the HB, the installation of another 2-hydroxybenzylidene moiety on the amino group of HB, results in a substantial decrease in background fluorescence, such that the compound HHB has the potential to exhibit higher sensitivity for zinc(Ⅱ) than that of HB.

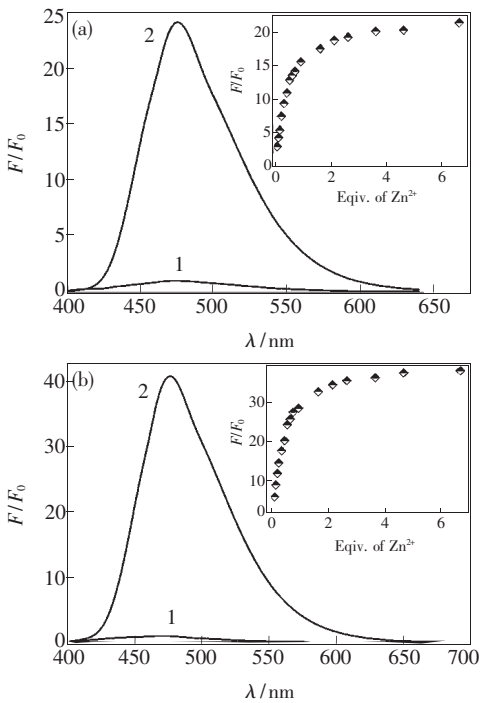

Fig. 3 Luminescence responses of HB (a) and HHB (b) in the presence 0.2 mmol · L^{-1} of Zn^{2+}. The lines-1 represent of the free probes only (20 mmol · L^{-1}, in DMSO-H_2O, 80∶20), the lines-2 represent the zinc-binding fluorescence enhancement. The insets exhibit the titration profile upon addition of Zn^{2+}, respectively. Excitation wavelength was 370 nm, the intensities were recorded at 475 nm.

As the representative data of the fluorescence response of a 10 mmol · L^{-1} solution of HB and HHB in the presence of metal ions shown in Fig. 4, the fluorescent intensities of HB and HHB are not increased by addition of various metal ions found at high concentrations (1 mmol) in cells, such as K^+, Na^+, Mg^{2+} and Ca^{2+}. Addition of transition metal ions (50 mmol · L^{-1}) such as Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Hg^{2+} does not increase the fluorescent intensity. The IIB group congener Cd^{2+} induces a weak enhancement of the fluorescent intensity for HB, but no significant responses for HHB upon the addition of Cd^{2+} can be observed.

One challenge in the development of Zn^{2+} sensors is to achieve Zn^{2+} selectivity over divalent first-row metal ions while maintaining the adequate fluorescent intensity upon metal binding. To further explore the utility of HHB and as ion-selective fluorescent chemosensors for Zn^{2+}, the selectivity of HHB and HB for Zn^{2+} over first-row transition metals were investigated, in addition to biologically relevant alkali and alkaline earth metals. These competitive experiments were performed in aqueous DMSO solutions (DMSO-H_2O, 80∶20), since the fluorescent signaling of a sensor in aqueous solution is crucial to practical application.

As the representative data shows (Fig. 5), HB (20 μmol·L^{-1}) readily detect Zn^{2+} (100 μmol·L^{-1})

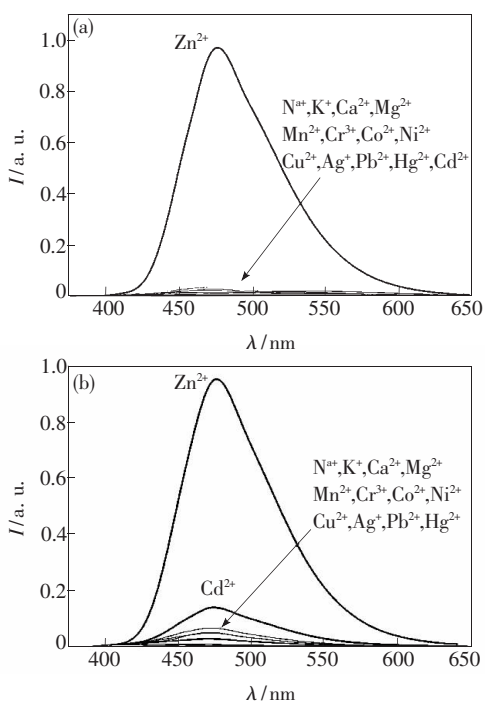

Fig. 4 Fluorescence responses of (a) HHB (10 mmol · L^{-1}) and (b) HB (10 mmol · L^{-1}) upon addition of Na^+, K^+, Mg^{2+}, Ca^{2+} (1 mmol · L^{-1}) and Cd^{2+}, Mn^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Ag^+, Hg^{2+}, Zn^{2+} (50 mmol · L^{-1}) in DMSO-H_2O (80∶20). Excitation wavelength was 370 nm.

in the presence of K^+, Na^+, Mg^{2+} and Ca^{2+} (0.5 mmol · L^{-1}), indicating that these physiologically relevant components will not interfere with the detection of Zn(Ⅱ) in such systems. But of the transition metal ions (0.5 mmol · L^{-1}) examined, luminescent intensities of the chemosensor HB were reduced to 15%, 60%, 25%, 15%, 5% and 10% by Cr^{3+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Hg^{2+}, and was increased 15% by Ca^{2+}, respectively. While for HHB, in the presence of transition metal ions as competing ions (0.5 mmol · L^{-1}), luminescent intensities of the zinc-HHB complexation were reduced to 85%, 60%, 45% and 5% by Mn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} (0.5 mmol · L^{-1}), respectively. No interference caused by Cr^{3+} and Hg^{2+} can be observed. Meanwhile, both the chemosensors can differentiate Zn^{2+} from Cd^{2+} and Hg^{2+}. Addition of Cd^{2+} results in negligible fluorescence enhancement, and the competitive experiment shows that Zn^{2+} readily displaces Cd^{2+} from the metal ion coordination sphere[54,55]. Furthermore, addition of excess salicylaldehyde (1 mmol · L^{-1}) to the solution of HB and Zn^{2+} in presence of the highly interfering metal ions (0.5 mmol · L^{-1}), such as Mn^{2+}, Ni^{2+} and Co^{2+}, except Cu^{2+}[56], the fluorescent intensities

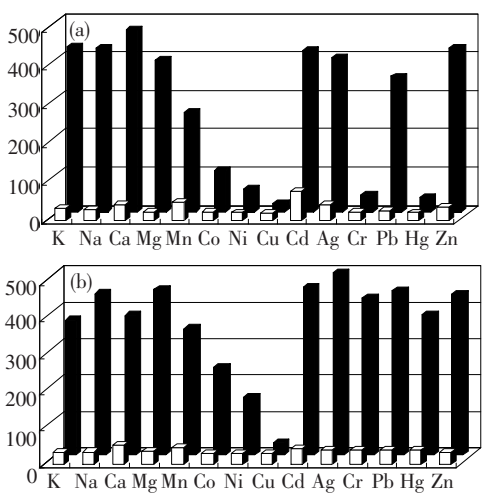

Fig. 5　Selectivity of the sensors for Zn(Ⅱ) over the metal ions of interest in an aqueous solution (DMSO-H_2O, 80∶20). In order: White bars: Sensors + 25 eq. cation of interest. Black bars: addition of 5 eq. Zn(Ⅱ) to the solution containing 1 eq. sensor and 25 eq. cation of interest. (a) HB (20 mmol · L^{-1}), (b) HHB (20 mmol · L^{-1}).

of the other cases increased to the level of corresponding HHB cases. It revealed that the better selectivity of HHB comparing to HB may attribute to the presence of another 2-hydroxybenzylidene group.

3.2　Metal-assisted Hydrolysis of HHB

In the ^1H NMR spectrum of HB, the peaks at about 11.73, 11.54 and 5.84 ppm are assigned to the OH proton, the C(O)-NH proton and the -NH_2 proton, respectively. After reaction with Zn^{2+}, the peak of hydroxy group disappears and the C(O)-NH proton becomes broader, suggesting the deprontonation and the coordination of the NO_2 chelating unit to Zn(Ⅱ) (Supporting information S3, S4). The signal of -NH_2 group disappears upon the addition of Zn^{2+}, probably due to the proton exchanges in the NMR time-scale. Other peaks in the Zn-HB complex could be assigned well to the protons on the aromatic rings.

The ^1H NMR spectum of metal-free HHB exhibits three peaks at about 12.81, 12.17 and 11.32 ppm, which are assigned to the two OH protons and one C(O)-NH proton, respectively. Upon addition of Zn^{2+}, the two OH proton peaks become weaker and broader with the C(O)-NH proton peak being broader. Two new peaks at 10.86 and 10.27 ppm are possibly assigned to the hydroxy and aldehyde group of salicylaldehyde, respectively (Supporting Material Figure S4). At the mean time, the addition of Zn^{2+} causes the peaks assigned to aromatic rings splitting and shifting.

A careful comparison of the spectra of salicylaldehyde and the Zn-HB complexation species suggests the co-existence of the Zn-HB complexation species, salicylaldehyde and free HHB compound within the titration mixture. That is: the peak at 7.64 ppm, which is marked by a star in Fig. 6, is the signal of salicylaldehyde. The peaks at about 9.06, 8.69 and 8.06 ppm, which are marked right cycle, are assigned to the protons of the HHB. And the peaks marked by rhombus at about 8.59, 7.74 and 6.67 ppm, are belong to the Zn-HB complexation (Fig. 6). Other protons of the salicylaldehyde, HHB and Zn-HB complexation are complete overlaped. The presence of the zinc(Ⅱ) ion causes the

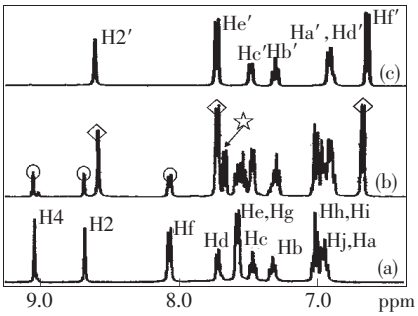

Fig. 6 ^{1}H NMR spectra (in d6-DMSO) of (a) HHB only, (b) HHB + $Zn(NO_3)_2 \cdot 6H_2O$ (1∶1), (c) HB + $Zn(NO_3)_2 \cdot 6H_2O$ (1∶1). The peaks marked by cycle, rhombus and star are assigned to the proton of HHB, HB + Zn complexation species, and salicylaldehyde, respectively.

significant hydrolysis of HHB, and at the same time the produced HB act as efficient chelator to coordinate the zinc (Ⅱ), such that the HHB and HB exhibit similar optical properties upon the recognition of zinc(Ⅱ).

Job plots shows a break at 0.5, suggesting a 1∶1 stoichiometry of the Zn(Ⅱ)-HHB complexation species (Supporting information Fig. S2). It seems that the hydrolysis reaction is a fast and irreversible step from which the titration behavior is controlled by Zn(Ⅱ)-HB complexation behavior. Although the metal-assisted hydrolysis of Schiff base has been well known, there are few examples that utilize such hydrolysis reaction on the design of metal sensor[57]. Despite such a metal-assisted hydrolysis process should be help to enhance the selectivity target metal ions.

Solid proof for the metal-hydrolysis reaction of the HHB comes from the single crystal analysis of the HHB-Zn complexation species. As shown in Fig. 7, the reaction of HHB with Zn^{2+} gives the complex 1 comprising the metal ions and deprotonated HB chelators. Hydrolysis reaction is happened, giving the residue fragment HB. Each zinc (Ⅱ) atom is coordinated to a NO_2 tridentate chelation group from one fragment and one phenoxyl O atom from another fragment to form the basic plane. The apical position is occupied by the O atom from the DMF solvent, comprising the five-coordination configuration of Zn(Ⅱ) centre. The C—O bond distance of 0.124 1(5) nm for C(7)-O(1), together with the C—N bond distance of 0.135 5(6) nm suggests the existence of the C(O)-NH unit[39]. And the C—O bond distance 0.133 8(6) nm for C(14)—O(2) reveals that the proton on O(2) is lost and the fragment acts as a monoanionic ligand during the coordination[58]. Two phenoxyl O atoms bridge two Zn atoms to form a dimeric unit with the Zn…Zn

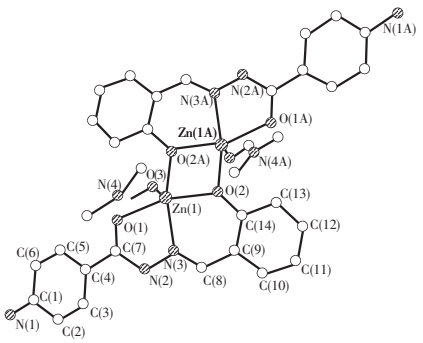

Fig. 7 Molecular structure of Zn-HHB complexation species, showing the hydrolysis of the ligand HHB. Hydrogen atoms, anions and solvent molecule are omitted for clarity. Selected bond distances (nm) and angle(°):
Zn(1)-O(1) 0.209 2(3), Zn(1)-N(3) 0.205 9(4), Zn(1)-O(2) 0.205 9(3), Zn(1)-O(2A) 0.198 1(3), Zn(1)-O(3) 0.210 8(4);
O(2)-Zn(1)-O(2A) 81.3(1), O(2A)-Zn(1)-N(3) 162.16(2), O(2)-Zn(1)-N(3) 86.5(1), O(1)-Zn(1)-O(2A) 113.2(1), O(1)-Zn(1)-O(2) 162.9(1), N(3)-Zn(1)-O(1) 77.2(1), O(2A)-Zn(1)-O(3) 97.1(1), O(2)-Zn(1)-O(3) 94.0(1), N(3)-Zn(1)-O(3) 96.8(2), O(1)-Zn(1)-O(3) 93.2(1).
Symmetry code: A, $-x$, $2-y$, $1-z$.

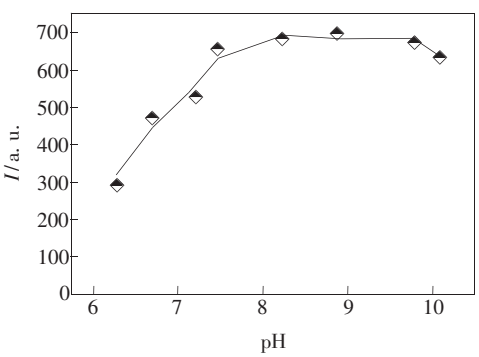

Fig. 8 The luminescent intensity pH-dependence of HHB (20 mmol · L^{-1} in DMSO-H_2O, 80∶20) in presence of Zn^{2+} (0.3 mmol · L^{-1})

separation of 0.306 nm. The dimeric units further connect each other to form one dimensional chains through weak coordination interactions between the Zn center and the amino N atom with the Zn…N distance of 0.378 nm (Supporting information S6).

HHB bears a wider pH range of 6.7 ~ 10.0. Under the biological pH window of 6.8 ~ 7.7, in the presence of 15 eq. of Zn^{2+} under aqueous conditions, HHB sensor shows very intense emission centered at 476 nm (Fig. 8). These results unambiguously prove that the sensor HHB can, at biological pH, selectively detect Zn^{2+} over most other metal ions that exist at high concentrations in living cells.

4 Conclusion

In a summary, we have reported a new strategy of preparation fluorescent chemosensor HHB for selective detection of Zn^{2+} in aqueous media. Compound HHB works upon the metal-assisted hydrolysis mechanism and exhibits higher selectivity for detection Zn^{2+} than that of compound HB. HHB features an excellent fluorescent enhancement with lower luminescence background and provides favorable sensitivity for Zn^{2+} under a large physiological pH range. Efforts to utilize current HHB sensor and prepare new ones for imaging applications are in progress.

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基于水解活性的 Zn^{2+} 荧光探针

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摘要： 合成了一种新颖的锌离子荧光探针 N′(2-羟基苄基)-4-(2-羟基苯亚甲基胺)-苯甲酰肼(HHB)，该探针本身的荧光较弱，但当它与 Zn^{2+} 配位时荧光增强，在 475 nm 处产生一较宽的发射光谱，量子产率 $f=0.59$（$\lambda_{ex}=370$ nm）。该化合物对 Zn^{2+} 具有良好的选择性能，环境生物体系中大量存在的碱金属和碱土金属离子 K^+，Na^+，Mg^{2+} 和 Ca^{2+} 以及过渡金属离子 Mn^{2+}，Co^{2+}，Ni^{2+} 和 Cu^{2+} 等对 Zn^{2+} 的检测没有明显影响。在生命体系的 pH 值范围(6.8～7.7)，配合物 HHB 显示出对 Zn^{2+} 检测较高的灵敏度。作为比较文中还研究了 HHB 水解的主要产物 4-氨基苄腙-水杨醛(HB)对 Zn^{2+} 的识别与传感性能。

关 键 词： 荧光传感器；Zn^{2+}；水解；选择性
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重要启示

本刊为方便广大作者的论文进行国际交流，并进一步加快我刊国际化进程，现向广大作者征集相关英语全文写作论文。对专家和编委审查合格的论文，我们将采取优先发表等优惠措施，欢迎广大作者踊跃投寄英语全文写作的学术论文。论文征集范围仍参见《发光学报》征稿简则。

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