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An unusual OFF–ON fluorescence sensor for detecting mercury ions in aqueous media and living cells†

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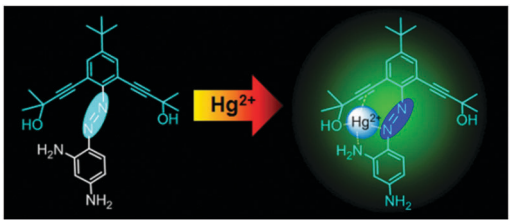
Maozhong Tian,*[ab] Libing Liu,[a] Yongjun Li,[a] Ruifeng Hu,[a] Taifeng Liu,[a] Huibiao Liu,[a] Shu Wang[a] and Yuliang Li*[a]

A novel azo derivative sensor (BDAA) based on alkynes was designed and utilized to direct detection of Hg^{2+} in aqueous solution and living cells. The new strategy achieved off to on switchable fluorescence. BDAA permits the highly selective and sensitive detection of Hg^{2+}. This sensor can be used for imaging of Hg^{2+} in living cells.

The design and synthesis of functionalized organic small molecules with controlled properties on the molecular level remains a challenging and attractive target of chemistry, materials science and biology.[1] The design of highly selective and sensitive fluorescence sensors based on organic conjugated molecules that are capable of detecting heavy- and transition-metal ions has been the subject of strong interest, because of the widespread use of these metal ions and their subsequent pollution triggering serious environmental and health problems.[2] Mercury is one of the most toxic and dangerous heavy metal elements in the environment, and can be easily bioaccumulated in the body.[3] Mercury compounds exhibit severe side effects on the nervous system, the endocrine system and kidneys.[4]

Mercury is a soft Lewis acid, and a lot of indicators for Hg^{2+} ions reported typically use sulfur as a soft donor atom to tightly bind the metal as part of fluorescence probes.[5] Only a few probes for Hg^{2+} based on alkynes have been developed according to the π electrophilicity of mercury ions.[5i,6] Furthermore, the recognition mechanisms of them were all attributed to irreversible chemical reactions of alkynes rather than to reversible interactions. Lee and co-workers reported a selective chemodosimeter for Hg^{2+} ions which were detected by a colorimetric channel in neutral aqueous solutions.[6b] Herein, we describe a new, highly selective and sensitive OFF–ON fluorescent Hg^{2+} sensor **BDAA** in aqueous solution (Scheme 1).

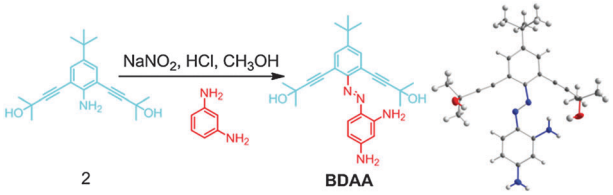


Scheme 1 Recognition mechanism of **BDAA** toward Hg^{2+}.

This sensor system exhibits significant advantage that the fluorescence output can be modulated from "off" to "on" in response to Hg^{2+} in aqueous media and living cells with little background interference. It also shows excellent selectivity for Hg^{2+} ions over relevant competing metal ions, and a 168-fold turn-on response was achieved with the sensitivity of ppb levels in aqueous solutions. The fluorescent response is insensitive to media pH. To the best of our knowledge, this is the first fluorescence sensor having the feature of chelation-enhanced fluorescence (CHEF) based on alkynes for Hg^{2+} ions.

To indeed obtain a highly sensitive and selective OFF–ON sensor for Hg^{2+} in aqueous solution, we introduced orthoethynyl and other yne groups onto the aryl ring of a *m*-phenylenediamine derived skeleton to define crescent-shaped ligand donor arrays as in **BDAA**. The introduction of amino groups can help to improve the solubility and chelation ability in aqueous solution. As shown in Scheme 2, **BDAA** can be easily synthesized (see ESI† for its synthesis, Table S1 for its crystal structure data).

The aqueous solubility of the **BDAA** has been determined to be 25 μM at least (Fig. S1, ESI†).[7] The **BDAA** in aqueous media does not exhibit fluorescence (Fig. S2 in ESI†) as typical azo dyes.[8]



Scheme 2 Synthesis of **BDAA** (right, crystal structure of **BDAA**).

[a] *Beijing National Laboratory for Molecular Science, Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China. E-mail: ylli@iccas.ac.cn; Tel: +86-010-62587552*

[b] *College of Chemistry and Environmental Engineering, Shanxi Datong University, Datong 037009, P. R. China. E-mail: tmz1994@@iccas.ac.cn*

† Electronic supplementary information (ESI) available: Synthetic details, UV-Vis and fluorescence spectra and fluorescence cell images. CCDC 961622 (**BDAA**). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3cc47915c

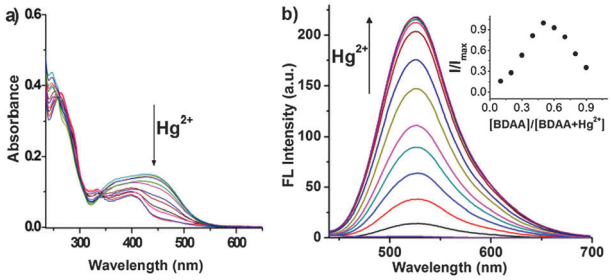


Fig. 1 (a) Absorption spectra of **BDAA** (10 μM) in HEPES (50 mM) solution (0.1 M KNO_3, pH = 7.4) upon addition of Hg^{2+}. (b) Emission spectra (excitation at 430 nm) of **BDAA** (10 μM) in the presence of Hg^{2+} (0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 50, 60 μM). Inset: Job's plot of **BDAA**. Excitation and emission slit widths were 3 and 6 nm, respectively.

In addition, the acid titration control experiments revealed that no obvious fluorescence change of **BDAA** could be observed from pH 3 to 11 (Fig. S3, ESI†). This phenomenon attested that no PET process emerged in this sensor. However, 3 equiv. of Hg^{2+} induced large increases of fluorescence intensity of **BDAA** in the pH range of 5–9. These results indicated that the **BDAA** can be utilized under physiological pH conditions for detection of Hg^{2+}.

We investigated the spectroscopic characteristics and Hg^{2+} response of the **BDAA** under physiological conditions (Fig. 1). It was found that free **BDAA** displayed very weak fluorescence (quantum yield: 0.008). The coordination of Hg^{2+} with the receptors blocked *cis–trans* transformation of azobenzene at the excited state upon Hg^{2+} binding, which lead to strong fluorescence. At the same time, the removal of intermolecular hydrogen bonding upon Hg^{2+} binding also contributes to the turn-on response.[9] The colorimetric and fluorescence responses of **BDAA** to Hg^{2+} in aqueous solution could be visible even with the naked eye (Fig. S4, ESI†). The fluorescence intensity at 525 nm dramatically enhanced by about 168-fold and the quantum yield increased ~52-fold (up to 0.419). Upon the addition of Hg^{2+}, a new absorption peak at 263 nm appeared with the concomitant distinct decrease of the peak at 446 nm. Furthermore, the fluorescence studies of the **BDAA**–Hg^{2+}complex were carried out in different solvents (Fig. S5, ESI†). The complex was sensitive to solvent effects, with the λ_{em} shifting from 461 nm in CH_2Cl_2 to 525 nm in water. In this molecular system, the amino groups functioned as the strong donors which led to more pronounced ICT in the excited state.[10] Standard density functional theory (DFT) was used to investigate the ICT effect. The spacial distributions of HOMO and LUMO are calculated at the B3LYP/6-31G (d) level (Fig. S6, ESI†). The calculated spatial distributions indicated that the density in the HOMO is concentrated in the *m*-phenylenediamine moiety, and the density in the LUMO is delocalized among the azo units, therefore, the aromatic amines work as the donating group for the D–A system, while the azo group acts as the acceptor. Meanwhile, the Stokes shift of **BDAA**–Hg^{2+} in CH_2Cl_2 was 55 nm, whereas the Stokes shift in water was increased to 118 nm. The very large Stokes shifts observed in protic solvents (water) were related to their ability to form hydrogen bonds. The large Stokes shift of **BDAA**–Hg^{2+} is a great advantage for fluorescence imaging, because a small Stokes shift can cause self-quenching and measurement error due to noise from the excitation and scattered light. More importantly, the enhancement of fluorescence intensity of **BDAA** corresponds to the concentration of Hg^{2+} in a linear manner (linearly dependent coefficient: R^2 = 0.9969). This indicated that the **BDAA** can be used to sensitively detect Hg^{2+} with a detection limit of *ca.* 46.5 nM (Fig. S7, ESI†). A Job's plot indicated that **BDAA** chelated Hg^{2+} ions with 1:1 stoichiometry (inset of Fig. 1). The association constant K was determined to be $3.96 \times 10^6\ M^{-1}$ (Fig. S8, ESI†), which is inferred from the Hg^{2+} titration curves.[11] The **BDAA**–Hg^{2+} solution was subsequently treated with excess Na_2S, the strong fluorescence of the **BDAA**–Hg^{2+} was almost quenched, indicating that the **BDAA** is a reversible sensor (Fig. S9, ESI†).

In order to elucidate the binding mode of **BDAA** and Hg^{2+}, we initially compared the ^{1}H NMR spectra of **BDAA** and its Hg^{2+} complex (Fig. S10, ESI†). When 0–1.1 equiv. of Hg^{2+} was added to a solution of sensor **BDAA** in DMSO-d_6, the signals of the six methyl protons adjacent to one oxygen atom were shifted downfield (Δppm = 0.34) more than that of the methyl protons of the *tert*-butyl group (Δppm = 0.11) and those of the other six methyl protons near the other oxygen atoms (Δppm = 0.19). This indicated that one oxygen atom of the sensor was coordinated in the first coordination sphere of the Hg^{2+} complex in the solution. It should be noted that the downfield shifts of the aromatic protons for the **BDAA**–Hg^{2+} complex suggested a stronger metal–nitrogen interaction in the **BDAA**–Hg^{2+} complex. This might improve the efficiency of the fluorescence enhancement. However, further insights into ^{1}H NMR titration spectra revealed that no further changes in ^{1}H NMR signals were observed at higher equivalents of Hg^{2+}. The ^{1}H NMR titration results also indicated 1:1 binding stoichiometry of **BDAA** with Hg^{2+} which supported the Job's plot results from fluorescence titration (inset of Fig. 1b).

To obtain additional information about the binding mode of the **BDAA** with Hg^{2+}, the ^{13}C NMR spectroscopic analyses in DMSO-d_6 solution were performed (Fig. 2). The chemical shifts of **BDAA** were assigned by HSQC and HMBC experiments (Fig. S11–S14, ESI†). The ^{13}C signals of one alkynyl group dramatically shifted downfield upon the addition of Hg^{2+}, which confirmed the coordination of the acetylenic group with Hg^{2+}. The peak of the C(t) atom and the C(g) atom also displayed an apparent downfield shift due to the binding of N, O atoms with Hg^{2+}. The binding mode of the sensor with Hg^{2+} was further confirmed through FT-IR spectroscopic analysis (Fig. S15, ESI†). The characteristic alkyne stretching frequency of the sensor appeared at 2219 cm^{-1}. In the **BDAA**–Hg^{2+} complex, it red shifted to 2013 cm^{-1}. In addition, the IR spectra of the **BDAA** confirmed the presence of an azo group (1420–1355 cm^{-1}). However, upon binding Hg^{2+}, the stretching frequency signal of the azo group weakened clearly. A weak band appeared in the vicinity of 461 cm^{-1} which was attributed to Hg^{2+}–N bonds in the complex. A strong band of 631 cm^{-1} should be assigned to Hg^{2+}–O bonds. Therefore, the fluorescence enhancement of the **BDAA** was mainly due to the blockage of *cis–trans* transformation of azobenzene at the excited

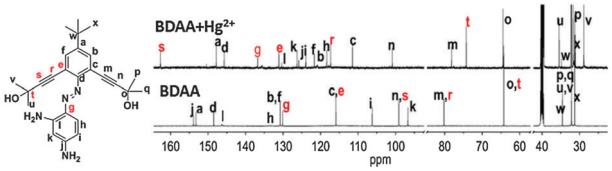


Fig. 2 ^{13}C NMR spectra of the free sensors **BDAA** and **BDAA** + Hg^{2+}.

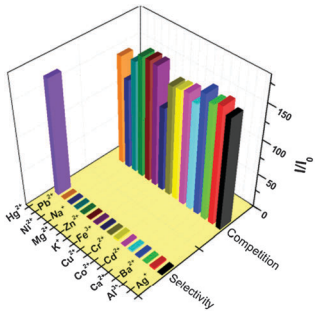


Fig. 3 Histogram showing selectivity of **BDAA** (10 μM) for Hg^{2+} in HEPES buffer (50 mM, containing 0.1 M KNO_3, pH = 7.4) solution. The pillars in the front row from left represent the I/I_0 value in the presence of various metal ions (5 equiv.). The pillars in the back row from left indicate the change in the emission intensity upon subsequent addition of Hg^{2+} (3 equiv.) to the solution containing **BDAA** and the metal ions of interest. For all measurements, λ_{ex} = 430 nm; T = 298 K. Excitation and emission slit widths were 3 and 6 nm, respectively.

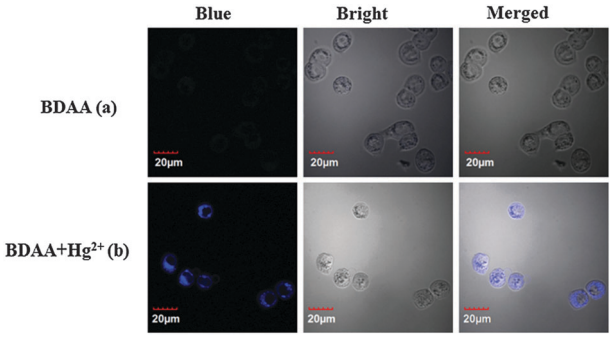


Fig. 4 Confocal fluorescence images of live HT-29 cells. (a) Cells incubated with 10 μM **BDAA** for 30 min. (b) Cells incubated with 30 μM Hg^{2+} for 30 min, washed two times, and then further incubated with **BDAA** for 30 min.

state upon the chelating of Hg^{2+} with one of the hydroxyl groups, azo group, one of the alkyne bonds and the amino group next to the azo group (Scheme 1). Further corroborative evidence for the mercury complex was observed in the MALDI-TOF mass spectrum which showed a mass peak corresponding to [**BDAA** + $Hg(ClO_4)_2{\cdot}3H_2O$ + $2Na^+$–H^+]$^+$ at m/z 931.3 (Fig. S16, ESI†).

The specificity of the sensor **BDAA** toward Hg^{2+} was determined by fluorescence screening. Nearly no fluorescence intensity changes were observed in emission spectra with various metal ions (Fig. 3) as well as anions such as ClO_4^-, CO_3^{2-}, SO_4^{2-}, F^-, Cl^-, Br^-, $H_2PO_4^-$ and Ac^- (Fig. S17, ESI†). However, under identical conditions, fluorescence intensity was enhanced significantly in the presence of Hg^{2+}. When 3 equivalents of Hg^{2+} were added into the solution of **BDAA** in the presence of 5 equivalents of other metal ions (10 equivalents of anions), the emission spectra displayed a similar pattern at near 525 nm to that with Hg^{2+} ions only, whereas Fe^{3+} and Ni^{2+} slightly quenched the fluorescence. These results clearly demonstrated that the **BDAA** sensor was highly specific for Hg^{2+} ions.

To further demonstrate the practical application of the **BDAA**, fluorescence imaging for Hg^{2+} was carried out in living cells using scanning confocal microscopy (Fig. 4). HT-29 cells were incubated with 10 μM of **BDAA** and 30 μM of Hg^{2+} ions (Fig. S18, ESI†) for 30 min at 37 °C, respectively. Both of them did not show intracellular fluorescence. However, after the addition of Hg^{2+} (30 μM), cells stained with **BDAA** were incubated for another 0.5 h, a marked increase in intracellular fluorescence was observed. These experiments indicated that **BDAA** is cell permeable and can respond to Hg^{2+} ions within living cells.

In summary, we have demonstrated a new strategy to direct detection of Hg^{2+} in aqueous solution and living cells which achieved "off" to "on" switchable fluorescence. The fluorescence intensity of the unusual sensor was significantly enhanced about 168-fold. Such a fluorescence sensor of the new structure expanded the functionality of the system, which revealed that the fluorescence output can be modulated from "off" to "on" in response to imaging of Hg^{2+} in living cells with little background interference. We believe that the unusual fluorescence sensor might have applicability for fundamental research and applications in the field of living sciences, with great potential to produce new structure fluorescence sensor devices.

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