Highly selective and sensitive twin-cyano-stilbene-based two-photon fluorescent probe for mercury (ii) in aqueous solution with large two-photon absorption cross-section

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ABSTRACT

The fluorophore derived from stilbene with two cyano groups in a single aromatic ring shows reasonably large two-photon absorption cross-section and emits strong two-photon fluorescence. A water-soluble two-photon sensor composed of the fluorophore containing bis[2-(2-hydroxyethylsulfanyl)ethyl]amino group (ionophore) has a very high sensitivity and selectivity for Hg^{2+} in water. It can be used to detect trace Hg^{2+} in near neutral aqueous solution (pH > 6.5), and its binding constant ($\log K$) for Hg^{2+} is as high as 5.72 ± 0.03. Nitrogen atom property and the sulfur atom number on the ionophore are believed to be responsible for the unique selectivity of probe **1** for Hg^{2+}.

1. Introduction

Recently, two-photon laser scanning microscopy (TPLM) [1] has been greatly developed in combination with suitable two-photon chemosensors to image the distribution of the guest molecules and ions in cellular processes with deeper penetration, better localized excitation, much less photodamage and photobleaching, smaller absorption coefficient of light in tissue, lower tissue autofluorescence [2], and higher 3-D spatial selectivity than that of the one-photon technique can attain [3–5].

For the maximum utilization of TPLM, it is essential to develop efficient two-photon probes. However, only a few two-photon fluorescent sensors for metal ions [6–9,12,14], fluoride ion [11], polarity [13], and pH [10] have been studied in organic solvents, model membranes or aqueous solution. The water-soluble two-photon sensors display rather small two-photon absorption cross-section (δ_{TPA}) ($\sim$10 GM; 1 GM $\equiv$ 1 $\times$ 10^{-50} cm^4 s photon^{-1} molecule^{-1}) [12,14], and their optimal excitation wavelengths do not fit the transmissive window for biological tissues, which lies between 600

and 1300 nm [15,16]. TPLM could greatly benefit from the development of the chromophores with large δ_{TPA} (>1000 GM) that can be excited efficiently in this range.

Hg^{2+}, known as one of the most prevalent components in the mercury family compounds (elemental, inorganic, and organic mercury), plays an important role in mercury biogeochemical cycling and mercury toxicology [17]. Hg^{2+} monitoring in environment and industrial waste streams will help us to attain a thorough evaluation of the character and process on mercury distribution and transformation [18].

Although many one-photon sensors for Hg^{2+} have been developed [19–23], only a two-photon fluorescence chemodosimeter exhibits the same response to Ag^+ as to Hg^{2+} and the value of its δ_{TPA} is no more than 13.4 GM [24]. Thus it can be seen that the development of two-photon fluorescence sensors for mercury ion is not only indispensable to biological chemistry but really challenging.

Higher values of δ_{TPA} make it possible for lower dye concentrations detection and laser power decrease needed for recording images, which result in less background fluorescence from endogeneous chromophores. This makes such chromophores interesting candidates for molecular two-photon excited fluorescence (TPEF) labels and probes. It is known that the push–pull chromophores have large δ_{TPA} values, especially when strong electron donors,

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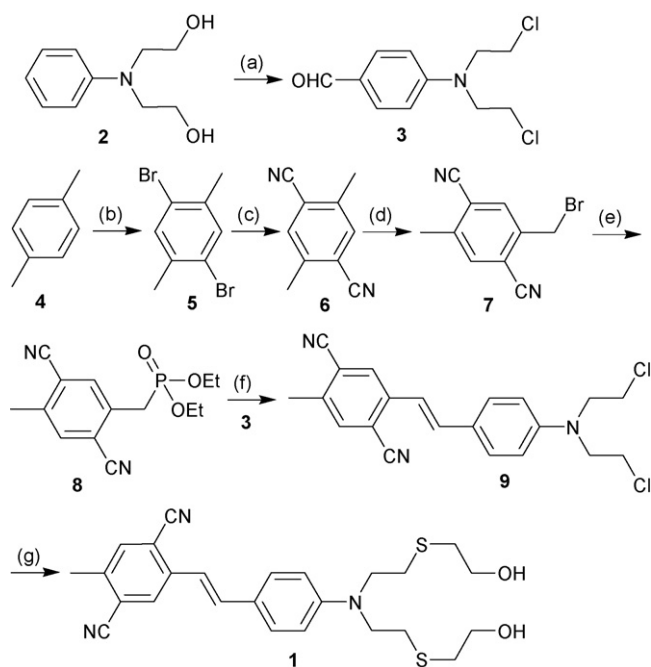
Scheme 1. Synthetic procedures of compound **1** (reagent and conditions: (a) POCl$_3$/DMF, 90 °C, 2 h (95%); (b) Br$_2$/CH$_2$Cl$_2$, no light, 20 °C, 24 h (90%); (c) CuCN/DMF, 150 °C, 48 h (78%); (d) NBS/CCl$_4$, 2 h (62%); (e) P(OEt)$_3$/toluene, 120 °C, 5 h (98%); (f) NaH (2 eq.)/THF, 12 h (58%); (g) K$_2$CO$_3$/MeCN, HS(CH$_2$)$_2$OH, 40 °C, 12 h (92%)).

such as *N,N′*-dialkylamino groups, are present at the extremities of the chromophores [18]. Beyond expectation, the δ_{TPA} value of a molecule with terminal amino groups is ten times of that with terminal alkoxy groups [25–28]. The substitution of hydrogens with cyano groups on the aromatic ring can dramatically boost the δ_{TPA} value of molecules [29], remarkably decrease transition energy, stabilize resonance structures and increase the excited state dipole moment. Herein we report our novel sensor, 1,7-bis(hydroxyethyl)-4-aryl-1,7-dithia-4-azaheptan (**1**, Scheme 1) as a two-photon fluorescent sensor for Hg^{2+}. It is composed of 4-methyl-2,5-dicyano-4′-amino stilbene (donor–bridge–acceptor, D–π–A) as a new two-photon fluorophore with remarkably large δ_{TPA} and 1,7-bis(hydroxyethyl)-1,7-dithia-4-azaheptane as a receptor (ionophore) with a very high sensitivity and selectivity for Hg^{2+}, and its photophysical properties with and without binding of the metal ions was studied using UV–vis spectrometry, fluorescence spectroscopy, and two-photon excitation spectroscopy.

2. Experimental

2.1. Materials and methods

NMR spectra were recorded on a VARIAN INOVA 400 MHz NMR spectrometer. Mass spectral determinations were made on an ESI-Q-TOF mass spectrometry (Micromass, UK). High resolution mass spectra measurements were performed on a GC-TOF mass spectrometry (Micromass, UK). Fluorescence measurements were performed on a PTI-C-700 Felix and Time-Master system. Fluorescence quantum yields were measured using standard methods [30] on air-equilibrated samples at room temperature. Quinine bisulfate in 0.05 M H$_2$SO$_4$ (Φ = 0.546) was used as a reference [30]. TPEF action cross-section spectra were measured according to the experimental protocol established by Xu and Webb [31], using a mode-locked Ti/sapphire laser that delivers ∼80 fs pulses at 76 MHz. Fluorescein (10^{-4} M in 0.1 M NaOH), whose TPEF action

cross-sections are well-known [31], served as the reference. The quadratic dependence of the fluorescence intensity on the excitation intensity was verified for each data point, indicating that the measurements were carried out in intensity regimes in which saturation or photodegradation does not occur. The measurements were performed at room temperature on air-equilibrated solutions (10^{-5} M). The experimental uncertainty on the absolute action cross-sections determined by this method has been estimated to be ±20% [31]. Absorption spectra were measured on a HP-8453 spectrophotometer. Solvents were generally dried and distilled prior to use. Reactions were monitored by thin-layer chromatography on Merck silica gel 60 F$_{254}$ precoated aluminum sheets. Column chromatography: Merck silica gel Si 60 (40–63 μm, 230–400 mesh). The pH-dependent fluorescence studies were performed according to the literature [32].

2.2. Synthesis

2.2.1. 2-Bromomethyl-5-methylterephthalonitrile (**7**)

2.0 g (13 mmol) 2,5-dimethylterephthalonitrile, 2.3 g (13 mmol) *N*-bromosuccinimide (NBS), 0.1 g benzoyl peroxide (BPO) and 100 mL of CCl$_4$ were placed into a 250 mL flask, the mixture was refluxed for 2 h. After cooled down to room temperature, the mixture was filtered, and the filtrate was concentrated by evaporating the solvent to get a viscous liquid. Flash chromatography on silica gel (20:1 v(CH$_2$Cl$_2$):v(*n*-hexane)) yielded compound **7** (1.9 g, 62%) as a white powder.

IR (KBr) cm^{-1}: 2226 (C≡N) and 3039 (C—H).
HRMS (EI) *m/z*: 233.9747 (calcd for C$_{10}$H$_7$BrN$_2$: 233.9793).
^{1}H NMR (CHCl$_3$-d, 400 MHz) ppm: 7.788 (s, 1H, Ph), 7.637 (s, 1H, ph), 4.594 (s, 2H, *CH$_2$*Ph), 2.604 (s, 3H, *CH$_3$*Ph).
Elemental analysis: calculated for C$_{10}$H$_7$BrN$_2$ (MW 233.98) C 51.09%, H 3.00%, Br 33.99%, N 11.92%; found C 51.13%, H 3.05%, Br 33.92%, N 11.90%.

2.2.2. 1-Diethylphosphorylmethyl-4-methyl-2,5-dicyanobenzene (**8**)

A solution of 2-bromomethyl-5-methylterephthalonitrile (1.4 g, 6 mmol) and P(OEt)$_3$ (2.5 g, 15 mmol) in 10 mL toluene were heated to 120 °C for 5 h. Excess P(OEt)$_3$ was removed in vacuo. Flash chromatography on silica gel (v(CH$_2$Cl$_2$):v(ethyl acetate) = 1:1) yielded compound **8** (1.7 g, 98%) as a white crystalline solid.

IR (KBr) cm^{-1}: 2227 (C≡N) and 1598–1345 (C=C).
HRMS (EI) *m/z*: 292.0977 (calcd for C$_{14}$H$_{17}$N$_2$O$_3$P: 292.0977).
^{1}H NMR (CHCl$_3$-d, 400 MHz) ppm: 7.758 (d, 1H, *J* = 2.8 Hz, Ph), 7.619 (s, 1H, Ph), 4.133 (m, 4H, 2 × *CH$_2$*CH$_3$), 3.377 (d, 2H, *J* = 22 Hz, *CH$_2$*Ph), 2.580 (s, 3H, *CH$_3$*Ph), 1.312 (t, 6H, *J$_1$* = *J$_2$* = 7.2 Hz, 2 × CH$_2$*CH$_3$*). ^{13}C NMR (CHCl$_3$-d, 100 MHz) ppm: 141.267, 134.723, 134.662, 134.283, 117.416, 116.391, 62.929, 62.861, 32.672, 31.298, 20.102, 16.527, 16.466.
Elemental analysis: calculated for C$_{14}$H$_{17}$N$_2$O$_3$P (MW 292.09) C 57.53%, H 5.86%, N 9.58%, O 16.42%, P 10.60%; found C 57.58%, H 5.91%, N 9.53%, O 16.32%, P 10.65%.

2.2.3. 2-(2-{4-[Bis(2-chloroethyl)amino]phenyl}vinyl)-5-methylterephthalonitrile (**9**)

Aldehyde **3** (1.25 g, 5.1 mmol), and NaH (130 mg, 5.4 mmol) were dissolved in 3 mL of tetrahydrofuran (THF), and the solution was cooled to 0 °C under N$_2$. To this solution, phosphonate **6** (1.46 g, 5.0 mmol) in 9 mL of THF was added dropwise. The reaction mixture was stirred for 1 h at 0 °C, and then for 12 h at room temperature, followed by the removal of THF under reduced pressure. Water was added to the reaction mixture, and the product was extracted with dichloromethane (4 × 10 mL). The organic layer was dried with dry Na$_2$SO$_4$ followed by evaporation of the solvent. The crude

product was separated by column chromatography with a gradient of hexane in dichloromethane (20–0%) and ethyl acetate in dichloromethane (0–20%). The resulting solid was recrystallized from acetone to give compound **9** (1.49 g, 78%) as a yellow powder.

IR (KBr) cm^{-1}: 2222 (C≡N) and 1596–1347 (C=C).

HRMS (EI) m/z: 383.0952 (calcd for $C_{21}H_{19}Cl_2N_3$: 383.0956).

^{1}H NMR (CHCl$_3$-d, 400 MHz) ppm: 7.975 (s, 1H, Ph), 7.555 (s, 1H, Ph), 7.491 (d, 2H, J = 8.8 Hz, Ph), 7.201 (d, 1H, J = 16.4 Hz, CH=CH), 7.146 (d, 1H, J = 16.4 Hz, CH=CH), 6.706 (d, 2H, J = 8.4 Hz, Ph), 3.778 (t, 4H, $J_1 = J_2 = 6.8$ Hz, 2 × CH$_2$Cl), 3.685 (t, 4H, $J_1 = J_2 = 6.4$ Hz, 2 × CH$_2$N), 2.556 (s, 3H, CH$_3$Ph). ^{13}C NMR (CHCl$_3$-d, 100 MHz) ppm: 147.132, 139.688, 139.505, 134.521, 134.435, 129.335, 128.909, 125.196, 118.227, 117.509, 116.980, 116.907, 114.119, 112.159, 53.530, 40.474, 20.109.

Elemental analysis: calculated for $C_{21}H_{19}Cl_2N_3$ (MW 383.09) C 65.63%, H 4.98%, Cl 18.45%, N 10.93%; found C 65.68%, H 5.05%, Cl 18.36%, N 10.90%.

2.2.4. 2-[2-(4-{Bis[2-(2-hydroxyethylsulfanyl)ethyl]amino}phenyl)vinyl]-5-methylterephthalonitrile (1)

Compound **7** (383 mg, 1 mmol), 2-mercaptoethanol (195 mg, 2.5 mmol), and anhydrous K$_2$CO$_3$ (414 mg, 3 mmol) were dissolved in acetone (25 mL), then the mixture was refluxed for 24 h with stirring under N$_2$. The resulting mixture was filtered, and the filtrate was concentrated by evaporating the solvent to get a viscous liquid. The crude product was purified by column chromatography using acetone/dichloromethane to afford compound **1** (260 mg, 58%) as a red solid. Further purification could be achieved by recrystallization from methanol to give needle solid.

IR (KBr) cm^{-1}: 3425 (OH), 2924 (C—H), 2224 (C≡N) and 1630–1348 (C=C).

^{1}H NMR (CHCl$_3$-d, 400 MHz) ppm: 7.958 (s, 1H, Ph), 7.679 (d, 2H, J = 8.8 Hz, Ph), 7.540 (s, 1H, Ph), 7.462 (d, 2H, J = 8.8 Hz, Ph), 7.184 (d, 1H, J = 16.0 Hz, CH=CH), 7.117 (d, 1H, J = 16.0 Hz, CH=CH), 3.783 (t, 4H, $J_1 = J_2 = 6.0$, 2 × CH$_2$O), 3.621 (t, 4H, $J_1 = J_2 = 7.2$, (CH$_2$)$_2$N), 2.788 (m, 8H, (CH$_2$SCH$_2$)$_2$), 2.548 (s, 3H, CH$_3$Ph), 2.282 (br s, 2H, 2 × OH). ^{13}C NMR (CHCl$_3$-d, 100 MHz) ppm: 147.612, 139.741, 139.232, 134.632, 134.351, 129.250, 128.749, 124.293, 117.507, 117.386, 116.908, 113.856, 111.981, 61.191, 51.634, 35.526, 29.287, 20.041.

Elemental analysis: calculated for $C_{25}H_{29}N_3O_2S_2$ (MW 467.17) C 64.21%, H 6.25%, N 8.99%, O 6.84%, S 13.71%; found C 64.30%, H 6.33%, N 8.95%, O 6.78%, S 13.64%.

3. Results and discussion

3.1. Design and synthesis of 2-[2-(4-{bis[2-(2-aminophenylsulfanyl)ethyl]amino}phenyl)vinyl]-5-methylterephthalonitrile (1)

4-[Bis(2-chloroethyl)amino]benzaldehyde (**3**) [33], 2,5-dibromo-p-xylene (**5**) [34], and 2,5-dimethylterephthalonitrile (**6**) [34] were synthesized according to literature procedures. In order to obtain **7**, we studied the alpha-bromination reaction of **6**, and found the optimum reaction time is 2 h. Reaction of **7** with phosphorous acid triethyl ester (P(OEt)$_3$) gave **8** in 98% yield (Scheme 1). Condensation of **8** with **3** afforded **9**, and then nucleophilic substitution of **9** with 2-mercaptoethanol gave **1** in high yield (86%). In the reaction of **9** and 2-mercaptoethanol, the substitution of chloro by mercapto group instead of hydroxy group was observed; the reason is that the nucleophilic ability of mercapto group is superior to that of hydroxy group. In addition, in the alpha-bromination reaction of **6**, we acquired four bromi-
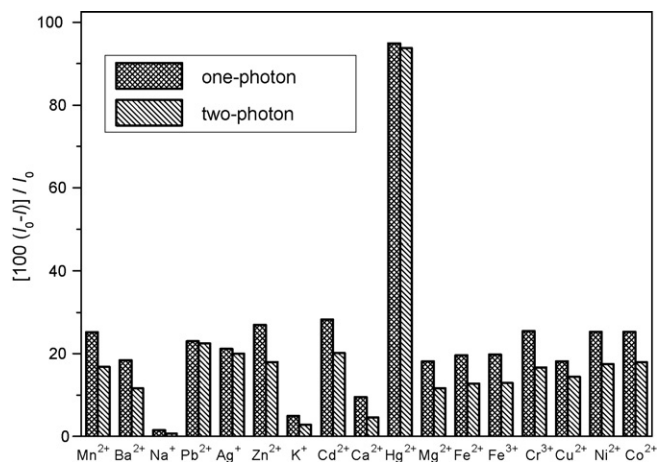

Fig. 1. A comparison of the percent quenching of one- and two-photon excited fluorescence of **1** (1 μM) in H$_2$O (10 mM tris–HCl buffer, pH 6.6) in the presence of 30 eq. of metal ions ($\lambda_{ex1} = 400$ nm, $\lambda_{ex2} = 790$ nm).

nation products: 2-bromomethyl-5-methylterephthalonitrile, 2,5-bis(bromomethyl)terephthalonitrile, 2-(1, 1-dibromo)methyl-5-methylterephthalonitrile and 2-bromomethyl-5-(1,1-dibromo)methylterephthalonitrile, respectively. Only 2,5-bis(bromomethyl)terephthalonitrile [34] has been reported.

3.2. Selectivities of sensor 1 for metal ions in one- and two-photon excited fluorescence

To investigate the binding properties of **1** toward metal ions, the fluorescent spectrum changes were detected upon addition of various metal perchlorates (Ag$^+$, Ca^{2+}, Cd^{2+}, Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Na$^+$, Cu^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Hg^{2+}, Pb^{2+}, K$^+$, and Ba^{2+}) to H$_2$O (10 mM tris–HCl (tris(hydroxymethyl)aminomethane hydrochloride) buffer, pH 6.6) solutions of **1**, respectively. The experimental results suggest that **1** shows a notable selectivity to Hg^{2+}. As depicted in Fig. 1, no pronounced response can be observed for **1** to other metal ions. Similar selectivities were also confirmed in the corresponding OPEF (Fig. 2) and TPEF (Fig. 3) experiments. The highly selective recognition of **1** for Hg^{2+} can be attributed to two factors. On the one hand, sulfur atom and Hg^{2+} are typical "soft base" and "soft acid", respectively, and so the very strong affinity between them is quite natural. On the other hand, nitrogen atom properties and the number of sulfur atoms of open chain
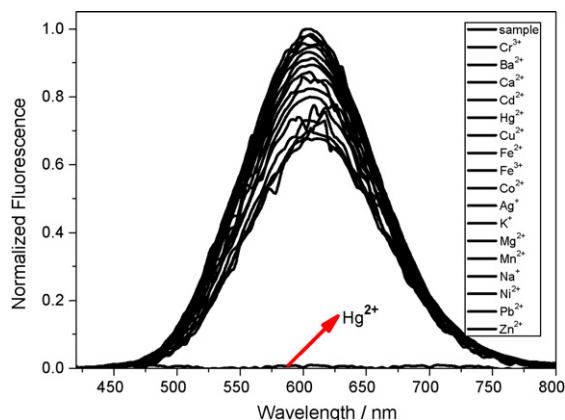

Fig. 2. The one-photon fluorescence spectra of **1** in H$_2$O (10 mM tris–HCl buffer, pH 6.6) upon the addition of 30 eq. metal ions.
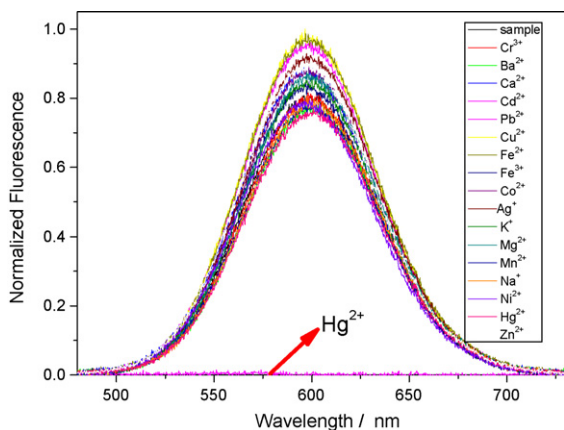
Fig. 3. The two-photon fluorescence spectra of **1** in H_2O (10 mM tris–HCl buffer, pH 6.6) upon the addition of 30 eq. metal ions.

Fig. 5. The two-photon fluorescence spectra of **1** in H_2O (10 mM tris–HCl buffer, pH 6.6) versus Hg^{2+} concentrations (0–6 eq.).

monoaza-dithiacrown ether may play an important role in chelating of heavy-metal-ion. The extent of TPF suppression by Hg^{2+} is smaller than that of the one-photon process (Fig. 1). Also, the two-photon process shows better selectivity for Hg^{2+}. Nevertheless, the trends observed in both experiments are more or less the same. These results provide additional support for the previous conclusion that efficient TPF probes for bioimaging applications could be designed by using the same strategy as that developed for the synthesis of one-photon fluorescent probes [4,5].

3.3. Sensitivity of sensor **1** to Hg^{2+} in UV–vis, one- and two-photon excited fluorescence spectra

Notably, upon complexation with Hg^{2+}, λ_{max} of **1** blue-shifted from 400 nm to 358 nm, and its absorption intensities decreased gradually (Fig. 4). The weak absorption band at 298 nm increased progressively. The binding constant of **1** for Hg^{2+}, expressed as $\log K$, was determined from the absorption-titration curves to be 5.72 ± 0.03 at 20 °C in water, which is comparable to those previously reported [19–24].

As shown in Figs. 4 and 5, the OPEF and TPEF spectra of **1** also displayed obvious changes when Hg^{2+} was added. The emission band of **1** at 613 nm progressively decreased upon the addition of Hg^{2+} to the solution in OPEF and TPEF. **1** exhibits comparatively high fluorescence quantum yields in toluene ($\Phi = 0.78$), MeCN ($\Phi = 0.42$)
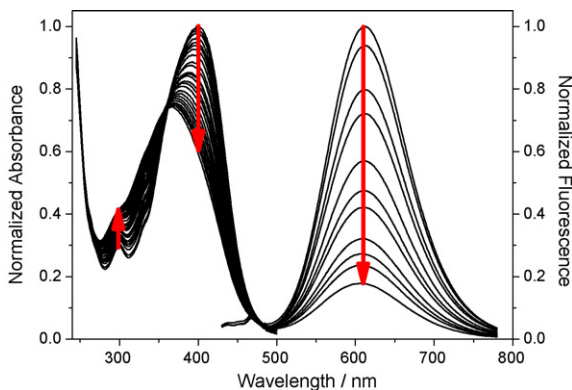

Fig. 4. Absorption and emission spectra of **1** (10 μM and 1 μM, respectively) in H_2O (10 mM tris–HCl buffer, pH 6.6, $\lambda_{ex1} = 400$ nm) upon addition of 0–2 and 0–6 eq. of $Hg(ClO_4)_2 \cdot 2H_2O$ predissolved in MeCN (0.01 M), respectively.
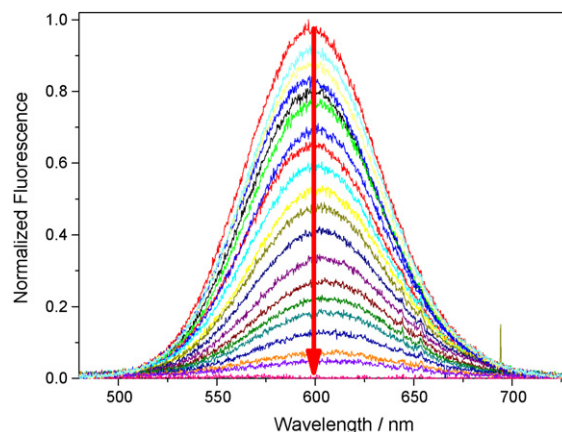
and H_2O ($\Phi = 0.20$). The binding constants of **1** for Hg^{2+}, expressed as $\log K$, was determined from the one- and two-photon emission-titration curves to be 5.47 ± 0.02 and 5.34 ± 0.02 at 20 °C in water (Fig. 6), respectively. Hence, **1** can serve as a good sensor for Hg^{2+} applied to OPEF and TPEF detection.

For the complexation ratio between the ligand and the metal ion, a Job plot experiment was conducted by varying the concentration of both **1** and Hg^{2+}. The appearance of isosbestic point at 360 nm and the value of the maximum point at the mole fraction for 0.52 suggest a typical ligand–metal complex ratio of 1:1 (Fig. 7).

3.4. Two-photon absorption cross-section of **1** versus two-photon excited wavelength

The δ_{TPA} of **1** was determined by using the two-photon-induced fluorescence measurement technique [15]. Fig. 8 shows that δ_{TPA} values of **1** in toluene and in H_2O are 2700 and 840 GM at 790 nm, respectively. δ_{TPA} decreases with the change in the solvent from toluene to H_2O, which should be attributed to the excited state charge separation. For dipoles molecule **1**, the charge separation is most favorable in a twisted conformation, where the donor D and acceptor A are orbitally decoupled. This not only led to intramolecular fluorescence quenching, but also attenuated π-electron conjugating and reduced δ_{TPA}. More polar solvent H_2O is favorable to balance positive and negative charge arising from the twisted conformation, so the value of δ_{TPA} for probe **1** in H_2O is comparatively small. Significant decrease in δ_{TPA} with increased solvent polarity has been reported [35]. Nevertheless, the intensity of the TPF was strong enough for the measurement. When excess Hg^{2+} was added, δ_{TPA} decreased even further, probably due to the electron-donating ability of the aromatic amino moiety attenuated upon complexation.

The fluorescence of **1** was also weakened by protonation of the tertiary amine in the twin-cyano stilbene skeleton, almost completely quenched at pH < 1. The fluorescence intensity for **1** had a sharp slump at pH < 6.5, and reached a maximum at pH > 7. The pH change fit to an apparent pK_a of 4.6 for **1** (Fig. 9). The plateau of the fluorescence corresponds to a molecular state in which tertiary amine is fully deprotonated. Therefore, when pH > 6.5, Hg^{2+}-induced changes can be readily measured in OPEF and TPEF. The fluorescence increasing at high pH and decreasing by addition of H^+ and Hg^{2+} are consistent with an intramolecular charge transfer (ICT) from the aromatic amine to the fluorophore.
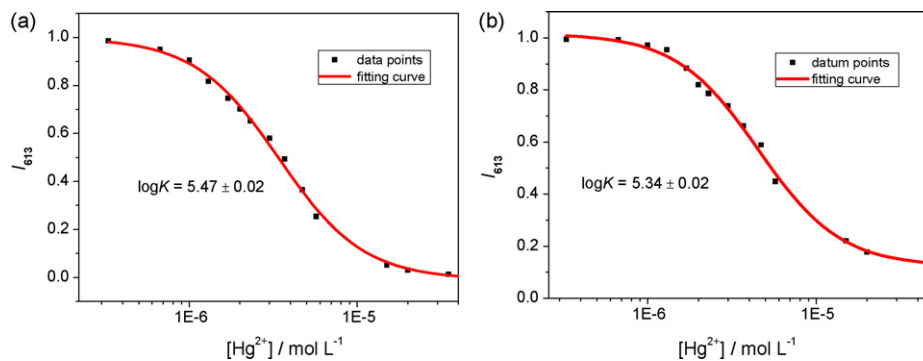
Fig. 6. The best fitting curve between change in (a) one- and (b) two-photon emission intensity of **1** at 613 nm and Hg^{2+} concentrations (10 mM tris–HCl buffer, pH 6.6, $\lambda_{ex1} = 400$ nm, $\lambda_{ex2} = 790$ nm).
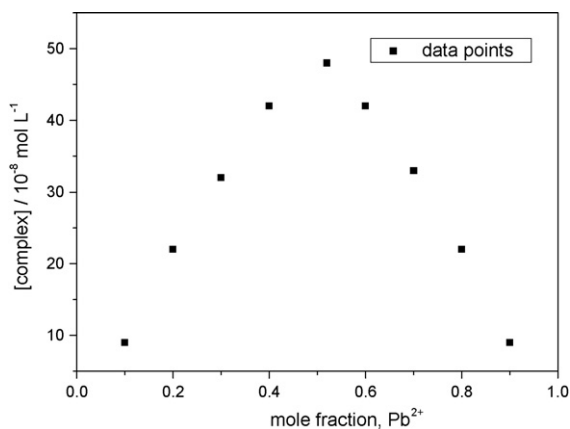

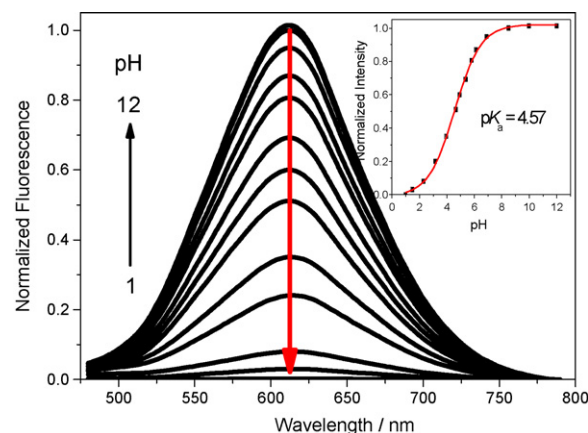**Fig. 7.** Job's plot for the complexation of **1** with Hg^{2+} in H_2O (10 mM tris–HCl buffer, pH 6.6).

Fig. 9. Fluorescence intensities of **1** (1 μM) in H_2O versus pH ($\lambda = 613$ nm). pH was adjusted with HCl, NaCl and NaOH. Inset: one-photon emission intensity of **1** at 613 nm as a function of pH.

3.5. The dependence of the up-converted fluorescence intensity on the incident intensity

To demonstrate that the fluorescence for **1** obtained at 790 nm is attributed to two-photon excitation, the experiment is conducted by changing the incident corresponding TPF intensity. The dependence of the up-converted fluorescence on the incident intensity for 1×10^{-5} mol L^{-1} of **1** is shown in Fig. 10. The nearly perfect quadratic dependence indicates that the up-converted fluorescence is induced by TPA.
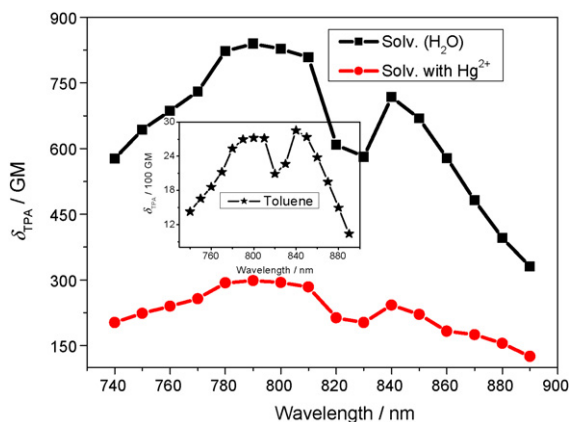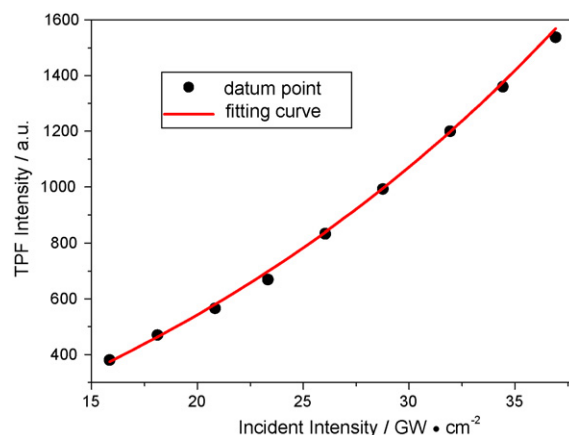

Fig. 10. The dependence of the up-converted fluorescence intensity for **1** in H_2O on the incident intensity.

4. Conclusion

In summary, a two-photon sensor for mercury (II) ion by using twin-cyano stilbene as the fluorophore and the open chain azathia-crown ether the receptor was synthesized. The sensor emits strong two-photon fluorescence when excited by 790 nm laser photons. Moreover, the sensor exhibits a fairly good sensitivity and selectivity for mercury (II) in water. Two cyano groups in one aromatic ring and an amino group in the other aromatic ring are recognized as

Fig. 8. Two-photon excitation spectra of **1** before (■) and after (●) addition of 5 eq. of Hg^{2+}.

important contributing factors for the large two-photon absorption cross-section of the sensor. The developed two-photon fluorescent sensor can be used to detect and determine trace Hg^{2+} near neutral aqueous solution (pH > 6.5) with a pK_a 4.6 and binding constant (log K) 5.72 ± 0.03.

Acknowledgments

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