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Highly selective colorimetric and fluorescent detection for Hg^{2+} in aqueous solutions using a dipeptide-based chemosensor

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A peptidyl chemosensor (**1**) showed sensitive colorimetric and fluorescent responses to Hg^{2+} and Ag^{+} among 16 metal ions in aqueous solutions. **1** detected sensitively Hg^{2+} and Ag^{+} by color change with a red shift of the maximum absorbance band at 452 nm; a red color for Hg^{2+} and an orange color for Ag^{+}. Furthermore, **1** showed a highly selective colorimetric response to Hg^{2+} among 16 metal ions in aqueous solutions containing NaCl. The selective colorimetric detection of Hg^{2+} was not interfered by other metal ions. About 2 equiv. of Hg^{2+} was enough for the saturation of color and fluorescent change of **1** in aqueous solutions. The dissociation constant for Hg^{2+} was 2.4×10^{-10} M^2 (R^2 = 0.993) and the detection limit for Hg^{2+} was 25.6 nM (R^2 = 0.994). The binding mode of **1** with Hg^{2+} ions was proposed on the basis of the results of pH titration experiment and mass spectrometry.

Introduction

There are high demands for new detection methods of heavy and transition metal ions (HTM) because these metal ions showed sever toxicities to living organisms. In particular, Hg^{2+} ion is regarded as one of the most toxic metal ions.[1-2] On the other hand, current detection methods for Hg^{2+} ions using atomic absorption spectrometry and inductively coupled plasma-mass spectrometry shared some drawbacks of expensive instruments and time consuming procedure.[3-4]

In recent years, colorimetric chemosensors for Hg^{2+} ion have attracted considerable attention due to their simple detection with naked eye, inexpensive instrumentation, and its potential applications in environment field.[5-20] Therefore, a range of colorimetric chemosensors for Hg^{2+} ions have been reported.[5-20] As the bioaccumulation of mercury in living organisms including humans occurs mostly through water contamination with Hg^{2+} ions, ideal colorimetric chemosensors for Hg^{2+} ions should dissolve well in aqueous solutions and sensitively detect low level of Hg^{2+} ions in aqueous solutions.[1-2] On the other hand, most colorimetric chemosensors required high percentages of organic solvents for their operations in aqueous solutions due to poor solubility in water and suffered from interference of other metal ions because of a low binding affinity for Hg^{2+} ions in aqueous solutions.[5-20] In addition, successful colorimetric detection for Hg^{2+} ion in aqueous solution was mostly achieved by chemodosimeters (reactive probes), however they irreversibly detected Hg^{2+} ions.[7-12,19] Therefore, it is highly challenging to synthesize new reversible colorimetric chemosensors for Hg^{2+} ions with high water solubility, high sensitivity, and high selectivity.

In recent years, fluorescent chemosensors for HTM based on amino acids or peptides have received many attentions because they dissolved well in aqueous solutions and exhibited selective and sensitive fluorescent responses to specific metal ions.[21-32] On the other hand, it is very rare to developing colorimetric chemosensors based on peptides for monitoring HTM. In the present study, we synthesized a new colorimetric chemosensor based on a dipeptide containing a disulfide bond as following reasons. Disulfide bonds of some proteins including human serum albumin participated in the binding site for Hg^{2+} ions with a specific geometry conformation.[33,34] In addition, Hg^{2+} ions interacted directly with a disulfide bond of the protein and sometimes formed a bis(mercapto) bond.[34] Thus, we synthesized a 7-nitro-2,1,3-benzoxadiazole (NBD) labelled dipeptide (**1**) containing a disulfide bond and investigated the detection ability for HTM ions in aqueous solutions. NBD has been used as a chromophore for *in vivo* and *in vitro* analysis of amino acid neurotransmitters and biological active peptides because NBD has a visible light absorption, high extinction coefficients, high quantum yield, and high chemical stability.[35-45]

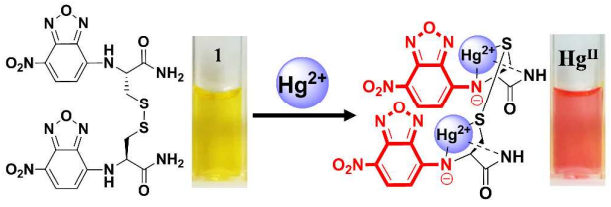

Fig. 1. Proposed binding mode and color change of **1** with Hg^{2+} ions.

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Interestingly, the NBD labelled dipeptide (**1**) selectively detected Hg^{2+} and Ag^{+} among 16 metal ions in aqueous solutions by a colorimetric and fluorescent change. In aqueous buffered solution containing NaCl (1 mM), **1** showed an exclusive selectivity for Hg^{2+} and the detection of Hg^{2+} was not interfered by other heavy metal ion. **1** as a colorimetric chemosensor showed promising properties for detecting Hg^{2+} ions such as fast, high water solubility, reversible detection, high selectivity, colorimetric and fluorescent response to Hg^{2+} ions. Further study indicates that the disulphide bond of **1** played a critical role for the selective recognition of Hg^{2+} ions among various heavy metal ions.

Materials and Methods

Materials

1-hydroxybenzotriazole (HOBt) and Rink Amide MBHA resin (100~200 mesh, 0.5 mmol/g) were purchased from Bead Tech. Fmoc-Cys(Trt)-OH was purchased from NovaBioChem. N, N'-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), tri-isopropylsilane (TIS), triethyl amine (TEA), diethyl ether, acetonitrile (CH_3CN), piperidine (pip) were purchased from Sigma Aldrich. 4-chloro-7-nitro-2,1,3-benzoxadiazole (4-ClNBD) and N, N-dimethylformamide (DMF) were purchased from Acros Organics. All perchlorate and chloride salts of metal ions were purchased from Sigma Aldrich and metal ion stock solution was prepared in high purity de-ionized water.

Solid phase synthesis of 1 and 2

1 was synthesized in solid-phase synthesis using Fmoc chemistry (Scheme 1). Activated Fmoc-Cys(Trt)-OH (176 mg, 0.3 mmol) with DIC (47 ul, 0.3 mmol) and HOBt (40 mg, 0.3 mmol) was loaded to Rink Amide MBHA resin (200 mg, 0.1 mmol) according to the reported procedure.[46] After deprotecting the Fmoc group with 25% piperidine in DMF followed by washing, NBD chloride (4-ClNBD, 60 mg, 0.3 mmol) was coupled with the amino group of the amino acid on resin in the presence of triethylamine (83 µl, 0.6 mmol). After complete of the coupling reaction, the cleavage of **2** from the resin was accomplished with TFA/TIS/H_2O (95:2.5:2.5, v/v/v) at room temperature for 4 h. After removing the excess TFA by N_2, the crude product was precipitated by addition of cold diethylether at -20°C into the cleavage solution. The product was collected by centrifugation. The product was washed with diethylether (-20°C) and then was dried under vacuum. **1** was synthesized from **2** by air oxidation instantly in 50% CN_3CN/H_2O. **1** and **2** was purified with semi-preparative HPLC using water (0.1% TFA) / acetonitrile (0.1% TFA).

1: Yield 80%, Orange solid; m.p. 135 °C; ^{1}H NMR (400 MHz, 50% CD_3CN/D_2O, 25℃) δ 9.23 (d, J=8.8 Hz, 2H), 7.16 (d, J=8.8 Hz, 2H), 5.56 (s, 2H), 4.56 (t, J=12 Hz, 2H), 4.36-3.97 (m, 4H); ^{13}C NMR (400 MHz, 50% CD_3CN/D_2O, 25℃) δ 172.49, 144.64, 137.58, 128.78, 124.28, 114.99, 101.16, 56.71, 40.12; ESI-HRMS Calcd: m/z 565.06 $[M+H^+]^+$, $C_{18}H_{17}N_{10}O_8S_2$. Found: m/z 585.07 $[M+H^+]^+$, $C_{18}H_{17}N_{10}O_8S_2$. (Fig. S1-4)

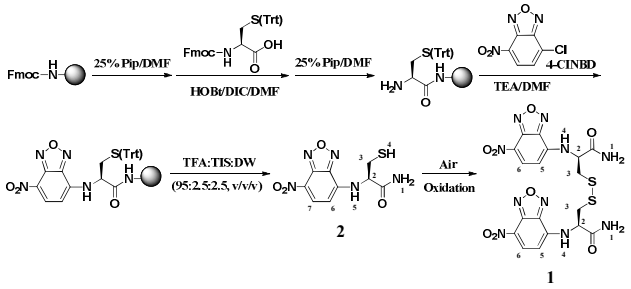
2: Yield 60%, Yellow Orange solid; m.p.142 °C; ^{1}H NMR (400 MHz, 50% CD_3CN/D_2O, 25℃) δ 8.48 (d, J=8.0 Hz, 1H), 7.54 (d, J=8.0 Hz, 1H), 3.8 (t, J=3.6 Hz, 2H), 3.09-3.05 (m, 1H); ^{13}C NMR (400 MHz, 50% CD_3CN/D_2O, 25℃) δ 179.88, 143.68, 132.10, 124.86, 99.77, 81.33, 51.07, 31.21; ESI-HRMS Calcd: m/z 284.04 $[M+H^+]^+$, $C_9H_{10}N_5O_4S$. Found: m/z 284.05 $[M+H^+]^+$, $C_9H_{10}N_5O_4S$. (Fig. S5-8)

Absorbance and Fluorescence Measurements

A stock solution of **1** with the concentration of 1 mM was prepared in CH_3CN and stored in a cold and dark place. The concentration of **1** was confirmed by the absorbance at 478 nm for NBD group (λ_{max} = 478 nm, ε = 18492 $cm^{-1}M^{-1}$). This stock solution was used for absorbance and fluorescence experiments after appropriate dilution. The Absorbance and fluorescence titration was carried out in aqueous buffered solution (10 mM HEPES, at pH 7.4). UV/Vis absorbance spectrum (300–650 nm) of a sample in a 10 mm path length quartz cuvette was measured using a Perkin-Elmer UV-Vis spectrophotometer (model Lambda 45). The emission spectrum (490–650 nm) of the sample was also measured using a Perkin-Elmer Fluorescence spectrometer (model LS 55). The absorbance and emission spectra of **1** were measured in the presence and absence of metal ions (Na^+, K^+ and A^{3+} as chloride anion and Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} as perchlorate anion). Emission spectra were measured by excitation with 469 nm. The slit width for excitation and emission was 5 and 5 nm, respectively.

Ellman's Test

Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) was used to quantify the thiol groups in the compound.[47] DTNB reacted with the thiol group in the compounds and formed mixed disulfides, resulting in the release of thionitrobenzoic acid (14,150 $M^{-1}cm^{-1}$ at 412nm), of which color was measured at 412nm with spectrophotometer to quantify the thiol group. A stock solution of the compound was diluted in 10 mM Tris buffer solution at pH 8. After mixing with DTNB solution, an absorbance at 412 nm was measured for the quantification of the thiol group.



Scheme 1. Solid phase synthesis scheme of **1** and **2**.

Determination of dissociation constant (K_d)

The dissociation constant (K_d) was calculated based on the titration curve of **1** with metal ions. The dissociation constant was determined by a nonlinear least squares fitting of the data with the the following equation, as referenced elsewhere.[48]

$$A = \frac{A_0 + A_\infty K_a[G]^2}{1 + K_a[G]^2}, \quad K_d = 1/K_a$$

Where A is absorbance signal, A_0 and A_∞ are the final absorbance signal, [G] is total concentration of metal ion (G).

Determination of Detection limit

To determine the S/N ratio, the absorbance of free **1** was measured ten times and the standard deviation of blank measurements was determined. Three independent measurements of the absorbance were performed in the presence of metal ions and each average value of the absorbance was plotted as a function of the concentration of metal ions for determining the slope. The detection limit was calculated using the following equation : detection limit = 3σ/m, where σ is the standard deviation of the intensity of free **1**, m is the slope between the absorbance at 464 nm vs. Concentration.[49]

Results

As shown in scheme 1, a NBD labelled peptide (**1**) was synthesized easily using solid phase synthesis with high yield (80%).[46] **1** and **2** were characterized by ^{1}H NMR, ^{13}C NMR, and ESI-HRMS data, respectively (Fig. S1-S8) and the high purity (>96%) was confirmed by analytical HPLC. As Ellman's test has been used to quantify the thiol groups of various compounds including peptides, Ellman's test was used to confirm the complete disulfide bond formation of **1**.[47] As shown in Fig. S9, an absorbance of **1** at 452 nm was not changed in the presence of DTNB, which indicated that **1** has a disulfide bond by complete oxidation of the thiol group. The disulfide bond of **1** was not reduced by 1 mM DTT as a reducing agent at room temperature for 1 hr. **1** (10~100 uM)was dissolved well in aqueous solutions containing small percentage of organic solvent.

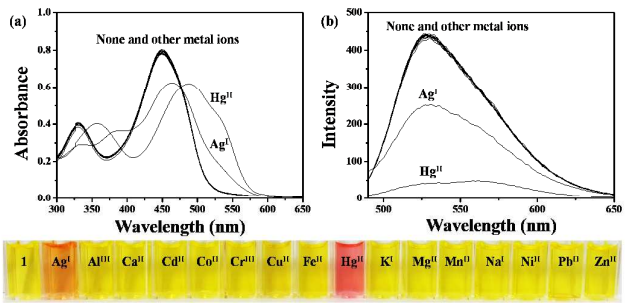


Fig. 2. (a) UV-VIS absorbance, (b) fluorescence emission spectra and visible color change of **1** (15 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% (v/v) CH_3CN in the presence of various metal ions (60 μM) (λ_{ex} = 469 nm, Slit 5/10 nm).

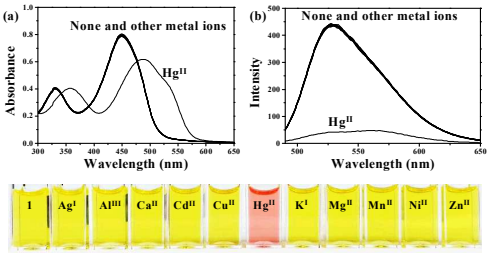


Fig. 3. (a) UV-VIS absorbance, (b) fluorescence emission spectra and visible color change of **1** (15 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4, 1 mM NaCl) containing 3% (v/v) CH_3CN in the presence of various metal ions (60 μM) (λex = 469 nm, Slit 5/ 10 nm)

UV-VIS spectra of **1** were measured in the absence and presence of various metal ions (Ag^+, Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, Pb^{2+}, and Zn^{2+}) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% (v/v) CH_3CN. **1** showed selective colorimetric responses to Hg^{2+} and Ag^+ among 16 metal ions; pink color for Hg^{2+} and orange color for Ag^+ (Fig. 2). Emission spectra of **1** in the presence of various metal ions revealed that **1** showed selective fluorescent responses to Hg^{2+} and Ag^+ among 16 metal ions in aqueous solutions.

To improve selective detection of Hg^{2+}, a colorimetric response of **1** to HTM was measured in aqueous buffered solution containing NaCl (1 mM). Interestingly, **1** showed an exclusively selective color change to Hg^{2+} ions, as shown in Fig. 3. In this condition, **1** showed pink color for Hg^{2+} ions and the absorbance induced by Hg^{2+} ions was almost same as that measured in aqueous solution without NaCl. Emission spectra of **1** in the presence of various metal ions indicated that **1** showed a turn-off response to Hg^{2+} ions in aqueous buffered solution containing NaCl (1 mM). To investigate the role of the disulfide bond of **1** for the selective detection of Hg^{2+} ions, we synthesized NBD labelled cysteine (**2**) and measured UV-VIS absorbance and emission spectra in the presence of metal ions. The presence of thiol group of **2** was confirmed by Ellman's test (Fig. S9). The colorimetric and fluorescent responses to metal ions were investigated in aqueous buffered solution (10 mM HEPES, pH 7.4). **2** showed sensitive colorimetric and fluorescent responses to several HTM such as Ag^+, Cd^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} (Fig. S10). This indicates that the thiol group of **2** acted as a ligand for various metal ions, whereas the disulfide bond of **1** acted as a selective ligand for Hg^{2+} ions. Cysteine containing a thiol group regarded as an important ligand for several heavy metal ions in various metalloproteines that played an important role in the detoxification and transport of HTM.[50-52]

UV-Vis titration experiments were carried out for measuring the binding affinity of **1** for Hg^{2+} (Fig. 4). Upon the addition of Hg^{2+}, the gradual red shift (30 nm) of the maximum absorbance at 452 nm with decreasing of the absorbance intensity was observed and the change of the absorbance was complete by 2 equiv. of Hg^{2+}. The clear isosbestic point at 480 nm in the titration of **1** with Hg^{2+} suggests that **1** may form one complex with Hg^{2+}. The Job's analysis of **1** with Hg^{2+} showed that the maximum absorbance was observed at 0.67 mole fraction (Fig. S11). This indicated that **1** might form a 1:2 complex with Hg^{2+}.

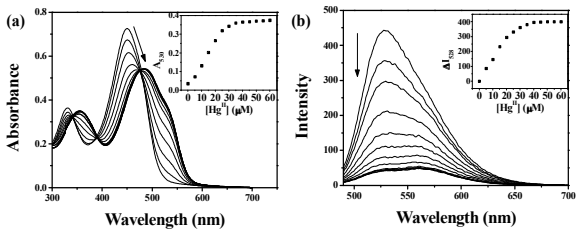


Fig. 4. (a) UV-VIS absorbance and (b) fluorescence emission spectra of **1** (15 μM) in the presence of Hg^{2+} (0, 5, ..., 60 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% CH_3CN. (λex = 469 nm, Slit 5/10 nm)

We also investigated the formation of the complex between **1** and Hg^{2+} by ESI-mass spectrometry (Fig. S12). After adding Hg^{2+} ions into the solution of **1**, a new peak $[M + Hg^{2+} - 3H^+]^-$ was observed in the mass spectrum with negative ion mode. This confirmed that **1** interacted strongly with Hg^{2+} and formed a 1:1 complex with Hg^{2+}. In general, it was difficult to observe a peak corresponding to the 1:2 complex with Hg^{2+} in mass spectrum due to an ionization process in gas phase. To confirm the 1:2 complex between the dipeptidyl chemosensor and Hg^{2+} in solution, we investigated the binding stoichiometry of the monomer form **2** of the dipeptidyl chemosensor with Hg^{2+}. As shown in Fig. S13, upon addition of Hg^{2+}, about 1 equiv. of Hg^{2+} required for the complete change of the absorbance. This result suggested that the monomer form of the dipeptide might form a 1:1 complex with Hg^{2+}. Furthermore, the data of the absorbance titration was well fitted by the equation for the 1:2 complex model rather than the 1:1 complex model. The dissociation constant (K_d) of **1** for Hg^{2+} was calculated to be 2.4×10^{-10} M^2 (R^2 = 0.993) (Fig. S14). Fluorescence titration experiments were carried out. The emission intensity change at 528 nm as the function of Hg^{2+} ions was fitted by a non-linear equation for the 1:2 complex model to calculate the dissociation constant. The dissociation constant was calculated to be 1.9×10^{-10} M^2 (R^2 = 0.993) for Hg^{2+} (Fig. S14). This K_d value was consistent with that measured in UV-Vis titration experiment.

The detecting ability of **1** for Hg^{2+} was investigated in the presence and absence of other metal ions (Fig. 5). The Hg^{2+}-dependent color change of **1** was not affected considerably by the presence of other metal ions. **1** showed a pink color to Hg^{2+} even in the presence of Ag^+, suggesting that **1** could allow the naked eye detection of Hg^{2+} even in the presence of Ag^+ because of the potent binding affinity for Hg^{2+}.

1 exhibited a sensitive colorimetric response to low levels of Hg^{2+} in an aqueous solution. The detection limit of **1** for Hg^{2+} was determined to be 25.6 nM (R^2 = 0.994) in aqueous solutions (Fig. S15). We also investigated the anion effect on the detection for Hg^{2+} in aqueous solutions. The colorimetric response of **1** to $Hg(ClO_4)_2$, $HgCl_2$, $Hg(OAc)_2$ and $Hg(NO_3)_2$ were measured (Fig. S16). **1** showed a similar colorimetric response to Hg^{2+} regardless of the species of counter anion. This indicates that **1** sensitively detected Hg^{2+} regardless of the species of counter anion.

To investigate the potential practical application of **1**, we tested whether **1** could detect Hg^{2+} in real groundwater from local mountain (Odae mountain) in Korea. As the analysis of mercury ions in this groundwater by cold vapor atomic absorption indicated that there was no Hg^{2+} in this groundwater, sample solutions containing Hg^{2+} were spiked into the groundwater at the concentrations ranged from 200 nM to 60 μM. As shown in UV-Vis titration experiments (Fig. S17), the addition of increasing concentration of Hg^{2+} induced the red shift (30 nm) of the maximum absorbance at 452 nm with decreasing of the absorbance intensity in real sample solution. The dissociation constant (K_d) of **1** for Hg^{2+} in real sample solution was calculated to be 3.9×10^{-10} M^2 (R^2 = 0.997) based on the UV/Vis titration result (Fig. S17). The K_d value for Hg^{2+} measured in groundwater was consistent well with that measured in aqueous buffer solution prepared by distilled water. We also measured the detection limit of **1** for Hg^{2+} in this groundwater. The detection limit of **1** for Hg^{2+} was determined to be 34.3 nM (R^2 = 0.987) in the groundwater (Fig. S18). These analytical data indicated that the dipeptidyl chemosensor **1** showed a sensitive response to Hg^{2+} ions in real groundwater and provided a simple method for the detection of Hg^{2+} in practical water samples.

The reversible sensing ability of **1** for Hg^{2+} ions was tested in aqueous solutions (Fig. S17). After adding Hg^{2+} to the solution of **1**, a red shift in the absorption was observed, indicating the formation of the complex between **1** and Hg^{2+}. And then the addition of EDTA as a chelating agent for Hg^{2+} ions resulted in the return of the metal free spectrum, which confirmed the reversible sensing ability of **1** for Hg^{2+} ions.

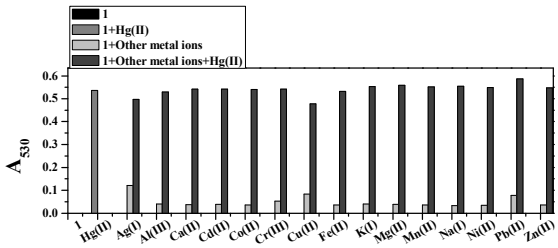


Fig. 5. UV-VIS absorbance of **1** (15 μM) in the presence of Hg^{2+} and other metal ions (60 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% CH_3CN

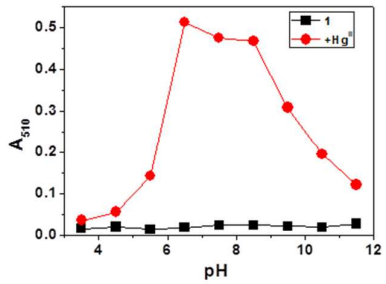


Fig. 6. UV/VIS absorbance spectra of **1** (15 μM) in different pH of 10 mM buffer solution in absence of these metal ions (■) and in presence of Hg^{2+} (●)

We proposed the binding mode of **1** with Hg^{2+} based on the results of pH titration experiment, ESI mass spectrometry, and UV titration experiment (Fig. 1). As shown in Fig. 6, **1** did not show a colorimetric response to Hg^{2+} in the acidic pH (< pH 4) but showed significant colorimetric responses to Hg^{2+} in neutral and basic pH. No response to Hg^{2+} in the acidic pH was due to the protonated amine group ($pK_a \cong 4$) of NBD fluorophore of **1** in acidic pH. This result confirmed that the amine group of **1** played a critical role in the binding with Hg^{2+}. The red shift in the absorption of **1** in the presence of Hg^{2+} revealed that the chelation of Hg^{2+} with **1** resulted in the increase of the conjugation of the NBD moiety. Thus, the

chelation of Hg^{2+} must induce the deprotonation of the NH group of **1**. The chelation of the amino group with Hg^{2+} and deprotonation were also confirmed by ESI mass spectrum (Fig. S12). The peak corresponding to $[M + Hg^{2+} - 3H^{+}]^{-}$ in negative ion mode also suggests that Hg^{2+} chelated the amine group of benzoxadizole moiety, resulting in the induction of the deprotonation process. The binding mode of **1** with Hg^{2+} was further investigated by NMR spectroscopy (Figure S20). ^{1}H NMR titration experiments were carried out in CD_3OD and DMSO-d^6 because this solvent system provided enough solubility for the complex between **1** and Hg^{2+} and **1** showed a colorimetric response to Hg^{2+} ions in this solvent system (Figure S21). When Hg^{2+} was added into the solution containing **1**, the downfield shifts in H(6) and H(5) corresponding to the protons of the benzoxadiazole moiety were observed. The downfield shifts of H(6) (Δ=0.18 ppm) and H(5) (Δ=0.16 ppm) may be attributed to the chelation of Hg^{2+} with the amine group of benzoxadizole moiety, resulting in the induction of the deprotonation process. The downfield shifts in H(3) and H(2) corresponding to the protons of α amino carbon and the methylene of -CH_2-S-S- suggested the chelation of Hg^{2+} with the amino and disulphide groups of **1**. Overall results proposed the binding mode of **1** with Hg^{2+}, as shown in Fig. 1.

Conclusions

1 showed sensitively colorimetric and fluorescent responses to Hg^{2+} and Ag^{+} among 16 metal ions in aqueous solutions. In aqueous solution containing NaCl, **1** showed exclusively selective colorimetric and fluorescent responses to Hg^{2+} among 16 metal ions. The colorimetric change of **1** was saturated by about 2 equiv. of Hg^{2+} and the sensitive detection of **1** for Hg^{2+} was not considerably interfered by other heavy metal ions. **1** showed nanomolar detection limit for Hg^{2+} (25.6 nM, R^2 = 0.994) in aqueous solutions. The binding mode study showed that the dipeptidyl chemosensor formed a 1:2 complex with Hg^{2+} and the disulphide bond of the sensor played a critical role in the selective binding of Hg^{2+} ions.

Acknowledgements

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