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Highly sensitive colorimetric detection of Hg^{II} and Cu^{II} in aqueous solution: From amino acids toward solid platforms

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A chemosensor (**NBD-H**) based on an amino acid with 7-nitro-2,1,3-benzoxadiazole detected Hg^{II} and Cu^{II} selectively among 15 metal ions in aqueous solutions by a colorimetric change. **NBD-H** sensitively differentiated Hg^{II} and Cu^{II} in aqueous solutions by a color change; a pink color to Hg^{II} and an orange color to Cu^{II}. **NBD-H** showed nanomolar detection limits for Hg^{II} (176 nM, R^2 = 0.996) and Cu^{II} (163 nM, R^2 = 0.996). The detection limit for Cu^{II} was much lower than the maximum allowable level of Cu^{II} in drinking water demanded by the U.S. EPA. The binding mode study showed that deprotonation of the NH group of **NBD-H** played a critical role in the binding and sensing of metal ions. **NBD-H** immobilized on PEG-PS resin maintained the potent binding affinity and sensing ability for the metal ions. The resin with **NBD-H** was recyclable for the detection of metal ions in 100% aqueous solutions.

Introduction

The detection of low levels of heavy and transition metal ions (HTM) contamination has become a significant issue due to the toxicity of metal ions to living organisms.[1] In particular, Hg^{II} ion is regarded as one of the most toxic metal ions among HTM.[1a] Although Cu^{II} plays an essential role in many biological processes in humans, the exposure of large amounts of Cu^{II} ions can cause liver or kidney damage and can generate reactive oxygen species with the potential to induce cellular damage.[1b]

In recent years, colorimetric chemosensors for HTM have attracted considerable attention due to their simple naked eye detection, inexpensive instrumentation, and its potential applications in the environment field.[2-4] Therefore, a range of colorimetric chemosensors for HTM have been reported.[2] As the accumulation of heavy metal ions in living organisms occurs mostly by water contamination,[1] ideal colorimetric chemosensors for HTM should operate properly in aqueous solutions. On the other hand, some colorimetric chemosensors require a high percentage of organic solvents for their operations in aqueous solutions and suffer from low sensitivity because of the low binding affinity for target metal ions in aqueous solutions.[2-4] In addition, most reported colorimetric detection for metal ions have used chemodosimeters (reactive probes) based mainly on rhodamine rather than chemosensors.[3,4] Therefore, new ideal colorimetric chemosensors will demand high water solubility, reversibility, high sensitivity to specific metal ions, and low detection limits.

Recently several research groups have reported fluorescent chemosensors for HTM based on the amino acids and peptides because these biomolecules showed high solubility in aqueous solutions, biological compatibility and potent binding affinity for specific metal ions.[5] On the other hand, to the best of the authors' knowledge, there is no report on the synthesis of colorimetric chemosensors based on amino acids for monitoring HTM. 7-nitro-2,1,3-benzoxadiazole (NBD), as a fluorophore, has been used for the *in vitro* and *in vivo* analysis of neurotransmitters based on amino acids using HPLC or capillary electrophoresis because of its interesting photophysical properties, such as visible light absorption, high extinction coefficients, high quantum yield, and high chemical stability.[6]

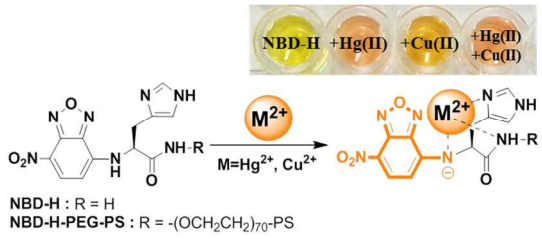


Fig. 1 Proposed binding mode and color change of **NBD-H** with the metal ions.

This study shows that NBD labelled histidine (**NBD-H**) detects Hg^{II} and Cu^{II} sensitively among the 15 tested metal ions in aqueous solution by a colorimetric response with a large red shift as well as

emission intensity change, as shown in Fig 1. Furthermore, the PEG resin conjugated with **NBD-H** showed a sensitive colorimetric response to Hg^{II} and Cu^{II} in aqueous solutions, suggesting that the amino acid-based chemosensor on the resin maintained the potent binding affinity and sensing ability for these metal ions in aqueous solutions.

Experimental

Materials and reagents

Fmoc-His(Trt)-OH, 1-hydroxybenzotriazole (HOBt) and Rink Amide MBHA resin (100~200 mesh, 0.5 mmol/g) were purchased from Bead Tech. Wang resin (100~200 mesh, 1.1 mmol/g) and Nova-Syn Tentagel resin (90 μm, 0.25 mmol/g) were purchased from NovaBioChem. N, N-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), tri-isopropylsilane (TIS), triethyl amine (TEA), diethyl ether, acetonitrile (ACN), and piperidine were supplied by Sigma Aldrich. 4-chloro-7-nitro-2,1,3-benzoxadiazole (4-ClNBD) and N, N-dimethylformamide (DMF) were obtained from Acros Organics. All perchlorate and chloride salts of metal ions were purchased from Sigma Aldrich and a metal ion stock solution was prepared in high purity de-ionized water.

Synthesis

NBD-H: NBD-H was synthesized in solid-phase synthesis using Fmoc chemistry (Scheme 1). Activated Fmoc-His(Trt)-OH (186 mg, 0.3 mmol) with DIC (47 ul, 0.3 mmol) and HOBt (40 mg, 0.3 mmol) was loaded to swollen Rink Amide MBHA resin (200 mg, 0.1 mmol) according to the reported procedure.[7] 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 deprotected amino group in the presence of triethylamine (83 μl, 0.6 mmol). After the coupling reaction was complete, the cleavage of **NBD-H** from the dried 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 the N_2, the crude product was precipitated by the addition of cold ether into the cleavage solution. The precipitated crude product was centrifuged, washed with ether, and lyophilized under vacuum. The crude product was purified further with semi-preparative HPLC using a water (0.1% TFA) / acetonitrile (0.1% TFA) gradient to provide **NBD-H** in an isolated yield of 75%. The product was characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, ESI-mass and FAB-HRMS data. **NBD-H:** Orange solid, m.p. 114 °C; ^1H NMR (400 MHz, [D_3]CAN, 25□) δ 8.40 (d, J=8.8 Hz, 1H), 8.36 (s, 1H), 7.89 (s, 1H), 7.32 (s, 1H), 7.20 (s, 1H), 6.2 (d, J=8.8 Hz, 2H), 4.95-4.85 (m, 1H), 3.5-3.3 (m, 1H); ^{13}C NMR (100 MHz, 50% [D_3]ACN/D_2O) δ 172.6, 144.3, 143.7, 137.6, 133.5, 128.1, 123.3, 117.5, 56.2, 26.6; ESI-Mass (m/z): $[M + H^+]^+$ calcd.: 318.09, obsd.: 317.98; FAB-HRMS Calcd: m/z 318.09 $[M+H^+]^+$, $C_{12}H_{11}N_7O_4$. Found: m/z 318.09 $[M+H^+]^+$, $C_{12}H_{11}N_7O_4$.

NBD-H-PEG-PS: NBD-H-OH (carboxylic form of **NBD-H**) was synthesized using the solid-phase synthesis using Fmoc chemistry (Scheme S1). Activated Fmoc-His(Trt)-OH (186mg, 0.3 mmol) with DMAP (11 mg, 0.02 mmol), DIC (47ul, 0.3 mmol) and HOBt (40mg, 0.3 mmol) was loaded into Wang resin (91mg, 0.1 mmol). After deprotecting the Fmoc group, the same procedure for the preparation of **NBD-H** was performed. The crude product was purified further with semi-preparative HPLC using a water (0.1% TFA) / acetonitrile (0.1% TFA) gradient to provide **NBD-H**-OH with an isolated yield of 70%. Purified **NBD-H**-OH (32 mg, 0.1 mmol) was activated by DIC (16 ul, 0.1 mmol) and HOBt (13 mg, 0.1 mmol). Activated **NBD-H**-OH was loaded onto tentagel resin (PEG-PS resin, 200 mg, 0.05 mmol) for 4 hours (ESI, Scheme S1†). The complete coupling was confirmed by a ninhydrin test and the resin was then washed 3 times with DMF and MeOH, and dried in a desiccator overnight. After weighing the **NBD-H-PEG-PS**, the resin was swollen in a 10 mM HEPES buffer solution (pH 7.4) for 1 hr. The swollen resin was transfer to the test cells for the detection of metal ions.

Absorbance and Fluorescence Measurements

A stock solution of **NBD-H** with a concentration of 1 mM was prepared in CH_3CN and stored in a cold and dark place. The concentration of **NBD-H** was confirmed by the absorbance at 478 nm for the NBD group (λ_{max} = 478 nm, ε = 18492 $cm^{-1}M^{-1}$). This stock solution was used for the absorbance and fluorescence experiments after the appropriate dilution. The absorbance and fluorescence titration was carried out using the above solutions at a pH of 7.4 using 10 mM HEPES buffer solution. The UV/Vis absorbance spectrum (300–650 nm) of a sample in a 10 mm path length quartz cuvette was measured in a 10 mM HEPES buffer solution at pH 7.4 using a Perkin-Elmer UV-Vis spectrophotometer (model Lambda 45). The emission spectrum (490–650 nm) of the sample was also measured in the same solution and cuvette using a Perkin-Elmer Fluorescence spectrometer (model LS 55). The absorbance and emission spectra of **NBD-H** in the presence of metal ions (Na^I, K^I, and Al^{III} as chloride anion and Ag^I, Ca^{II}, Cd^{II}, Co^{II}, Cu^{II}, Fe^{II}, Fe^{III}, Hg^{II}, Mg^{II}, Mn^{II}, Ni^{II} and Zn^{II} as perchlorate anion) were measured. The emission spectra were measured by excitation with 469 nm. The slit widths for excitation and emission were 10 and 8 nm, respectively.

Determination of Dissociation constant (K_d)

The dissociation constant was calculated based on the titration curve of **NBD-H** with metal ions. The dissociation constants were determined by a nonlinear least squares fitting of the data with the following equation, as referenced elsewhere.[8]

$$A = A_\infty \times \frac{([H] + [L] + K_d) - \sqrt{([H] + [L] + K_d)^2 - 4[H][L]}}{2[H]}$$

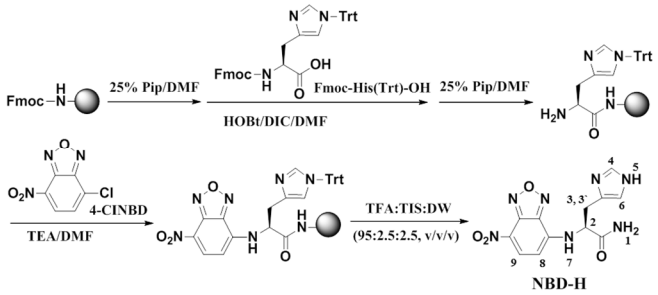
where A is the absorbance signal and A_∞ is the final absorbance signal, [H] and [L] is the total concentration of the host (H) and metal ion (L).

Determination of detection limit

To determine the S/N ratio, the absorbance of free **NBD-H** was measured ten times and the standard deviation of the blank measurements was determined. Three independent measurements of the absorbance were taken 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 **NBD-H**, and m is the slope between the absorbance at 464 nm vs. concentration.

Results and discussion

As histidine was reported to act as an important ligand in several metalloproteins,[9] NBD-labelled histidine (**NBD-H**) was synthesized readily in high yield (75%) by solid phase synthesis (Scheme. 1) The detailed procedure for the synthesis and characterization of **NBD-H** is described in the supporting information (ESI, Fig. S1-S3†).



Scheme 1. Solid phase synthesis of **NBD-H**.

NBD-H was dissolved well in aqueous solutions containing a small percentage of organic solvent. The UV-VIS spectra of **NBD-H** were measured in the presence of various metal ions (Ag^I, Al^{III}, Ca^{II}, Cd^{II}, Co^{II}, Cu^{II}, Fe^{II}, Fe^{III}, Hg^{II}, K^I, Mg^{II}, Mn^{II}, Na^I, Ni^{II} and Zn^{II}) in an aqueous buffer solution (10 mM HEPES, pH 7.4) containing 3% CH_3CN. As shown in Fig. 2, free **NBD-H** showed a strong absorption band at 478 nm (λ_{max} = 478 nm, ε = 18492 $cm^{-1}M^{-1}$), corresponding to a yellow color. Interestingly, **NBD-H** showed a sensitive colorimetric response to Hg^{II} and Cu^{II} among 15 metal ions; pink color to Hg^{II} and orange color to Cu^{II}. The addition of Ag^I induced a decrease in the absorbance and the disappearance of a yellow color due to precipitation (ESI, Fig. S4†).

UV-Vis titration experiments were carried out to obtain the binding affinity of **NBD-H** to Hg^{II} and Cu^{II} (Fig. 3). Upon the addition of Hg^{II}, the gradual red shift (12 nm) of the maximum absorbance band at 478 nm with decreasing intensity was observed and the change in the absorbance was complete by less than 2 equiv. of Hg^{II}. The addition of Cu^{II} induced a red shift (24 nm) of the maximum absorbance band with a small increase in intensity. Approximately 2 equiv. of Cu^{II} was required for a complete change in the absorbance. The clear isosbestic points in the titration of **NBD-H** with Hg^{II} and Cu^{II} suggests that **NBD-H** may prefer to form only one complex with Hg^{II} and Cu^{II}, respectively (Fig. 3).

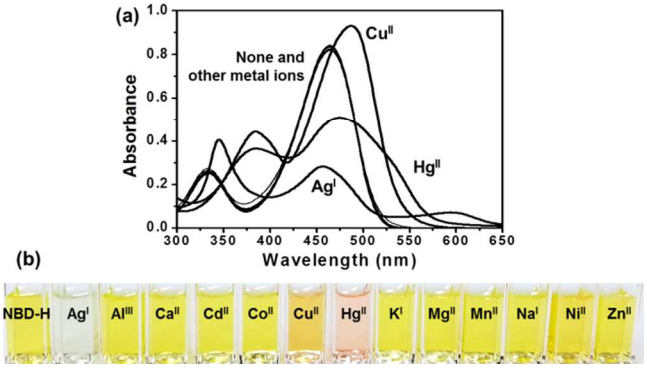


Fig. 2 (a) UV-VIS absorbance spectra (b) visible color change of **NBD-H** (30 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% CH_3CN in the presence of 2 equiv. of various metal ions.

The Job's plot of **NBD-H** with Hg^{II} and Cu^{II} showed that the maximum absorbance was observed at 0.5 mole fraction (ESI, Fig. S5†). This suggests that **NBD-H** may form a 1:1 complex with Hg^{II} and Cu^{II}, respectively. Assuming the formation of a 1:1 complex, the dissociation constant of **NBD-H** was calculated to be 3.13 μM (R^2 = 0.985) for Hg^{II} and 9.67 μM (R^2 = 0.990) for Cu^{II}, respectively, from the UV/VIS titration (ESI, Fig. S6†). As **NBD-H** exhibited a sensitive linear response to low levels of Hg^{II} and Cu^{II} in an aqueous solution (ESI, Fig. S7†) **NBD-H** showed nanomolar detection limits for Hg^{II} (176 nM, R^2 = 0.996) and Cu^{II} (163 nM, R^2 = 0.996) in aqueous solutions. The detection limit for Cu^{II} was much lower than the maximum allowable level (20 μM) of Cu^{II} in drinking water demanded by the EPA.[1c]

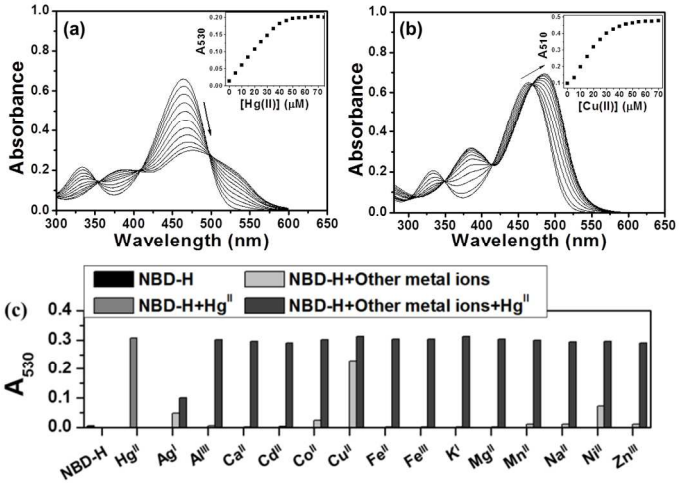


Fig. 3 UV-VIS absorbance of **NBD-H** (30 μM) in the presence of (a) Hg^{II}, (b) Cu^{II} (0, 5, 10, ..., 75 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 3% CH_3CN. (c) UV/VIS absorbance (530 nm) of NBD-H (30 μM) in the absence of metal ions (black), in the presence of Hg^{II} (gray), in the presence of other metal

ions (bright gray), and in the presence of other metal ions and Hg^{II} (dark gray). The concentration of metal ions was 60 μM.

Fluorescence titration experiments were carried out in aqueous solutions. **NBD-H** detected Hg^{II} and Cu^{II} from the turn off response (ESI, Fig. S8-S9†). The emission intensity change at 526 nm as a function of the metal concentration was fitted by a non-linear equation for the 1:1 complex model to calculate the dissociation constant. The dissociation constant was calculated to be 0.04 μM (R^2 = 0.922) for Cu^{II} and 2.89 μM (R^2 = 0.970) for Hg^{II}**.** The K_d value for Cu^{II} was lower than that measured using the absorbance spectra, whereas the K_d value for Hg^{II} was similar to that measured using the absorbance spectra, which can be explained by the more potent quenching effect of Cu^{II} than Hg^{II} for emission.[10]

The interference effect of other metal ions on the detecting ability of **NBD-H** for Hg^{II} was investigated because Hg^{II} is the most toxic metal ion among HTM (ESI, Fig. S10†). The Hg^{II}-dependent absorbance of **NBD-H** was not changed considerably by the presence of other metal ions except for Cu^{II} and Ag^{I}. The Hg^{II}-dependent absorbance at 530 nm increased slightly in the presence of Cu^{II} because Cu^{II} also induces an increase in absorbance. On the other hand, the chemosensor showed a pink color to Hg^{II} in the absence and presence of Cu^{II}, suggesting that **NBD-H** had a more sensitive response to Hg^{II} than Cu(II) and **NBD-H** could allow naked eye detection of Hg^{II} even in the presence of Cu(II). The Hg^{II}-dependent absorbance decreased in the presence of Ag^{I} possibly due to precipitation. To remove the interference effect of Ag^{I}, colorimetric response of **NBD-H** for Hg^{II} was measured in an aqueous solution containing NaCl (1 mM). In this case, the chemosensor could detect Hg^{II} properly without inference from Ag^{I} (ESI, Fig. S11†). The anion effect on the detection for Hg^{II} in aqueous solutions was investigated. The colorimetric response of **NBD-H** to $Hg(ClO_4)_2$, $HgCl_2$, $Hg(OAc)_2$, and $Hg(NO_3)_2$ were compared (ESI, Fig. S12†). **NBD-H** showed a similar colorimetric response to Hg(II) regardless of the species of counter anion.

ESI-Mass spectrometry and NMR spectroscopy were used to examine the binding stoichiometry and binding mode of **NBD-H** for the metal ions. First, the binding mode of **NBD-H** with Hg^{II} or Cu^{II} was investigated by mass spectrometry. After adding Hg^{II} and Cu^{II} into the solution of **NBD-H**, respectively, a new peak at m/z 516.05, corresponding to $[M + Hg^{2+} - 3H^{+}]^{-}$ was observed in the mass spectrum for negative ion mode and a new peak at m/z 380.06, corresponding to $[M + Cu^{2+} - H^{+}]^{+}$ was observed in the mass spectrum for positive ion mode (Fig. 4). This confirmed that **NBD-H** interacted strongly with the metal ions and formed a 1:1 complex with Hg^{II} and Cu^{II}, respectively.

The binding mode of **NBD-H** with Hg^{II} was investigated further by NMR spectroscopy (Fig. 5). ^1H NMR titration experiments were carried out in CD_3CN/D_2O because **NBD-H** showed a red shift to Hg^{II} in this solvent system. When the concentration of Hg^{II} increased, considerable upfield shifts in the protons corresponding to the benzoxadiazole moiety (H9 and H8) and imidazole moiety (H4 and H6) were observed. Small upfield shifts in the protons (H2 and H3) of **NBD-H** were also observed. This result suggested the chelation of Hg^{II} with the amino group of the NBD moiety and imidazole part of the chemosensor.

The pH titration experiment also provides additional information regarding the binding mode of **NBD-H** with Hg^{II} and Cu^{II} (ESI, Fig. S13†). In the acidic pH, **NBD-H** did not show colorimetric responses to Hg^{II} and Cu^{II}, but exhibited sensitive responses to these metal ions in neutral and basic pH. This also confirmed that the imidazole group (pK_a = 6) of **NBD-H** played a critical role in binding with these metal ions because the protonated imidazole group of **NBD-H** in acidic pH did not interact with the metal ions.

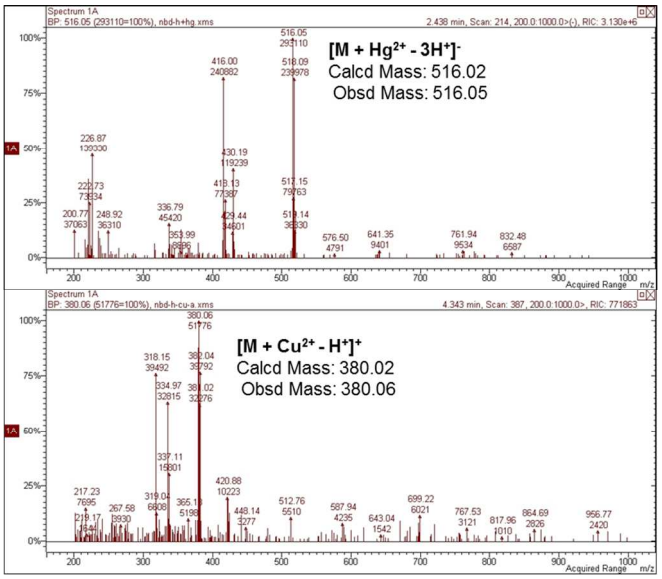


Fig. 4 ESI-MS spectrum of **NBD-H** (100 μM) in 50% CH_3CN/H_2O in presence of (a) Hg^{II} (10 equiv.) for negative ion mode and (b) Cu^{II} (10 equiv.) for positive ion mode.

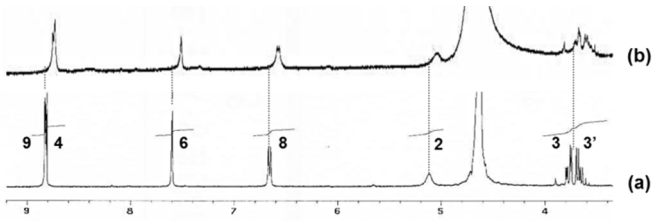


Fig. 5 ^1H NMR spectra of **NBD-H** (30 mM) in 50% CD_3CN /D_2O (v/v, 50:50) in the (a) absence and (b) presence of Hg^{II} (1 equiv.).

The binding mode of Hg^{II} to **NBD-H** was proposed by the organic spectroscopic data. The red shift in the absorption in the presence of Hg^{II} indicated that the chelation of Hg^{II} with **NBD-H** resulted in an increase in the conjugation of the NBD part. Considering the possible ligands for Hg^{II} and the red shift, NH may chelate Hg^{II} with deprotonation. The deprotonation of NH might induce an increase in the conjugation of the benzoxadiazole part, resulting in an up field shift of the aromatic protons (H8 and H9) and the aliphatic proton (H2) in

NMR titrations and a red shift of the UV/VIS titration. The deprotonated complex between **NBD-H** and Hg(II) was also confirmed by the ESI mass spectrum (Fig. 4). In negative ion mode, the mass corresponding to $[M + Hg^{2+} - 3H^{+}]^{-}$ was clearly observed. This result strongly suggests that the NH of the benzoxadiazole moiety might chelate Hg^{II} with deprotonation (Fig. 1).

The reversible sensing ability of **NBD-H** for Hg^{II} or Cu^{II} ions in aqueous solutions was tested (Fig. 6). After adding Hg^{II} or Cu^{II} to the **NBD-H** solution, a red shift in absorption was observed, indicating the formation of a complex between **NBD-H** and the target metal ion. The addition of EDTA as a chelating agent for the metal ions into the solution resulted in the return of the metal free spectrum, which confirmed the reversible sensing ability of **NBD-H**.

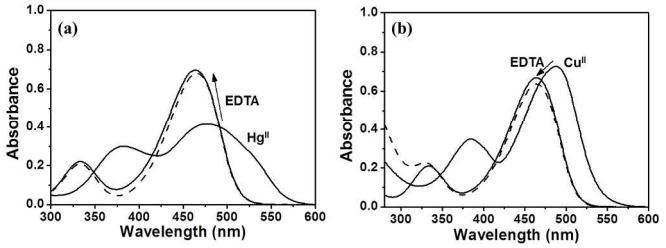


Fig. 6 UV-VIS absorbance spectra of **NBD-H** (30 μM) with (a) Hg^{II} and (b) Cu^{II} (5 equiv.) in the presence and absence of 5 equiv. of EDTA.

For further applications, **NBD-H** was immobilized on the resin (PEG-PS) and the sensing ability of the resin (**NBD-H**-PEG-PS) for metal ions was investigated.[11] The PEG-PS resin was selected due to the high swelling property in water and the easy conjugation of **NBD-H** into the resin. The resin showed a sensitive colorimetric response for Hg^{II} and Cu^{II} in a 100% aqueous solution (Fig. 7 and ESI, Fig. S14†).

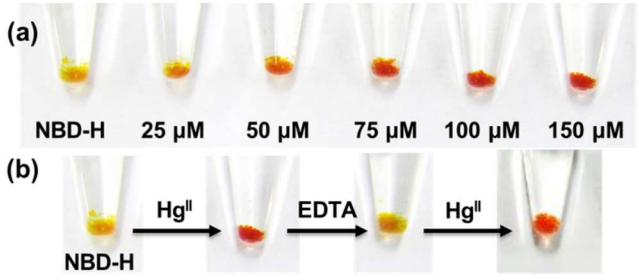


Fig. 7 (a) Color change in **NBD-H**-PEG-PS (100 μM) with increasing concentration of Hg^{II} in a 100% aqueous solution (10 mM HEPES, pH 7.4) and (b) color change of regenerated **NBD-H**-PEG-PS in the presence of Hg^{II} (150 μM).

Upon the addition of the solution containing micro-molar concentrations of Hg^{II}, the color of the resin was changed from yellow to red. The red color on the resin depended on the concentration of Hg^{II} in the solution. After filtering the resin followed by a treatment with EDTA, the color of the resin returned to yellow (Figure 7b), indicating that the resin was regenerated as metal free resin. Upon the addition of a mercury solution, the color of the regenerated resin changed to red. This suggests that the regenerated resin also showed sensitive sensing ability for Hg^{II} ions.

Conclusions

A **NBD** labelled amino acid (**NBD-H**) sensitively and selectively detected Hg^{II} and Cu^{II} among metal ions in aqueous solution from the colorimetric and fluorescent changes. Approximately 2 equiv. of the metal ions were sufficient to saturate the colorimetric change in the chemosensor. **NBD-H** showed nanomolar detection limits for Hg^{II} (176 nM, R^2 = 0.996) and Cu^{II} (163 nM, R^2 = 0.996). The detection limit for Cu^{II} was much lower than the maximum allowable level (20 μM) of Cu^{II} in drinking water demanded by the EPA. The binding mode of **NBD-H** showed that the NH of the benzoxadiazole moiety of the chemosensor chelated Hg^{II} with a deprotonation, resulting in a red shift of the absorption band. The resin conjugated with **NBD-H** showed a sensitive colorimetric response to Hg^{II} ions in an aqueous solution, suggesting the solid supports conjugated with the amino acid-based chemosensor can be used as a sensing platform for heavy metal ions in aqueous solutions.

Acknowledgements

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis, additional spectroscopic data of compounds, and additional experiment result. See DOI: 10.1039/b000000x/

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Graphical Abstract

Highly sensitive colorimetric detection of Hg (II) and Cu(II) in aqueous solutions: From amino acids toward sold platforms

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A chemosensor (**NBD-H**) based on an amino acid with 7-nitro-2,1,3-benzoxadiazole selectively detected Hg^{II} and Cu^{II} among 15 metal ions in aqueous solutions by colorimetric change and fluorescent change.