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PAPER

Selective and sensitive ratiometric detection of Hg^{2+} in 100% aqueous solution with triazole-based dansyl probe†

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An amino acid-based fluorescent sensor (**3**) bearing a triazole group was synthesized by solid phase synthesis using click chemistry. **3** showed highly sensitive ratiometric response to Hg^{2+} in 100% aqueous solution. The dissociation constant for Hg^{2+} in aqueous solution was calculated to be 672 nM. **3** exhibited selective response to Hg^{2+} among 13 tested metal ions and the sensitive detection of Hg^{2+} by **3** was not interfered by other heavy metal ions, Group I and II metal ions. ^{1}H NMR analysis revealed that two sulfonamide and triazole groups of **3** played a critical role in the interactions with Hg^{2+}. **3** penetrated live cells and detected intracellular Hg^{2+}. **3** allowed selective and sensitive ratiometric detection of Hg^{2+} in live cells as well as in 100% aqueous solution.

Introduction

Mercury has been regarded as one of the most toxic metals and it exists in a variety of different forms. The mercury(II) ion (Hg^{2+}), the most common oxidation form and methylmercury, the metabolized form of Hg^{2+} by aquatic microbes have caused serious problems for human health and ecology.[1] Therefore, rapid and efficient detection of Hg^{2+} is essential for environmental monitoring of waters and evaluating the safety of aquatic foods. As fluorescence provides a powerful way for detecting metal ions because of its low detection limit and simple instrumentation, considerable efforts have been devoted to design fluorescent chemical sensors for detecting Hg^{2+}.[2–4] There are some successful achievements in fluorescent chemosensors for Hg^{2+}, however most of the reported Hg^{2+} sensors display at least one of the shortcomings including low sensitivity and low selectivity.[2–4] In particular, most of them suffer from interference of other metal ions such as Ag^+, Cu^{2+}, and Cd^{2+}, due to the similar size or softness of these metal ions to Hg^{2+}. As Hg^{2+} quenches well the emission of many fluorophores, turn on or ratiometric response has been proved to be effective because the quenching process is not selective and also occurs in the presence of other external factors.

In recent years, considerable efforts have been made to design fluorescent sensors that detect Hg^{2+} by ratiometric response because ratiometric sensing makes it possible to measure the analytes more accurately with minimization of background signal.[5] However, only a few ratiometric fluorescent sensors for Hg^{2+} have been reported.[6] As exposure of humans and fish to Hg^{2+} occurs mainly through water contamination, a new fluorescent sensor for Hg^{2+} will demand a sensitive response to Hg^{2+} in aqueous solution. However, most ratiometric fluorescent sensors for Hg^{2+} have been studied in organic solvents or organic/aqueous mixtures,[6d–o] limiting their practical application. In addition, to the best of our knowledge, all ratiometric sensors except one have not been demonstrated to detect intracellular Hg^{2+} in live cells.[6h] Therefore, it is highly challenging for the synthesis of new ratiometric sensors that detect Hg^{2+} in live cells as well as Hg^{2+} in 100% aqueous solution.

The majority of fluorescent chemical sensors are comprised of a receptor part and a fluorophore part. Recently, click reactions were used to synthesize fluorescent sensors for metal ions because the resulting 1,2,3 triazole group has served as an efficient covalent linker as well as a binding site for specific metal ions.[7,8] For example, a cyclam-based fluorescent sensor bearing 1,2,3 triazole formed by click reaction showed complete turn off response to Cu^{2+} among various metal ions.[8a] Triazole-based thiacalix[4]aren showed response to Ag^+, Hg^{2+}, Pb^{2+} and Cu^{2+} over other metal ions in organic solvent.[8b] However, most triazole-based fluorescent sensors did not operate well in 100% aqueous solution due to their low solubility in water and suffered from interference effects of other heavy metal ions. In recent years, several research groups including us have reported fluorescent sensors based on amino acids because amino acids were highly water soluble and acted as a ligand to specific metal ions.[9] We expect that amino acid based sensors containing triazole groups can be easily synthesized using solid phase synthesis and click reaction and the sensors containing triazole groups may

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† Electronic supplementary information (ESI) available: Experimental procedure and characterization data of compound **3**. UV absorbance, HPLC chromatogram, ESI mass spectra, ^{1}H NMR and ^{13}C NMR spectra, UV absorbance in the presence of Hg^{2+}, Job plot, titration curve with Hg^{2+}, and fluorescence spectra at different pH. See DOI: 10.1039/c2jm15664d

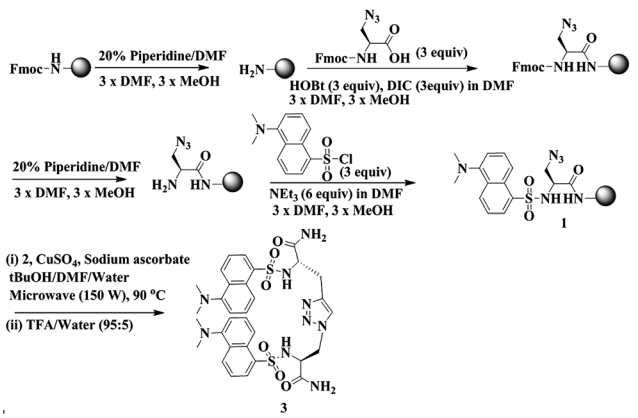
detect specific heavy metal ions in aqueous solution. In addition, the sensors can be conjugated into various materials such as calixarenes, cyclodextrins, and polymers by using click chemistry or covalent bonding of the C-terminal of amino acids in future applications. However, there is no report about amino acid based sensors containing triazole group for sensing heavy metal ions.

Herein, we report a novel fluorescent sensor (**3**) of which two dansyl labelled amino acids are conjugated by a triazole group acting as a linker as well as a binding site for heavy metal ions (Scheme 1). Dansyl was used as a fluorophore for the construction of this sensor because the dansyl fluorophore is sensitive to micro-environmental change by an internal charge transfer (ICT) mechanism and the dansyl fluorophore can be easily introduced at the N-terminal of amino acids in solid phase synthesis.[9a–e,10] We synthesized the amino-acid based fluorescent sensor (**3**) bearing a triazole group by solid phase synthesis and click reaction in high overall yield (70%) and investigated the response of **3** to metal ions in 100% aqueous solution. Interestingly, the compound **3** showed selective response to Hg^{2+} among various metal ions in 100% aqueous solution. Furthermore, **3** detected Hg^{2+} in aqueous solution by ratiometric response and the ratiometric response to Hg^{2+} was not interfered by competing metal ions such as Cd^{2+}, Ag^{+}, and Cu^{2+}. Most importantly, **3** penetrated live HeLa cells and detected intracellular Hg^{2+} by ratiometric response. Compound **3**, easily synthesized by solid phase synthesis and click reaction, displays desirable properties for sensors such as great selectivity and sensitivity for Hg^{2+}, good solubility in aqueous solution, easy synthesis, and no interference of competing metal ions.

Experimental

Reagents

N,*N*-diisopropylcarbodiimide, 1-hydroxybenzotriazole, and Rink Amide MBHA resin were purchased from Advanced Chem. Tech. Trifluoroacetic acid (TFA), dansyl chloride, triethylamine, *N*,*N* dimethylformamide (DMF) and piperidine were purchased from Aldrich. Fmoc-L-Dap(N3)-OH was purchased from IRIS Biotech GmbH and Fmoc-Pra-OH was purchased from Bachem.



Scheme 1 Synthesis of **3** by using solid phase synthesis and click reaction.

Solid phase synthesis of Compound 3

Compound **3** was synthesized by solid phase synthesis with Fmoc chemistry and click chemistry.[11,12] Fmoc protected L-Pra-OH and L-Dap(N3)-OH were separately assembled on Rink Amide MBHA resin (ESI†, Scheme S1). After deprotection of the Fmoc group, dansyl chloride was coupled by the following procedure. To the resin each of amino acid (200 mg, 0.1 mmol), dansyl chloride (80 mg, 0.3 mmol, 3 equiv) in DMF (3 ml) and triethylamine (80 μl, 0.6 mmol, 6 equiv.) were added and the resulting solution was stirred for 3 h at room temperature. After washing of the resin, cleavage of the resin bound compound **2** was achieved by treatment with a mixture (3 ml) of TFA:H_2O (95 : 5, v/v) at room temperature for 3 h. The click reaction was performed by the following procedure. The crude product of **2** (1 equiv, 34.51 mg), $CuSO_4$ (0.5 equiv, 12.48 mg), and sodium ascorbate (1 equiv, 19.81 mg) dissolved in DMF/t-butanol/water (4.5 : 4.5 : 1, v/v/v) were mixed with resin bound compound **1** and then the solution was heated for 10 min at 90 °C with microwave irradiation (150 W). Cleavage of the resin bound compound **3** was achieved by treatment with a mixture (3 ml) of TFA:H_2O (95 : 5, v/v) at room temperature for 3 h. After removing TFA from the solution by N_2 stream, addition of diethyl ether at −20 °C and centrifugation at 3,000 rpm for 10 min provided **3** in overall yield of 70%. The product was further purified by HPLC with a Vydac C_{18} column using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. The successful synthesis was confirmed by ESI mass spectrometry (platform II, micromass, Manchester, UK) and its homogeneity (>98%) was confirmed by reversed phase analytical HPLC with C_{18} column: The retention time of compound **3** is 29 min. The products were characterized by ^{1}H and ^{13}C NMR and ESI-mass data. The melting point, ^{1}H-NMR, ^{13}C-NMR and IR spectra of **3** are given below.

Yellowish solid, m.p. 130–135 °C, IR (KBr): 3398, 3294, 3010, 2820, 1685, 1316 cm^{-1}. NMR (400 MHz, D_2O/CD_3CN) δ 8.462–8.42(m, 2H), 8.38 (d, $J = 8.8$ Hz, 1H), 8.32(d, $J = 7.3$ Hz, 1H), 8.26 (d, $J = 7.3$ Hz, 1H), 8.09 (dd, $J = 7.6$, 1.8 Hz, 2H), 4.21(dd, $J = 10.255$, 4.4, 1H), 4.02 (dd, $J = 14.3$, 4.0, 1H), 4.11 (dd, $J = 14.2$,10.2 Hz, 1H), 3.81 (dd, $J = 10.9$, 4.4 Hz, 1H), 3.52 (s, 12H), 2.45 (dd, $J = 15.0$, 10.9), 2.37 (dd, $J = 15.0$, 4.4 Hz, 1H); ^{13}C NMR (100 MHz, CD_3CN) δ 173.547, 170.909, 148.889, 142.347, 136.161, 135.896, 130.862, 130.756, 130.453, 130.165, 130.059, 129.991, 129.513, 129.407, 129.119, 128.884, 125.367, 125.283, 125.003, 122.372, 122.153, 118.385, 117.597, 117.506, 57.356, 52.522, 51.628, 46.372, 29.180; ESI-Mass (*m*/*z*): $[M + H^+]^+$ calcd. for $C_{32}H_{37}N_9O_6S_2$: 708.23, obsd: 708.23.

General fluorescence measurements

A stock solution of compound **3** at the concentration of 1.12×10^{-3} M was prepared in 0.2% TFA containing distilled water and stored in a cold and dark place. This stock solution was used for all fluorescence measurements after appropriate dilution. All fluorescence measurements were carried out in 100% aqueous solution containing 10 mM HEPES buffer at 7.0 pH. The fluorescence emission spectrum of compound **3** in a 10 mm path length quartz cuvette was measured using a Perkin- Elmer luminescence spectrometer (Model LS 55). Emission spectra of

the sensor in the presence of various metal ions (Hg(II), Ca(II), Cd(II), Co(II), Pb(II), Ag(I), Mg(II), Cu(II), Mn(II), Ni(II) and Zn(II) as perchlorate anion and Na(I), Al(III) and K(I) as chloride anion) were measured by excitation with 330 nm. The slit size for excitation and emission was 10 and 5, respectively. The concentration of the probe was confirmed by UV absorbance at 330 nm for the dansyl group.

Determination of the dissociation constant

The dissociation constant was calculated based on the titration curve of the probe with Hg^{2+}. The fluorescence signal, F, is related to the equilibrium concentration of the complex (HL) between Host (H) and metal ion (L) by the following expression:[13]

$$[HL] = 0.5 \times [K_D + L_T + H_T - \{(-K_D - L_T - H_T)^2 - 4\,L_T\,H_T\}^{1/2}].$$

Fluorescent images of Hg^{2+} in HeLa cells with 3

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All cells were supplemented with an antibiotic antimycotic solution (100 units ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, and 0.25 mg ml^{-1} amphotericin B) and grown at 37 °C in standard cell culture conditions (5% CO_2, 95% humidity). Cell imaging experiments were performed with a LSM 510 META confocal laser-scanning fluorescent microscope (ZEISS, German) with 40× objective lens. HeLa cells were attached to the plate 24 h before study. Cells were first loaded with 30 μM compound **3** in DMEM (containing 2% DMSO) at 37 °C for 30 min, washed three times with 20 mM HEPES buffer solution (155 mM NaCl, pH 7.0) to remove the free compound **3**. The weak fluorescent intensity of the cells was confirmed and then the cells were further incubated with 5 equiv of $Hg(ClO_4)_2$ for 30 min in 20 mM HEPES (pH 7.0) containing 150 mM NaCl.

Results and discussion

Synthesis of 3 in solid phase synthesis using click chemistry

Compound **2** was readily synthesized by solid phase synthesis as follows (Scheme S1†). Fmoc-L-Pra-OH was assembled on resin. After the Fmoc group was deprotected in basic conditions, the dansyl fluorophore was conjugated into the amino acid on the resin. After cleavage of **2** from the resin, compound **2** was precipitated by adding diethyl ether. Compound **2** was used in the next reaction without further purification. Fmoc protected amino-3-azidopropanoic acid (Fmoc-L-Dap(N_3)-OH, **1**) was assembled on the resin. **3** was synthesized by solid phase synthesis using a click reaction between the resin bound azide **1** and alkyne **2**.[11,12] (Scheme 1). After the cleavage of **3** by TFA solution, ether precipitation provided **3** in overall yield of 70%. **3** was further purified by semi-preparative HPLC with a C_{18} column. The successful synthesis and high purity of **3** (>98%) were confirmed by analytical HPLC with a C_{18} column and ESI mass spectrometer (Fig. S1†).

Fluorescence emission response of 3 to various metal ions

As the mM concentration of **3** was dissolved well in distilled water in acidic conditions, all photochemical experiments using μM concentration of **3** were carried out in 100% aqueous solution without any co-solvent. Fig. 1 shows the fluorescence response of **3** to various metal ions (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, as perchlorate anion and Na^{+}, Al^{3+}, K^{+}, as chloride anion) in 100% aqueous solution at neutral pH by excitation with 330 nm. **3** exhibited a ratiometric response to Hg^{2+} among various tested metal ions in this condition, whereas no significant response was observed for any of the other metals tested except for Cu^{2+}. The emission intensity was slightly decreased by Cu^{2+}, however the previous sensors bearing triazole groups commonly showed a considerable response to Cu^{2+} mainly due to the interaction between triazole and Cu^{2+}.[7(a), 8]

Fig. 2 shows the fluorescent response of **3** to the amount of Hg^{2+} in 10 mM HEPES buffer solution at neutral pH. Upon the addition of increasing concentration of Hg^{2+}, a considerable blue-shift of the emission maximum from 530 nm to 503 nm with a decrease of the emission maximum was observed.

The ratiometric changes at 475 nm and 533 nm as a function of Hg^{2+} concentration were plotted. The ratiometric changes of **3** (5 μM) are proportional to the concentration of Hg^{2+} (0–2 equiv). About 2 equiv of Hg^{2+} was enough for the saturation of the emission intensity change, suggesting that **3** is highly sensitive to Hg^{2+} in aqueous solution. Even though the ratiometric changes induced by Hg^{2+} are not great, **3** shows selective ratiometric response to Hg^{2+} in 100% aqueous solutions, whereas most of the ratiometric sensors for Hg^{2+} required organic solvents for ratiometric response.[6d–o]

A Job's plot which exhibits a maximum at a 0.5 mole fraction, indicates that **3** forms a 1 : 1 complex with Hg^{2+} (Fig. S2†). The maximum intensity changes of **3** correlated well with the concentration of Hg^{2+} (Fig. S3†). Assuming the formation of a 1 : 1 complex, the dissociation constant of **3** with Hg^{2+} was calculated to be 6.72×10^{-7} M ($R^2 = 0.93$) by the non-linear curve fitting procedure.[13] The dissociation constant indicated that **3** has potent binding affinity to Hg^{2+} in 100% aqueous solution. The sensitivity of **3** for Hg^{2+} was calculated on the basis of the linear relationships between the maximum emission intensity at 530 and the concentration of Hg^{2+}. The sensor has a detection limit of 96 nM, based on $3\sigma_{bi}/m$, where σ_{bi} is the

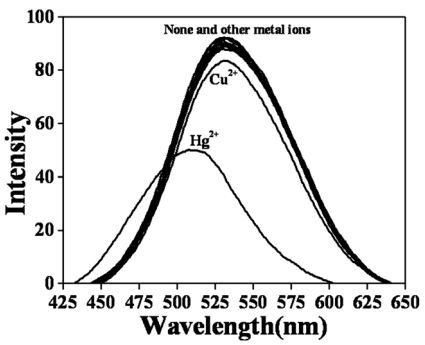


Fig. 1 Fluorescence spectra of **3** (5 μM) in 10 mM HEPES buffer solution (pH 7.0) in the presence of various metal ions (2 equiv) except Na^{+}, K^{+}, Mg^{2+} and Ca^{2+},which were 2000 equiv (λ_{ex} = 330 nm, slit = 10/5 nm).

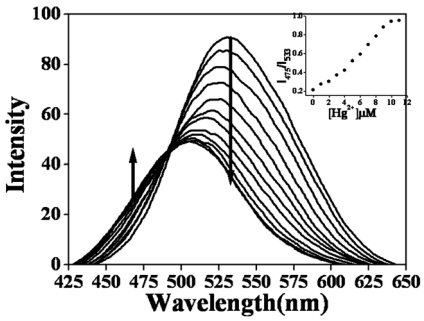


Fig. 2 Fluorescence titration spectra of **3** (5 μM) by adding Hg^{2+} (0, 1, 2, 3, 4,… 10 μM) in 10 mM HEPES buffer solution at pH 7.0. ($\lambda_{ex} = 330$ nm, slit = 10/5 nm).

standard deviation of the blank measurements, and *m* is the slope of the intensity *versus* the sample concentration plot (Fig. S4†). This confirms that **3** can be used to detect qualitatively low levels of environmental or biological contamination of Hg^{2+}. The fluorescence quantum yield (Φ_F) of **3** was determined in HEPES buffer solution in the absence of Hg^{2+} and in the presence of Hg^{2+} with reference to an anthracene standard.[14] A quantum yield ($\Phi_F = 0.15$) was observed for **3** in the absence of Hg^{2+} and upon addition of 2 equiv of Hg^{2+}, the quantum yield decreased to 0.044.

Fluorescence study in the presence of other metal ions and at different pH

To investigate the interference effect of other metal ions on the detection ability of **3** for Hg^{2+}, the fluorescence response of **3** to Hg^{2+} was measured in the presence of other metal ions (Fig. 3). Interestingly, the ratiometric response of **3** to Hg^{2+} was not affected by the other heavy metal ions including Cd^{2+} and Ag^{+}. Furthermore, **3** detects Hg^{2+} by ratiometric response without any interference from 10 mM of Group I and Group II metal ions such as Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}. Most of the reported ratiometric sensors for Hg^{2+} displayed cross-sensitivities toward other heavy metal ions such as Ag^{+}, Cd^{2+}, and Cu^{2+}.[6]

We investigated the influence of pH on the response of **3** to Hg^{2+} (Fig S5†). At pH < 4, free **3** itself showed very weak emission intensity and **3** exhibited little response to Hg^{2+} because the protonated dimethylamino group ($pK_a \cong 4$) may prevent the charge transfer from the dimethylamino group to the naphthalene moiety. At pH = 4.5, free **3** showed higher emission intensity than that measured at pH < 4 and **3** showed ratiometric response to Hg^{2+} at this pH condition. Until the pH of the solution reached 7.4, **3** showed ratiometric response to Hg^{2+}. As the pH increased from 8.5 to 11.5, the emission intensity of free **3** increased. This may be due to the deprotonation of the sulfonamide group ($pK_a \cong 10$) of **3**. However, unexpectedly, **3** showed turn off response to Hg^{2+} in this pH range. The overall results indicated that **3** showed sensitive ratiometric response to Hg^{2+} in mild acid and neutral pH (4.5–7.4). **3** may be suitable for monitoring Hg^{2+} in live cells at physiological pH.

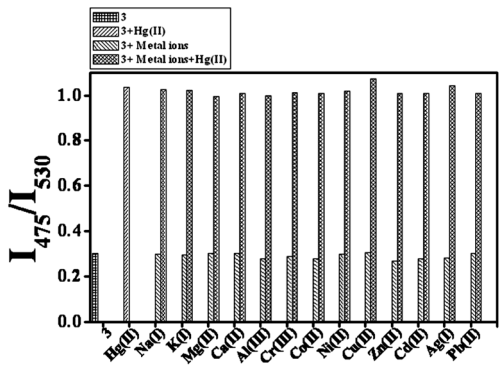


Fig. 3 Fluorescence response of **3** (5 μM) in the presence of additional metal ions (2 equiv) and Hg(II) (2 equiv) in 10 mM HEPES buffer solution at pH 7.0. Group I and Group II metal ions (10 mM) were used.

Binding mode of 3 with Hg^{2+}

The binding mode of **3** with Hg^{2+} was investigated by ESI mass spectrometry, NMR spectroscopy, and UV-vis absorption. The interaction between **3** and Hg^{2+} was investigated by using ESI mass spectrometry (Fig. 4). When 2 equiv of Hg^{2+} was added into the solution containing **3**, the new peak at 908.19 (*m*/*z*) corresponding to $[3 + Hg^{2+} - H^{+}]^{+}$ appeared with the peak at 708.30 (*m*/*z*) corresponding to $[3 + H^{+}]^{+}$. The result confirms that **3** directly interacts with Hg^{2+} and forms a 1 : 1 complex with Hg^{2+}.

^{1}H NMR studies provided the binding mode of **3** with Hg^{2+} (Fig. 5). As about a 10 mM concentration of **3** did not dissolve well in D_2O, ^{1}H NMR spectra of **3** upon addition of various concentrations of Hg^{2+} were measured in D_2O/CD_3CN (4 : 1, v/v). We confirmed that **3** showed decreased emission intensity with 10 nm blue shift of maximum intensity in the presence of Hg^{2+} in H_2O/CH_3CN (4 : 1, v/v).

Comparing the spectra of **3** in the presence and absence of Hg^{2+}, a downfield shift is apparent in aromatic and triazole proton peaks due to the deshielding effect of Hg^{2+}. This shift is most pronounced in H5 of the triazole and H15 and H22 of the dansyl moiety. The large downfield shift and disappearance of H5 of triazole is related to the strong binding of the triazole moiety to Hg^{2+}. Coordination between Hg^{2+} and the sulfonamide group may induce significant downfield shifts of H15 and H22 in the dansyl moiety by 0.60 and 0.59 ppm, respectively. A little shift of dimethyl protons in the dansyl moiety also suggests direct interactions between Hg^{2+} and the sulfonamide groups of the dansyl fluorophores (Fig. S6†). The NMR titration study revealed that **3** strongly interacted with Hg^{2+} through the binding

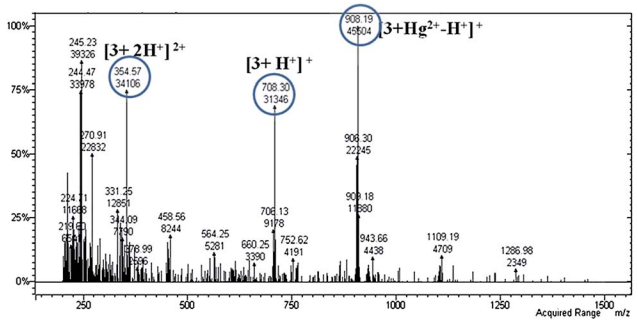


Fig. 4 ESI mass spectrum of **3** in the presence of Hg^{2+} (2 equiv) in CH_3CN/H_2O (1 : 1, v/v).

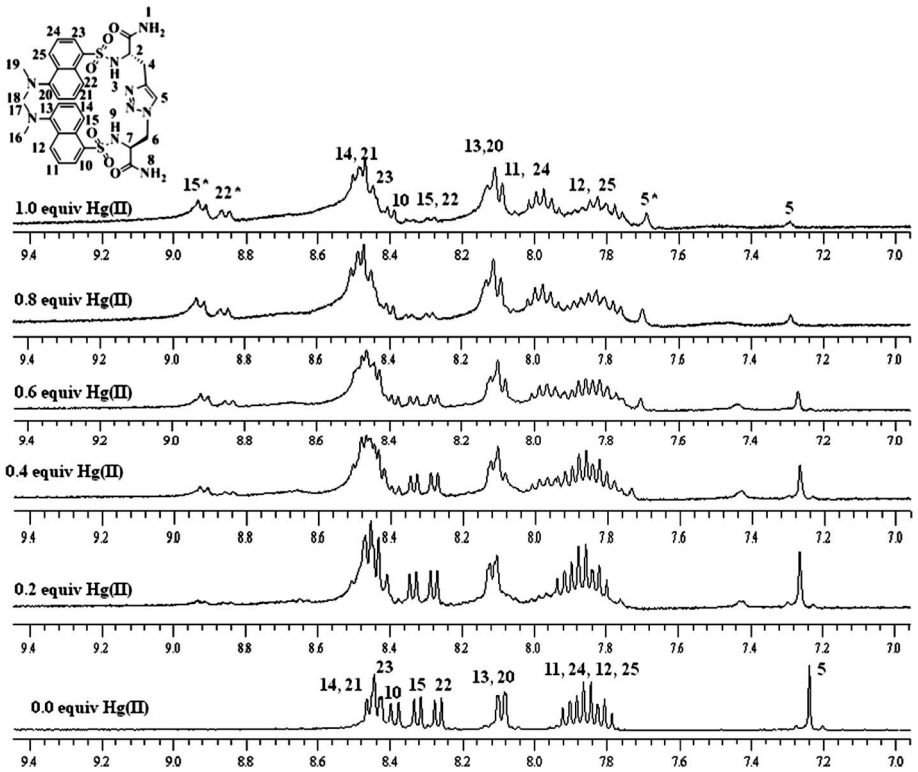


Fig. 5 Partial ^{1}H NMR spectra of **3** in the presence of Hg^{2+} in D_2O/CD_3CN (4 : 1, v/v).

of triazole and two sulfonamide groups of dansyl moieties to Hg^{2+}.

The binding mode of **3** with Hg^{2+} was also confirmed by UV-vis absorption. Upon addition of Hg^{2+}, the absorbance at 330 nm gradually red-shifted and simultaneously, the absorbance increased (Fig. S7†). This result suggests that the complex formation between Hg^{2+} and **3** has an influence on the sulfonamide group of the dansyl moiety, resulting in the increase of electron density of the napthyl chromophore.

Sensing mercury ions in live cells

As **3** displays sensitive ratiometric response to Hg^{2+} in aqueous solution at neutral pH, we investigate whether **3** can detect intracellular Hg^{2+} in live cells. The fluorescent images of HeLa cells incubated with **3** (30 μM) were monitored by confocal microscopy (Fig. 6). **3** was incubated with HeLa cells for 30 min at 37 °C, and then the HeLa cells were washed with PBS. The fluorescent change of the cells was monitored by confocal microscopy. The light blue colour image of the cells indicates that **3** penetrated HeLa cells in this condition. After addition of Hg^{2+} into **3** in the loaded cells, the fluorescent images of the cells were monitored by confocal microscopy. A considerable colour change of the cells from deep sky blue to blue colour was observed. This result demonstrates that **3** penetrated into live cells and detected intracellular Hg^{2+} by ratiometric response.

Conclusion

We reported a new fluorescent amino acid based sensor bearing a triazole group synthesized by solid phase synthesis and click reaction. The sensor detected Hg^{2+} by ratiometric response in 100% aqueous solutions. In addition, the sensor allowed a sensitive detection of Hg^{2+} even in the presence of other heavy metal ions and Group I and II metal ions. The sensor penetrated HeLa cells and detected intracellular Hg^{2+} in live cells. Considering the desirable properties for the sensor and easy synthesis,

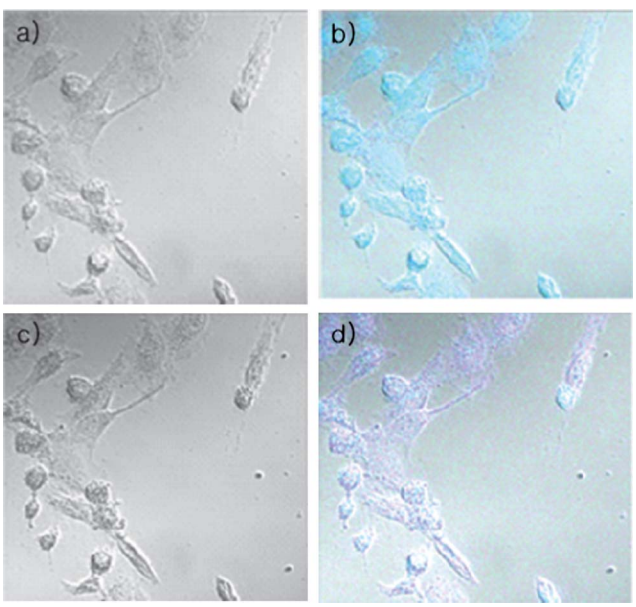


Fig. 6 Bright field and fluorescent and bright field images of HeLa cells with **3** (30 μM) (a, b), and **3** loaded HeLa cells incubated with Hg^{2+} (5 equiv) (c, d).

the sensor can be used to monitor low level Hg^{2+} in environmental or biological samples.

Acknowledgements

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