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Highly selectively monitoring heavy and transition metal ions by a fluorescent sensor based on dipeptide

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ABSTRACT

Fluorescent sensor (**DMH**) based on dipeptide was efficiently synthesized in solid phase synthesis. The dipeptide sensor shows sensitive response to Ag(I), Hg(II), and Cu(II) among 14 metal ions in 100% aqueous solution. The fluorescent sensor differentiates three heavy metal ions by response type; turn on response to Ag(I), ratiometric response to Hg(II), and turn off detection of Cu(II). The detection limits of the sensor for Ag(I) and Cu(II) were much lower than the EPA's drinking water maximum contaminant levels (MCL). Specially, **DMH** penetrated live cells and detected intracellular Ag^+ by turn on response. We described the fluorescent change, binding affinity, detection limit for the metal ions. The study of a heavy metal-responsive sensor based on dipeptide demonstrates its potential utility in the environment field.

1. Introduction

Detection and quantification of a low contamination of heavy and transition metal ions (HTM) in waters have become significant due to the toxicity of these metal ions to living organisms and humans. Specially, the use of silver and silver compounds has increased in electrical industry. Recently, bioaccumulation and potential toxicity of Ag(I) to fishes, invertebrates, and bacteria in waters have been reported [1–5]. Hg(II) has been regarded as the most toxic metal ions among HTM [6,7]. Thus, the development of fluorescence chemical sensors for Ag(I) and Hg(II) ions has received attention because fluorescence is a most powerful optical way for detecting low concentration of metal ions in waters. However, most of fluorescent chemical sensors for Ag(I) and Hg(II) ions displayed one of the following drawbacks such as low sensitivity, low selectivity, turn off response, or low water solubility [8–18]. Since Ag(I) and Hg(II) ions induced quenching of fluorescence emission intensity [19,20], chemical sensors that detect them by turn on response or ratiometric response are highly demanded. Furthermore, synthesis of fluorescent chemical sensors that differentiate Ag(I) and Hg(II) ions is highly challenging because both ions have similar size and belong to soft ions.

The receptor part of fluorescent chemical sensors mainly decides which kind of analysts can be detected. The selectivity and sensitivity of chemical sensors are mostly determined by recognition ability of the receptor part. The receptor part also contributes to converting a recognition event into a fluorescent signal. Generally, macrocyclic compounds such as crown ether and calixarene have been used as a receptor part in various fluorescent chemical sensors [14,15,19]. However, macrocyclic compounds are usually available only through tedious syntheses with low yield and frequently show poor solubility in 100% aqueous solution. Thus, we focus on dipeptide as a receptor because amino acid and peptide are highly water soluble and environmentally compatible and amino acid and peptide can be easily conjugated into fluorophores in solid phase synthesis with high yield.

Several research groups including us reported amino acid based fluorescent sensors for HTMs [21–25]. In comparison to the chemical sensors, amino acid based sensors were highly water soluble and showed sensitive response to specific metal ions in 100% aqueous solution. The selectivity of the sensors based on amino acids strongly depends on metal chelating ability of the amino acid of the sensors. For example, fluorescent sensors based on Trp, Met, or Asp acid showed an exclusive response to Hg(II) ions in aqueous solution [21–25] because the amino acids were regarded as an effective chelator for Hg(II) ions. Fluorescent sensor based on Cys amino acid showed response to several HTMs such as Hg(II), Pb(II),

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Cu(II), Cd(II), Ag(I), and Zn(II) because Cys is an effective chelator for several HTMs [26–28].

In the present study, we chose dipeptide as a receptor of the sensors and synthesized fluorescent sensor containing methionine (Met) and histidine (His). Both Met and His were frequently found in metal binding sites of several metalloproteins and these were well known as a metal chelating amino acid [27,29,30]. Specially, a receptor part containing thioether group has been extensively used in the design of fluorescent sensors for mercury or/and silver ions [16,31–33]. A fluorescent sensor containing Met as a receptor showed an exclusive selectivity to Hg(II) ion in aqueous and mixed organic–aqueous solvent system [25]. Histidine containing imidazole group interacts with several heavy metal ions such as Cu(II), Zn(II), Ag(I), and Ni(II) [29,30]. Dansyl fluorophore was conjugated into the dipeptide to convert metal binding events into fluorescent signals. Dansyl fluorophore of various fluorescent sensors was used for monitoring metal ions through chelation enhanced fluorescence (CHEF) effect and dansyl fluorophore is sensitive to the polarity of its microenvironment via an internal charge-transfer (ICT) mechanism [34–37].

The sensor (**DMH**) based on dipeptide shows response to Ag(I), Hg(II), and Cu(II) among 14 metal ions in 100% aqueous solution (Scheme 1).

Interestingly, **DMH** differentiates three heavy metal ions by response type. **DMH** shows turn on response to Ag(I), ratiometric response to Hg(II), and turn off response to Cu(II) in 100% aqueous solution. We investigated binding affinity, binding stoichiometry, and detection limit of the sensor for these metal ions. Furthermore we described the pH dependent sensitivity of **DMH** for Ag(I) and the interfering effect of other metal ion on detection of Ag(I) because only few chemical sensors showed turn on response to Ag(I) in 100% aqueous solution [8–10]. In comparison to chemical sensors, the dipeptide sensor shows several advantages such as easy synthesis with solid phase synthesis, high solubility in aqueous solution, sensitive response to heavy metal ions, and differentiation of three heavy metal ions by response type in aqueous media. To the best of our knowledge, this is the first example of fluorescent sensor based on dipeptide for monitoring heavy metal ions in 100% aqueous solution.

2. Experimental

2.1. Reagents

Fmoc–His(Trt)–OH, Fmoc–Met–OH, *N*,*N*-diisopropylcarbodiimide, 1-hydroxybenzotria-zole, and Rink Amide MBHA resin were purchased from Advanced Chem. Tech. Trifluroacetic acid (TFA), dansyl chloride, triethylamine, triisopropylsilane (TIS), *N*,*N* dimethylformamide (DMF) and piperidine were purchased from Aldrich.

2.2. Solid phase synthesis of dansyl–methionine–histidine (DMH)

DMH was synthesized in solid phase synthesis with Fmoc chemistry [38]. Fmoc protected L–His(Trt)–OH was assembled on Rink Amide MBHA resin. After deprotection of Fmoc group of resin bound His, Fmoc–L–Met–OH was coupled (Fig. S1). After deprotection of Fmoc group, dansyl chloride was performed by the following procedure. To the resin bound dipeptide (200 mg, 0.1 mmol), dansyl chloride (80 mg, 0.3 mmol, 3 equiv.) in DMF (3 ml) and triethylamine (40 μl, 0.3 mmol, 3 equiv.) were added. Cleavage of the peptide from resin was achieved by treatment with a mixture of 3 ml TFA:TIS:H_2O (95:2.5:2.5, v/v/v) at room temperature for 3 h. The crude **DMH** was triturated with diethyl ether chilled at −20 °C and then centrifuged at 3000 rpm for 10 min at −10 °C. The crude product was purified by HPLC with a Vydac C_{18} column using a water (0.1% TFA)–acetonitrile (0.1% TFA) gradient to give 82% of **DMH**. 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, mp 95–98 °C, ESI mass calculated: 519.18 [M+H$^+$]. Observed: 519.05 [M+H$^+$].

2.3. General fluorescence measurements

A stock solution of **DMH** at the concentration of 1.85×10^{-3} M was prepared in 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 phase containing 10 mM HEPES buffer at pH 7.4. Fluorescence emission spectrum of **DMH** in 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^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+} Pb^{2+}, Ag^{+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} as perchlorate anion and Na^{+}, Al^{3+} and K^{+} 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 dansyl group.

2.4. Determination of dissociation constant

The dissociation constant was calculated based on the titration curve of the probe with metal ion [39]. 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:

$$F = F_o + \Delta F \times [HL]$$

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

where F_o is the fluorescence of the probe only and ΔF is the change in fluorescence due to the formation of *HL*, L_T and H_T are total concentrations of metal ion (*L*) and host (*H*), respectively.

2.5. Determination of detection limit

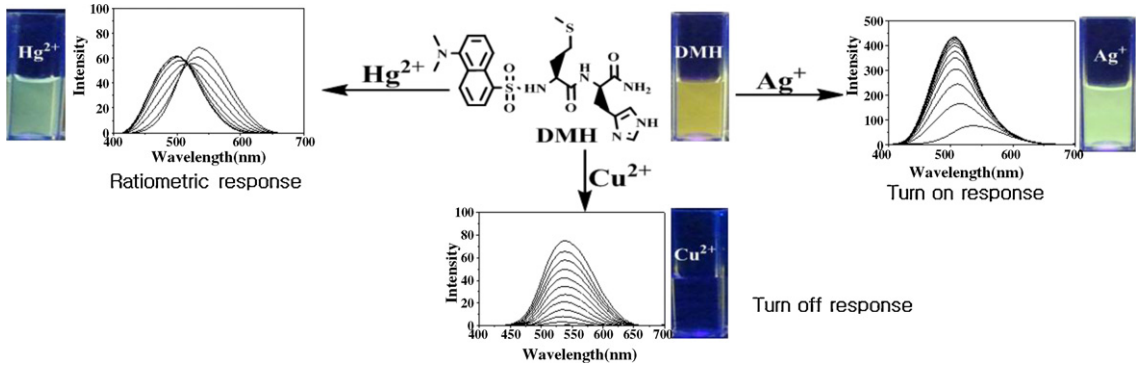
The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of **DMH** without any metal ions was measured by ten times and the standard deviation of blank measurements was determined. Three independent duplication measurements of emission intensity were performed in the presence of metal ions and each average value of the intensities was plotted as a concentration of metal ions for determining the slope. The detection limit is then calculated with the following equation:

$$\text{detection limit} = \frac{3\sigma_{bi}}{m}$$

where σ_{bi} is the standard deviation of blank measurements, *m* is the slope between intensity versus sample concentration.

2.6. Fluorescent images of Ag(I) in HeLa cells with DMH

The 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 penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) and grown at 37 °C in standard cell culture conditions (5% CO_2, 95% humidity). For cell imaging experiments were performed with a LSM 510 META confocal laser-scanning fluorescent microscope (ZEISS, German) with 40× objective lens. Excitation at 405 nm was



Scheme 1.

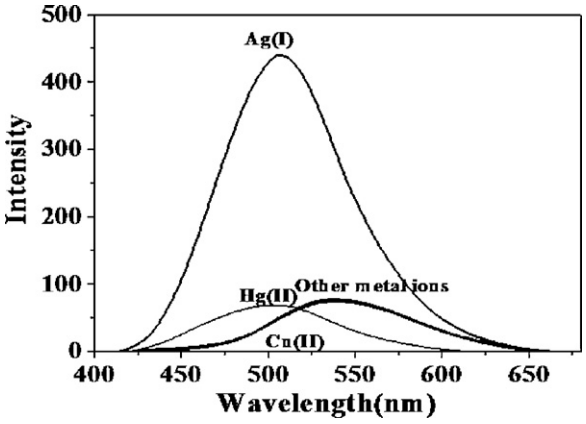


Fig. 1. Fluorescence spectra of **DMH** (10 μM) in the presence of each metal ions (5 equiv).

carried out with an argon ion laser. HeLa cells were attached to the plate 24 h before study. Cl^--free buffer of 20 mM HEPES (pH 7.4) containing 155 mM $NaNO_3$ was choose to avoid the formation of insoluble AgCl precipitates [40]. Cells were first loaded with 30 μM DMH in DMEM (containing 2% DMSO) at 37 °C for 30 min, washed three times with 20 mM HEPES buffer solution (155 mM $NaNO_3$, pH 7.4) to remove the free DMH. The weak fluorescent intensity of the cells was confirmed and then the cells were further incubated with 150 μM $Ag(ClO_4)$ for 20 min in 20 mM HEPES (pH 7.4) containing 155 mM $NaNO_3$.

3. Results and discussion

3.1. Solid phase synthesis of fluorescent sensor based on dipeptide

Fluorescent sensor based on dipeptide was efficiently synthesized in solid phase synthesis with Fmoc-chemistry [38]. After cleavage of the product from resin, **DMH** was purified from crude product by semi-preparative HPLC with a C_{18} column. The successful synthesis and purity of **DMH** (>98%) were confirmed by analytical HPLC with a C_{18} column and ESI mass spectrometer (Fig. S2). Details on the synthesis and characterization of **DMH** are described in Section 2.

3.2. Fluorescence response of DMH with various metal ions

As **DMH** has good solubility in water, the stock solution of **DMH** was prepared in 100% distilled water and all photochemical experiments were carried out in 100% aqueous solution without any co-solvent. Fig. 1 shows fluorescence spectra of **DMH** in the presence of each metal ions (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ag^+, Mg^{2+}, Mn^{2+},

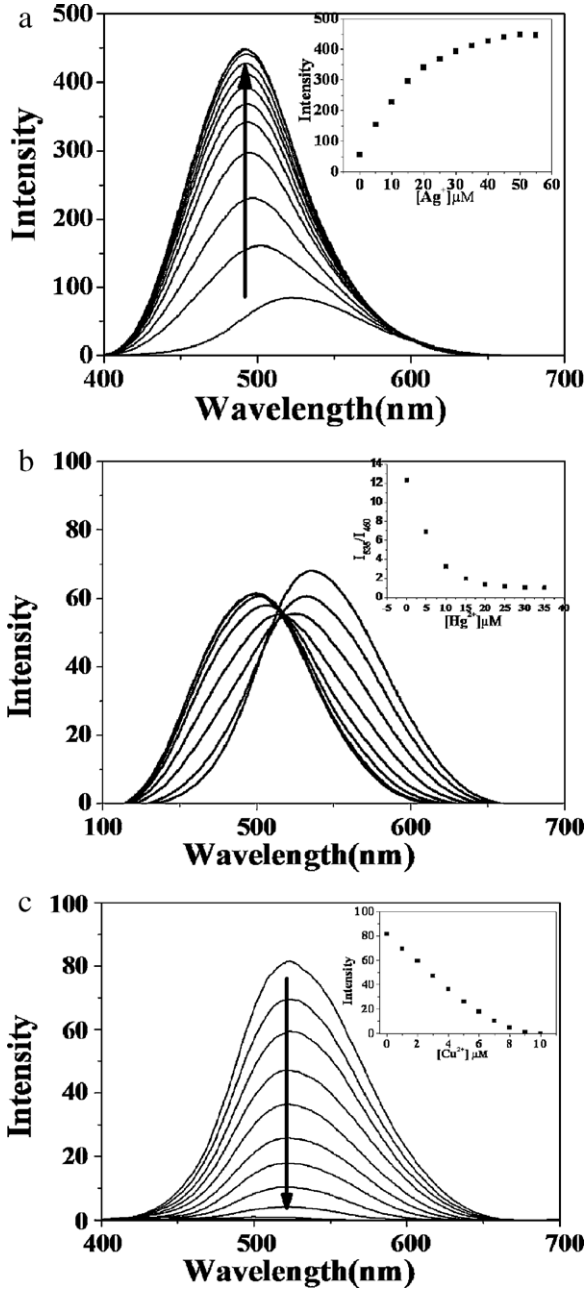


Fig. 2. Fluorescence emission spectra of **DMH** (10 μM) in the presence of increasing concentration of (a) Ag(I), (b) Hg(II), and (c) Cu(II) in 10 mM HEPES buffer at pH 7.4 (slit 10/5 nm, λ_{ex} = 330 nm).

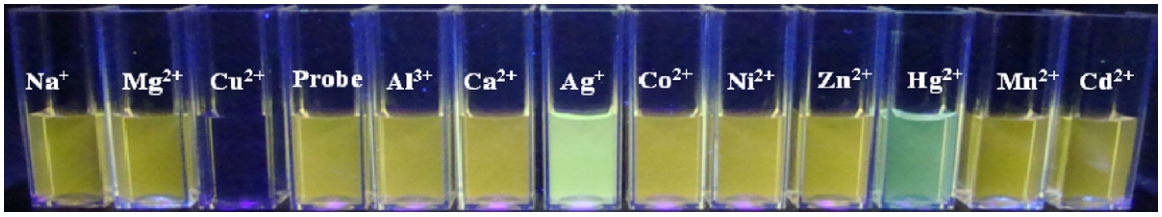


Fig. 3. Visible emission observed in 10 mM HEPES buffer solutions at pH 7.4 containing **DMH** (10 μM) and various metal ions (5 equiv).

Ni^{2+}, Hg^{2+}, Zn^{2+}, as perchlorate anion and Na^{+}, Al^{3+}, K^{+}, as chloride anion) by excitation with 330 nm.

DMH shows fluorescent response to Ag(I), Hg(II), and Cu(II) among test metal ions. Interestingly, the sensor differentiates three heavy metal ions by response type. **DMH** exhibits turn on response to Ag(I) and ratiometric response to Hg(II), while the emission intensity of **DMH** decreased with the addition of Cu(II). The fluorescent responses of **DMH** to the amount of Ag(I), Hg(II), and Cu(II) were measured in 10 mM HEPES buffer solution at pH = 7.4, respectively (Fig. 2).

Upon the addition of increasing concentration of Ag(I), about 9 fold enhancement and 30 nm blue shift from 536 to 506 nm of the maximum emission intensity were observed. About 5 equiv. of Ag(I) was required for the saturation of the emission intensity change. Upon the addition of increasing concentration of Hg(II), the intensity at 536 nm decreased and the intensity at 500 nm increased and the maximum intensity was shifted from 536 to 500 nm, which indicates that **DMH** is a ratiometric sensor for Hg(II) ion in 100% aqueous solution. In the titration curve, just 3 equiv. of Hg(II) was sufficient for saturation of shift of the maximum emission intensity. A comparison of the I_{536}/I_{500} ratios before and after Hg(II) addition provides an ~16-fold ratiometric change. Only few fluorescent chemical sensors are reported to show ratiometric response to Hg(II) in 100% aqueous buffer solution and most of fluorescent ratiometric sensors for Hg(II) required organic solvents for operation [15,41,42]. As shown in Fig. 2, the emission intensity was continuously decreasing with increasing concentration of Cu(II) and the emission spectrum completely disappeared in the presence of 1 equiv. of Cu(II). The decrease of the emission intensity can be tentatively explained in terms of electron transfer from the excited dansyl fluorophore to the complexed copper cation [20,43].

Fig. 3 presents a visible emission change of **DMH** (10 μM) in the presence of various metal ions (5 equiv.) in 10 mM HEPES buffer solution at pH 7.4. **DMH** solution containing Ag(I) displayed light green color. The addition of Hg(II) into **DMH** solution changed fluorescence color from yellow to blue. **DMH** solution containing Cu(II) shows no color, whereas the **DMH** solution containing other metal ions showed yellow color. (For interpretation of the references to

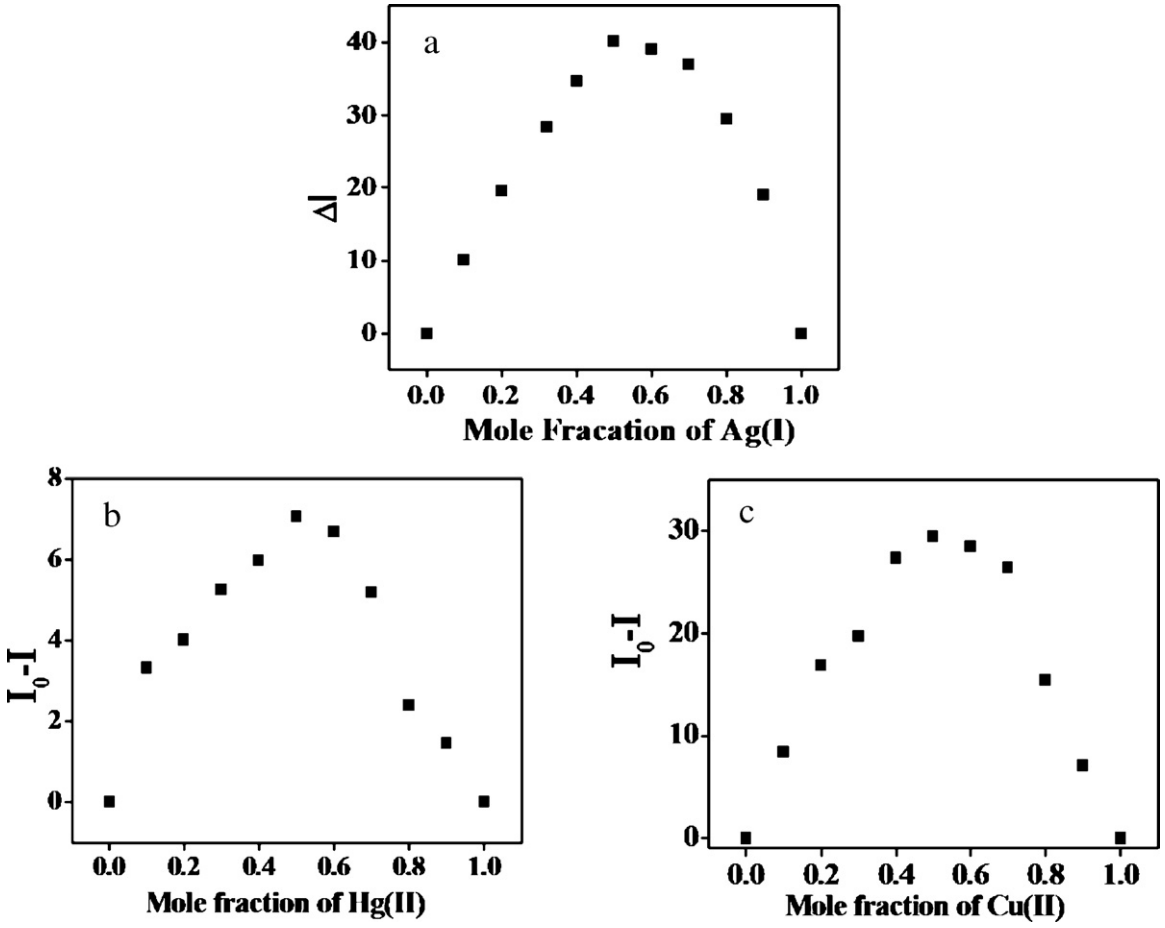


Fig. 4. A Job plot for **DMH** with (a) Ag(I), (b) Hg(II), and (c) Cu(II) in 10 mM HEPES buffer solution at pH 7.4.

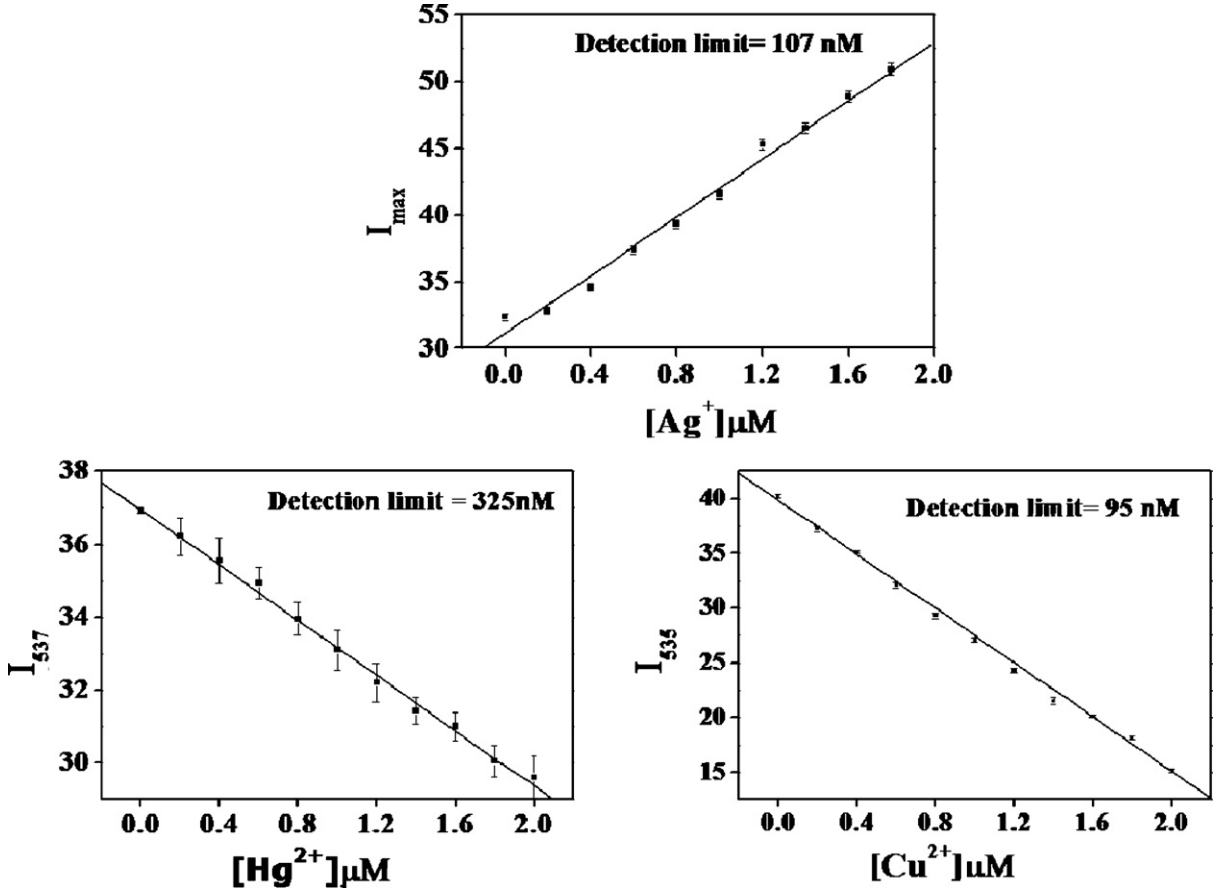


Fig. 5. Linear emission intensity change as a function of the concentration of HTM ions.

color in this figure legend, the reader is referred to the web version of this article.)Visible emission change showed what metal ion among Ag(I), Hg(II), and Cu(II) was included in **DMH** solution.

3.3. Binding stoichiometry and binding affinity

We investigated the binding stoichiometry and binding affinities of the dipeptide sensor for Ag(I), Hg(II), and Cu(II). A Job's plot analysis and ESI mass spectrometry were used to determine binding stoichiometry.

As shown in Fig. 4, a Job's plot exhibits a maximum at 0.5 mole fraction of Ag(I) in aqueous solution at low concentration (1–10 μM) of the sensor. This result suggests that **DMH** forms a 1:1 complex with Ag(I) in aqueous solution. The binding stoichiometry with **DMH** and Ag(I) was further analyzed by ESI mass spectrometry. When 5 equiv. of Ag(I) was mixed with the sensor (100 μM) in 100% aqueous solution, the new peak at 627.04 and 1145.05 corresponding to [**DMH**+Ag^+]$^+$ and [2**DMH**+Ag^+]$^+$ appeared, as shown in Fig. S3. According to mass spectrum, the dipeptide sensor may form 1:1 and 2:1 complexes with Ag(I) in aqueous solution depending on the concentration of **DMH**. It is reported that peptide sensors for heavy metal ions formed mixed type of the complex with heavy metal ions (1:1 and 2:1) depending on peptide concentration [44,45]. **DMH** may form a 1:1 complex with Ag(I) predominantly at low concentration (10 μM). Assuming 1:1 complex formation, the dissociation constant was calculated based on the titration curve with Ag(I) by nonlinear least square fitting. The dissociation constant for Ag(I) was calculated as 2.41×10^{-6} M, which indicates that **DMH** has a potent binding affinity for Ag(I) in 100% aqueous buffer solution.

A Job's plot analysis exhibited a maximum at 0.5 mole fraction of Hg(II), which indicated that **DMH** (10 μM) forms a 1:1 complex with Hg(II) in aqueous solution (Fig. 4b). The binding stoichiometry with **DMH** and Hg(II) was analyzed by ESI mass spectrometry (Fig. S3). When 3 equiv. of Hg(II) was added into the solution containing sensor (100 μM), the new peak at 719.27 corresponding to [**DMH**+Hg^{2+}]$^{2+}$ appeared. Overall results indicate that **DMH** forms a 1:1 complex with Hg(II). Assuming 1:1 complex formation, the dissociation constant for Hg(II) was calculated as 2.63×10^{-6} M based on the titration curve with Hg(II).

In the case of Cu(II) sensing, a Job's plot, which exhibits a maximum at a 0.5 mole fraction, indicates that **DMH** forms a 1:1 complex with Cu(II) (Fig. 3C). As shown in Fig. S3, ESI mass spectrum shows the peak at 580.08 corresponding to [**DMH**+Cu^{2+}]$^{2+}$, which confirms that **DMH** forms a 1:1 complex with Cu(II). The dissociation constant of **DMH** for Cu(II) was calculated as 3.70×10^{-9} by fitting of the titration curve with Cu(II) with 1:1 complex model. As His is a well known amino acid to interact with Cu(II) [27,29,30], almost all fluorescent peptide sensors containing His showed a response to Cu(II) [36,45,46]. The binding affinity of **DMH** for Cu(II) is much more potent than that of the other peptide sensors containing His. For example, as GlyGlyHis is a peptide motif from the amino terminal

Table 1
Type of response, detection limit and dissociation constant of DMH.

Metal ions	Type of response	Detection limit (nM)	Dissociation constant (1:1 binding)
Ag(I)	Turn-on	107	2.41×10^{-6} M
Hg(II)	Ratiometric	325	2.63×10^{-6} M
Cu(II)	Turn-off	95	3.70×10^{-9} M

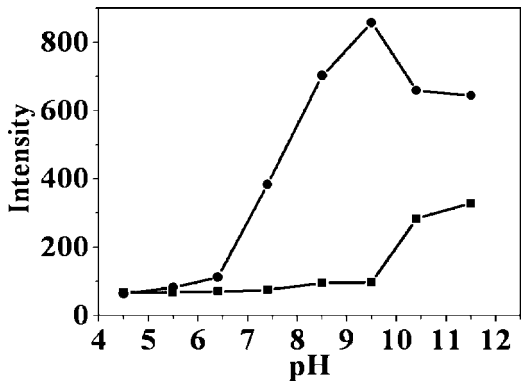


Fig. 6. Emission intensity of **DMH** in the presence (●) and absence of (■) Ag(I) (5 equiv.) at different pH.

Cu and Ni-binding (ATCUN) site [47], the fluorescent peptide sensor based on this motif showed a selective and sensitive response to Cu(II) [46]. The dissociation constant of the peptide sensor for Cu(II) was 263 nM. However, **DMH** consisting of just two amino acids was 90 times more sensitive than the peptide sensor based on GlyGlyHis. The dissociation constants of **DMH** for Ag(I), Hg(II), and Cu(II) were summarized in Table 1.

3.4. Detection limit of DMH for heavy metal ions

DMH showed instant response to heavy metal ions in 100% aqueous solution. Upon the addition of increasing concentration of Ag(I), the blue shift of maximum intensity was observed (Fig. 2). The maximum intensity increased in proportion to the concentration of Ag(I). Fig. 5 displays a linear response of the emission intensity as the concentration of Ag(I). A linear measurement is possible until 0.4 equiv. of Ag(I).

Upon the addition of Hg(II) to the solution of **DMH**, the large shift of maximum intensity was observed. The emission ratio (I_{536}/I_{470}) did not change linearly with the concentration of Hg(II). Instead, the emission intensity at 536 nm (I_{536}) decreased in proportion to the concentration of Hg(II) (Fig. 5b). In the case of Cu(II) titration, the intensity at 535 nm also gradually decreased with increasing concentration of Cu(II) and a linear measurement is possible until 0.4 equiv. (Fig. 5c). The detection limits of **DMH** for three metal ions were calculated on the basis of the linear relationships between the emission intensity and concentration of metal ions, as shown in Fig. 5. Table 1 summarized the detection limits of **DMH** for target metal ions. The detection limits for Ag(I) (107 nM) and Cu(II) (95 nM) were much lower than the EPA's drinking water maximum contaminant levels of Ag(I) and Cu(II), respectively [48]. However, the detection limit for Hg(II) is higher than the EPA's drinking water maximum allowable level. We expect that the detection limits for three heavy metal ions can be further optimized by amino acid replacements and can be improved by optical techniques such as a more intense light source, a longer integration time, and/or large slit size.

3.5. Detection of Ag(I) by DMH in different pH and in the presence of other metal ions

In comparison to the chemical sensors for Hg(II) and Cu(II), relatively few chemical sensors for Ag(I) have been reported [10,12,49–52]. Furthermore, most fluorescent chemical sensors for Ag(I) suffer from low sensitivity, turn off response, and/or low water solubility. As **DMH** shows sensitive turn on response to Ag(I) in aqueous solution, we further characterize the detection ability of **DMH** for Ag(I) in different pH. Fig. 6 shows the influence of pH on the response of **DMH** to Ag(I).

At acidic condition (pH < 6), **DMH** and **DMH**–Ag^+ complex showed very weak emission intensity, respectively. This was attributed to the protonated dimethylamino group ($pK_a \cong 4$) of dansyl fluorophore [53]. The protonated dimethylamino group prevents the charge transfer from dimethylamino group to napthyl moiety [54], resulting in weak emission intensity. As the imidazole ring of His is mostly protonated at pH less than 6, His cannot interact with silver ion in this pH range. Thus, **DMH** did not show considerable response to Ag(I). **DMH** showed sensitive turn on response to Ag(I) with ~10 fold enhancements in the 7.4–9.5 pH range. At pH > 9.5 the intensity of **DMH**–Ag(I) complex decreased with increasing pH. This may be due to the deprotonation of sulfonamide group ($pK_a \cong 10$) of the sensor in basic condition. Overall results confirm that **DMH** shows sensitive response to Ag(I) in the 7.4–9.5 pH range.

To test reversibility, EDTA was added to solutions of **DMH** and Ag(I) that exhibited a strong emission intensity. Addition of EDTA to solutions of **DMH** and Ag(I) caused an immediate fluorescence decrease (Fig. S4). Addition of EDTA (~100 equiv.) restored the original, metal-free spectrum, which confirms that the fluorescence response of **DMH** for Ag(I) is reversible and **DMH** has a potent binding affinity to Ag(I) in aqueous solution because EDTA has a reported K_d value of ~50 nM for Ag(I).

To investigate the interference effect of other metal ions on the detection ability of **DMH** for Ag(I), the fluorescence response of **DMH** to Ag(I) in the presence of other metal ions was measured (Fig. 7).

The Ag(I)-dependent fluorescence change of **DMH** seemed to be affected by Na(I), K(I), Ca(II), and Mg(II). However, this is due to the formation of insoluble AgCl precipitate by interaction of counter anion, Cl^- with Ag(I). The fluorescence spectrum of **DMH**–Ag(I) was not changed by heavy metal ions except Hg(II) and Cu(II). The addition of Hg(II) or Cu(II) affected Ag(I)-dependent fluorescence spectrum of **DMH**. As the EPA's drinking water maximum allowable level of Cu(II) is much higher than Hg(II), we investigate the effect of Cu(II) on detection ability of **DMH** for Ag(I). The addition of Cu(II) into the solution containing **DMH** and Ag(I) caused an immediate decrease of emission intensity (Fig. S5). It is due to the fact that **DMH** has a more potent-binding affinity for Cu(II) than dose Ag(I). However, the decreased emission spectrum of **DMH** in the presence of Ag(I) and Cu(II) was somewhat different from that of **DMH** in the presence of Cu(II), which indicates that **DMH** may interact with Cu(II) and Ag(I) simultaneously. After addition of 1 equiv. of Cu(II) into **DMH** solution that shows little emission intensity, the addition of Ag(I) caused an fluorescence increase (Fig. S6). This indicates that **DMH** can monitor Ag(I) ion in aqueous solution even in the presence of small amount of Cu(II).

3.6. Detection of intracellular Ag(I) with DMH

As **DMH** displays sensitive turn on response to Ag(I) in waters at physiological pH, we investigate whether **DMH** can penetrate live cell and then detect intracellular Ag(I) ions. Generally, fluorescent imaging Ag(I) in live cells has a main difficulty that mM concentration of cellular chloride ions may cause the precipitation of Ag(I) as AgCl. To solve this problem, HeLa cells were incubated with **DMH** (30 μM) for 30 min at 37 °C and then the HeLa cells were washed with 20 mM HEPES buffer solution (pH 7.4) containing $NaNO_3$ instead of NaCl [40].

The fluorescent image of the cells incubated with **DMH** was monitored by confocal microscopy, as shown in Fig. 8. The weak green fluorescent image of the cells indicates that even though **DMH** is a hydrophilic dipeptide, it can penetrate HeLa cells in this condition.

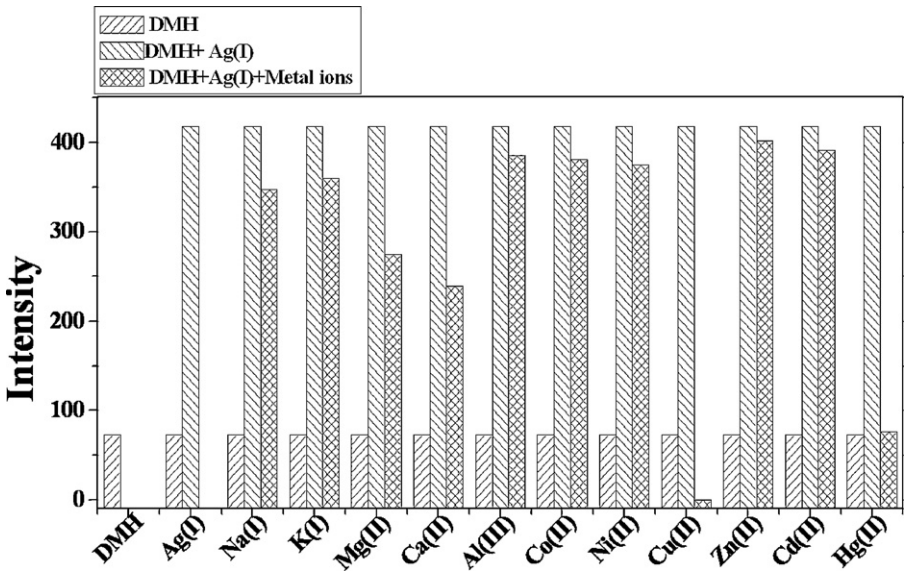


Fig. 7. Emission intensity response of **DMH** (10 μM) in the presence of Ag(I) (5 equiv.) and additional various metal ions (5 equiv.) in 10 mM HEPES buffer at pH 7.4.

After addition of $AgClO_4$ into the **DMH** loaded cells, we observed a considerable enhancement of emission intensity in HeLa cells, which supports that **DMH** is suitable for detecting Ag(I) ions in live cells.

3.7. Binding mode of DMH with Ag(I) and Hg(II)

To determine the binding mode of **DMH** with Ag(I) and **DMH** with Hg(II), ^{1}H NMR experiments were carried out in DMSO-d6. As **DMH** operated at neutral and basic pH, NMR spectra of **DMH** with metal ions were measured in the presence of small amount of ammonium formate (Fig. 9).

When 5 equiv. of Ag(I) ions was added, large and small chemical shifts in H(4), H(5), H(2), H(10), and H(9) were observed, which indicates that Ag(I) ion coordinates imidazole group and thioether group of **DMH**. Small chemical shifts of aromatic protons of the dansyl moiety suggest that Ag(I) ion interacts with the dansyl moiety. However, a little shift of H(15) suggests that Ag(I) ion coordinates sulfonamide group of the dansyl fluorophore. Unfortunately, we could not investigate the binding mode of **DMH** with Hg(II) by NMR because mM concentration of **DMH** and Hg(II) precipitated in the presence of ammonium formate or NaOD in DMSO-d6.

Previously, we synthesized dansyl conjugated methionine (Met) that showed turn on detection of Hg(II) in 100% aqueous solutions and investigated the binding mode of this sensor with Hg(II) by organic spectroscopy techniques including NMR [25]. According to the studies, Hg(II) coordinates with the thioether group of Met and the sulfonamide group of the dansyl fluorophore. Considering the binding mode of dansyl conjugated Met with Hg(II), the role of imidazole group of **DMH** for interactions with Hg(II) should be investigated. Thus, we measured emission spectrum change of **DMH** with Hg(II) and Ag(I) in acidic and neutral pH (Fig. S7) because the protonated imidazole group ($pK_a \cong 6$) cannot be interacted with the metal ions. At pH = 5, **DMH** showed turn off response to Hg(II), whereas **DMH** showed a little response to Ag(I). At pH = 6 and 7.4, **DMH** displayed ratiometric response to Hg(II), whereas **DMH** dis-

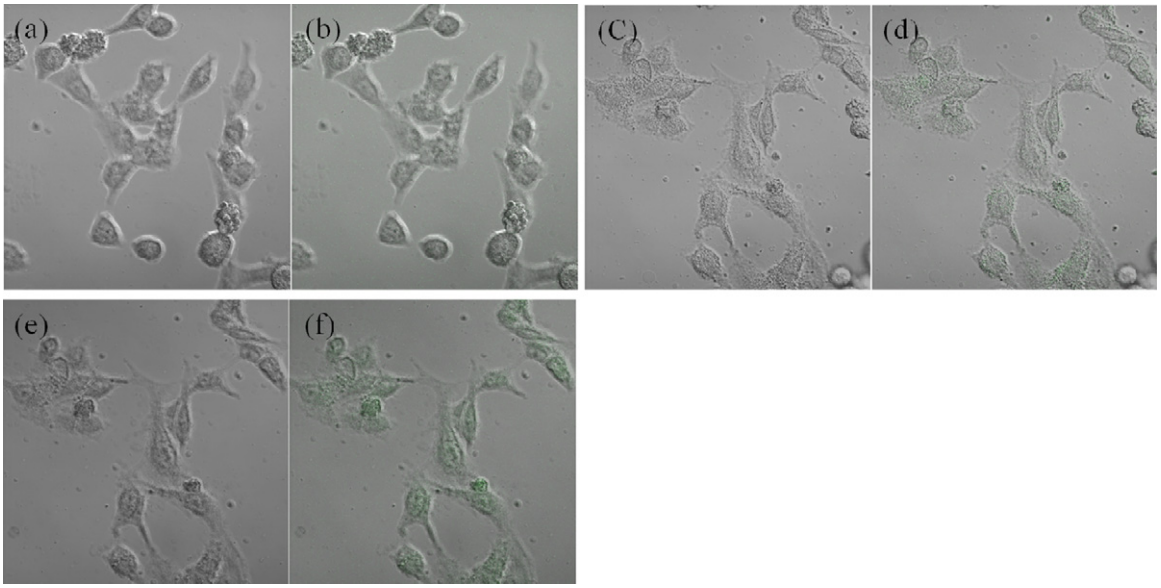


Fig. 8. Bright field (a, c, e) and fluorescent and bright field (b, d, f) images of HeLa cells (a, b), HeLa cells with **DMH** (30 μM) (c, d), and HeLa cells with **DMH** (30 μM) in the presence of Ag(I) (150 μM) (e, f).

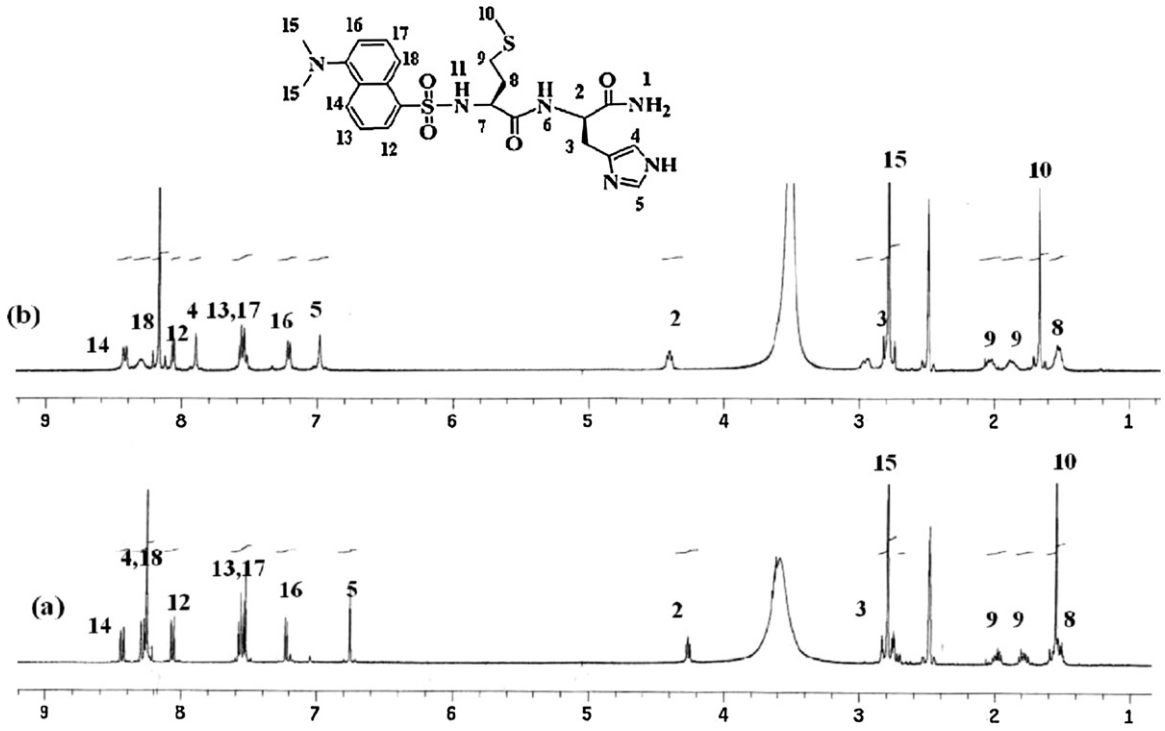


Fig. 9. Partial ^{1}H NMR (400 MHz) of **DMH** (5 mM) in DMSO-d6 at 25 °C (a) in the absence and (b) presence of $AgClO_4$ (5 equiv.) and ammonium formate (2 equiv).

played turn on response to Ag(I). This result suggests that the His residue of **DMH** plays an important role in the ratiometric response to Hg(II). Accordingly, on the basis of the other results, it can be proposed that Ag(I) and Hg(II) interact with the imidazole, thioether, and sulfonamide groups of **DMH**, respectively.

The addition of Ag(I) ions as well as Hg(II) ions to **DMH** caused a decrease in intensity of the absorption bands in the UV/Vis spectra (Fig. S8). However, the addition of Ag(I) ions led to a hypsochromic shift (blue shift) of the absorption band at 330 nm, whereas the addition of Hg(II) ions caused a bathochromic shift (red shift) of the absorption band at 330 nm. This result strongly suggests that even though the metal ions interact with the same sulfonamide group of the dansyl fluorophore, the binding effect on the fluorophore seems to be different depending on the property of metal ion itself. Thus, we conclude that the difference response of **DMH** to the metal ions seemed to be dependent on the property of the metal ions such as charge and size.

Even though several fluorescent sensors based on an amino acid for heavy metal ions were reported [21–26], there is no report of fluorescent sensors based on dipeptide for monitoring heavy metal ions. The dipeptide based sensor (**DMH**) employed in this study shows interesting properties for detection of heavy metal ions. The sensor consisting of environmental compatible amino acids is highly water soluble and shows sensitive response to specific metal ions in 100% aqueous solution. Fluorescent sensors based on an amino acid shows an exclusive response to Hg(II) or a response to several heavy metal ions, whereas **DMH** based on dipeptide (Met–His) shows response to specific heavy metal ions such as Ag(I), Hg(II), and Cu(II). Furthermore, **DMH** differentiates the three metal ions by response type. In addition, **DMH** penetrates live cells and detect intracellular Ag^+ by turn on response. This result suggests that if we will change the sequence of dipeptide of the sensor, we can develop new fluorescent sensors for detecting various metal ions in aqueous solutions. Considering the unique property of the sensor based on dipeptide and possible sequences of dipeptides, fluorescent sensors based on dipeptide will provide a valuable tool for monitoring specific heavy metal ions in aqueous solutions.

4. Conclusion

High purity of a fluorescent dipeptide sensor (**DMH**) was efficiently synthesized in solid phase synthesis. The dipeptide sensor shows potent binding affinities and sensitive response to specific heavy metal ions in 100% aqueous solution. The sensor differentiates three heavy metal ions by response type; turn on response to Ag(I), ratiometric response to Hg(II), and turn off response to Cu(II). Considering the potent binding affinity and detection limit for these metal ions, the dipeptide sensor is useful for detection of trace amounts of Ag(I), Hg(II), and Cu(II) in environmental samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.06.052.

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