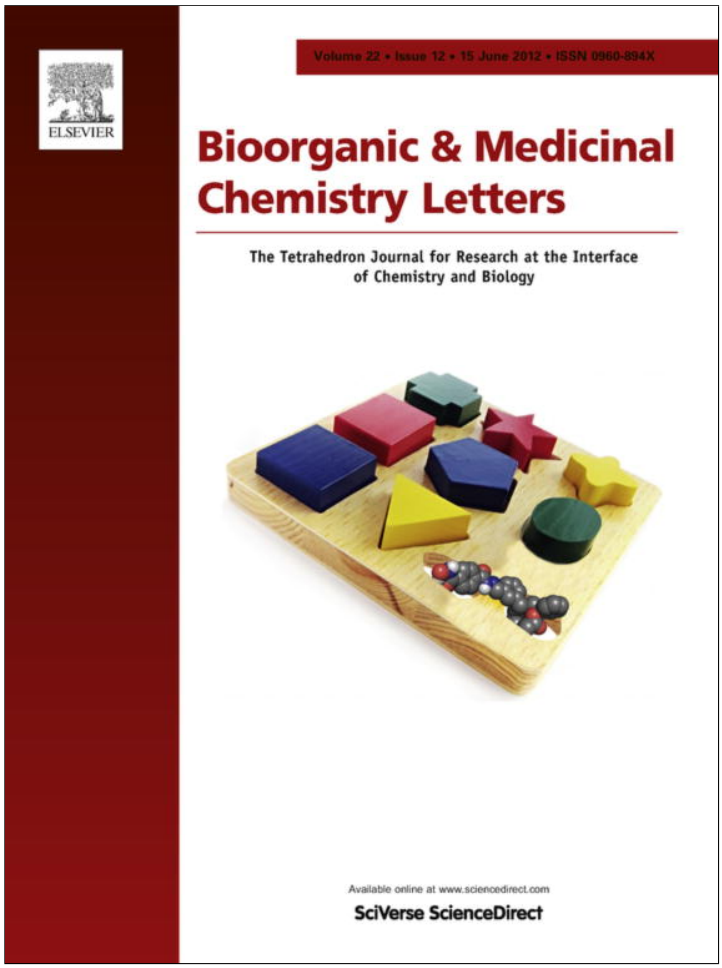

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Synthesis of a ratiometric fluorescent peptide sensor for the highly selective detection of Cd^{2+}

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ABSTRACT

A novel ratiometric fluorescent peptidyl chemosensor (Dansyl-Cys-Pro-Gly-Cys-Trp-NH_2, D-P5) for metal ions detection has been synthesized via Fmoc solid-phase peptide synthesis. The chemosensor exhibited a high selectivity for Cd^{2+} over other metal ions including competitive transition and Group I and II metal ions in neutral pH. The fluorescence emission intensity of D-P5 was significantly enhanced in the presence of Cd^{2+} by fluorescent resonance energy transfer (FRET) and chelation enhanced fluorescence (CHEF) effects. The binding stoichiometry, detection limit, binding affinity, reversibility and pH sensitivity of the sensor for Cd^{2+} were investigated.

Cadmium, an important heavy and transition metal, has been recognized as a highly toxic heavy metal and listed as high as the seventh on the Top 20 Hazardous Substances Priority List by the Agency for Toxic Substances and Disease Registry and US Environmental Protection Agency (EPA).[1] Chronic exposure to Cd^{2+} sources can potentially cause mutations and canceration in higher animals, such as lung, prostatic, and renal cancers.[2] Therefore, it is vitally important to develop some analytical methods for detecting and monitoring cadmium in vitro and in vivo. Fluorescent sensors for monitoring metal ions, owing to its simplicity, high sensitivity and high selectivity, have attracted a great deal of attention in recent years.[3] Recently, some fluorescent sensors for detecting cadmium have been reported.[4] Although these sensors exhibited good selectivity and good aqueous solubility, most of them were chelating units composed of organic molecules. Furthermore, the synthesis of these chelating units was rigorous and their binding was not always reversible.[5] Considering of the drawbacks of organic molecules, peptide sensors for detecting metal ions has drawn great attention due to its following advantages[6]: (1) Peptide sensors, consisting of natural amino acids, can be easily synthesized by Fluorenylmethoxycarbonyl(Fmoc) solid-phase peptide synthesis (SPPS).[7] (2) The sensitivity and selectivity of peptide sensors can be optimized by further amino acid replacement.[8] (3) Peptide sensors can be used in aqueous solution.[9] One of the early examples of the fluorescent peptidyl chemosensor was a small peptide sequence (25 residues) based on the zinc finger protein.[10] Other fluorescent peptide probes for the detection of Zn^{2+}, Cu^{2+}, Hg^{2+} have also been designed successfully.[11] However, very few fluorescent peptide sensors for Cd^{2+} detection have been explored up to now.[5f,12]

Furthermore, ratiometric fluorescent sensors for heavy metal ions become attractive because they make it possible to measure the analytes more accurately with minimal background signal.[13] Lee and his co-workers have reported a novel ratiometric fluorescent peptide sensor containing tryptophan (donor) and dansyl fluorophore (acceptor) for monitoring several heavy metal ions such as Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+} and Ag^{+} in aqueous solution.[14] However, this sensor suffered from limitations due to its lower selectivity. Therefore, it is necessary to develop new peptide-based ratiometric fluorescent sensors with a high selectivity for Cd^{2+}. Here, we report a new peptide sensor (**D-P5**: Dansyl-Cys-Pro-Gly-Cys-Trp-NH_2) containing dansyl group (acceptor) and Trp residue (donor) for detecting Cd^{2+}. As shown in Scheme 1, when the peptide sensor interacts with metal ions, it may fold and the Trp residue and dansyl group will get closer to each other, resulting in an increase of emission intensity of dansyl group by fluorescent

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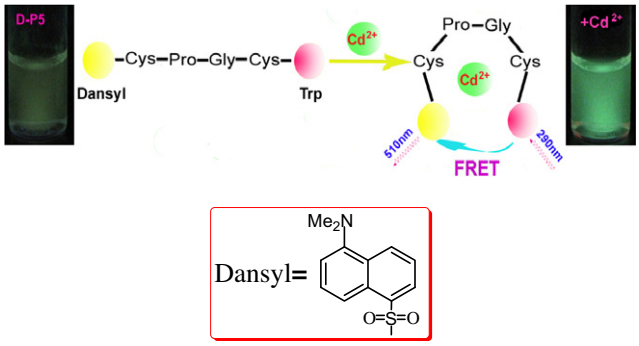

Scheme 1. Proposed possible Cd^{2+} binding mode of **D-P5**.

resonance energy transfer (FRET) effect. The results showed that the peptide sensor exhibited a high selectivity to Cd^{2+} with ratiometric response.

The peptide with sequence of Dansyl-Cys-Pro-Gly-Cys-Trp-NH_2 (D-P5) was synthesized on Rink Amide resin by standard Fmoc solid-phase peptide synthesis (SPPS) using a CS136 peptide synthesizer.[15] The Dansyl group was coupled as reported previously.[14] The identity of D-P5 was confirmed by ESI-MS (D-P5 calcd 796.16; obsd 796.12). (Fig. S1) The fluorescence emission spectra were measured in 50 mM HEPES buffer solution (pH 7.0). The excitation wavelength was 330 nm for monitoring dansyl fluorophore emission and 290 nm for monitoring both of Trp and dansyl fluorophores emission (Fig. 1). As shown in Figure 1A, with stepwise addition of Cd^{2+} to the solution of D-P5, the fluorescence emission intensity at 510 nm gradually increased while the intensity at 360 nm decreased. The fluorescence intensity ratio at 510–360 nm (F_{510}/F_{360}) increased from 7.2 to 25.3 with the Cd^{2+} concentration from 1 to 10 μM. This result showed the FRET effect of D-P5 in the presence of Cd^{2+}, indicating that Cd^{2+} induces shortening the distance between Trp and dansyl and causes the folding of D-P5.[14] We proposed one possible Cd^{2+} binding mode of D-P5 (Scheme 1). In fact, FRET signals can be attributed from the intramolecular and intermolecular energy transfer from Trp to the dansyl group.

Figure 1B shows that, while exciting at 330 nm, only one emission peak appeared at 510 nm with the Cd^{2+} addition. This result suggests that direct interaction may occur between the dansyl group and Cd^{2+}, because the complexation of the sulfonamide group of dansyl fluorophore by cations may inhibits photoinduced electron transfer (PET), that is, there exists a chelation enhanced fluorescence (CHEF) effect. It has been proven that the emission spectra of dansyl group is sensitive to its microenvironments.[15]

In the titration curve, the saturation of the fluorescence emission intensity of D-P5 (5 μM) was achieved at 0.5 of $c(Cd^{2+})/c$(D-P5), indicating that the binding ratio between the peptide and Cd^{2+} was 2:1. We also explored the coordination stoichiometry between D-P5 and Cd^{2+} by Job's plot (Fig. S2).[13b,16] It showed a maximal peak at 0.3 mole fraction of Cd^{2+}, which confirms the binding stoichiometry of 2:1 between D-P5 and Cd^{2+}. This result implies that D-P5 with Cys-X-X-Cys structure interacted with Cd^{2+} by two Cys residues and exhibits a fluorescence response with Cd^{2+} by FRET as well as CHEF. The association constant was obtained from the fluorescence titration curve. The high binding affinity ($K_a = (1.4 \pm 0.2) \times 10^{11}\ M^{-2}$) of D-P5 for Cd^{2+} might be due to the strong interaction between sulfuric groups of two Cys residues and Cd^{2+}. This result shows that D-P5 can bind to Cd^{2+} strongly and is suitable for the Cd^{2+} detection.

To evaluate the selectivity of **D-P5**, the fluorescence responses of **D-P5** was measured towards different metal ions, including some related heavy, transition and main group metal ions. Due to the interaction of these ions with D-P5 showed the degree of

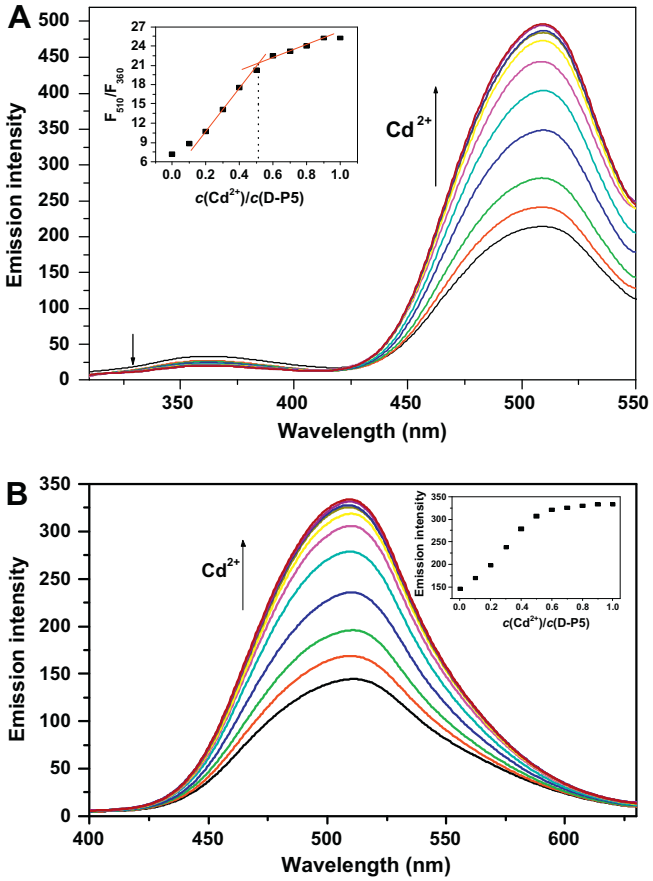

Figure 1. Fluorescence emission spectra of D-P5 (10 μM) in the presence of Cd^{2+} (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM) in 50 mM HEPES (100 mM $NaClO_4$, pH 7.0) with an excitation at (A) 290 nm (B) 330 nm. Inset: (A) the titration curve based on the fluorescence emission ratio at 510 nm to 360 nm (F_{510}/F_{360}). (B) the titration curve based on the fluorescence emission at 510 nm.

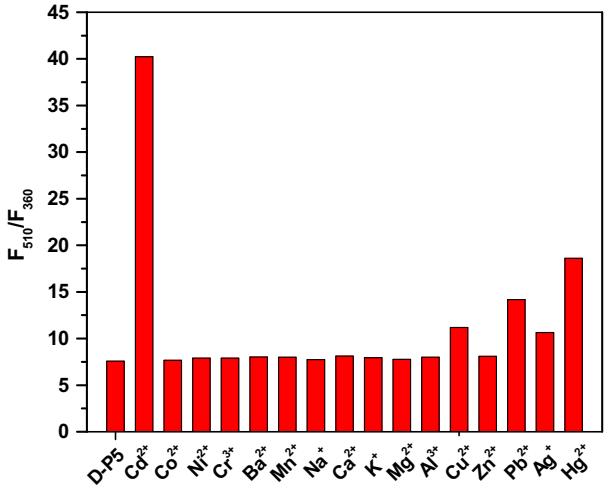

Figure 2. Fluorescence intensity ratio (F_{510}/F_{360}) of D-P5 (10 μM) in the presence of various metal ions (1 equiv) in 50 mM HEPES buffer (pH 7.0) with an excitation at 290 nm.

fluorescence emission intensity at 510 nm and 360 nm, we chose F_{510}/F_{360}, which can eliminate most or all ambiguities by built-in calibration of the two emission bands, in the fluorescent measurement of Cd^{2+} ion instead of F_{510} for analyzing the selectivity (Fig. 2).

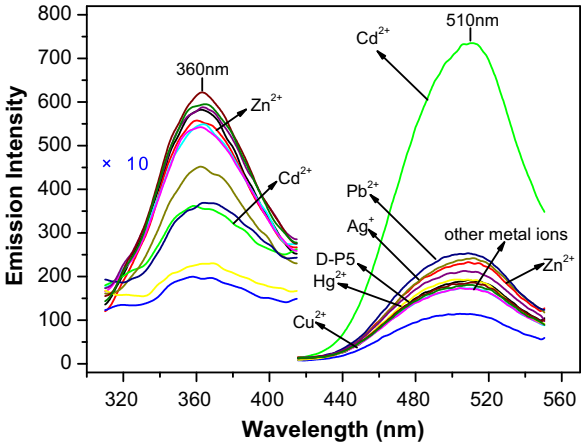

Figure 3. Fluorescence spectra of D-P5 (10 μM) in the presence of various metal ions in 50 mM HEPES buffer solution (pH 7.0) with an excitation at 290 nm. The molar ratio of metal/D-P5 is 1:1.

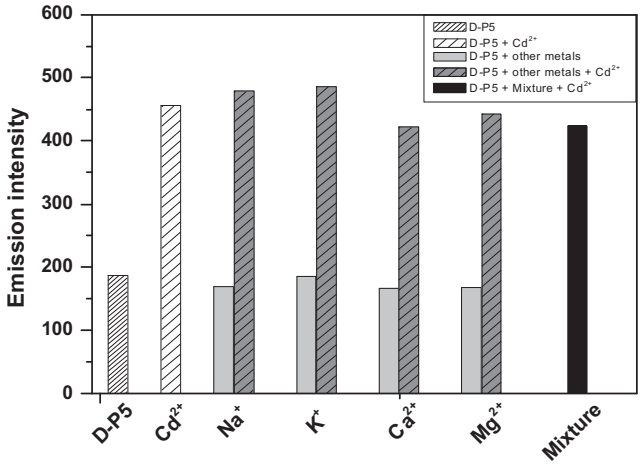

Figure 4. Fluorescence intensity of D-P5 (10 μM) in the presence of various metal ions in 50 mM HEPES buffer (pH 7.0), λ_{ex} = 290 nm. The concentration of Cd^{2+} was 10 μM and Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} were 10 mM, respectively.

Results showed that 1 equiv of Co^{2+}, Ni^{2+}, Cr^{3+}, Ba^{2+}, Mn^{2+}, Al^{3+} and 1000 equiv of Na^{+}, Ca^{2+}, K^{+} and Mg^{2+} had negligible influence on the ratio, whilst 1 equiv of Cu^{2+}, Zn^{2+}, Pb^{2+}, Ag^{+} and Hg^{2+} showed very limited enhancement of the ratio. It should be noted that, although the ratio of F_{510}/F_{360} of Cu^{2+}, Zn^{2+}, and Hg^{2+} is moderate, these ions do not interfere the selectivity for Cd^{2+} if we compare the intensity (F_{510}) on fluorescence spectra of D-P5 (Fig. 3). These metal ions, including Cu^{2+}, Pb^{2+}, Ag^{+} and Hg^{2+} had very little influence on the fluorescence intensity of D-P5 at 510 nm, showing negligible interference to Cd^{2+}. In addition, Cd^{2+}, Zn^{2+}, Pd^{2+} and Ag^{+} ions have the similar property, showing the similar wavelength with a slight shift. But their emission intensities were different respectively.

For Zn^{2+}, the emission intensity at 510 nm was stronger. To further eliminate this interference, we have tried some methods and chose citric acid as a masking agent. If citric acid was added to the D-P5/Zn^{2+} system and D-P5/Cd^{2+} system respectively, the spectrum of D-P5/Zn^{2+} system was reversed to the Zn-free state but the spectrum of D-P5/Cd^{2+} system was small change. This shows that addition of citric acid can eliminate the influence of Zn^{2+} (Fig. S3). In addition, the titration curve of Zn^{2+} was also different from that of Cd^{2+} (Fig. S4). The fluorescence emission intensity at 360 nm gradually decreased with the addition of Cd^{2+}. However, there was no apparent change at 360 nm in the titration curve of Zn^{2+}. The high selectivity can be explained by Hard–Soft Acid–Base Theory, that is, D-P5 has two Cys residues as soft ligands which tends to bind the softer Cd^{2+} other than Zn^{2+}.[17] The results indicated that D-P5 possess a high selectivity for Cd^{2+}, thus it might be a potential sensor candidate for Cd^{2+}.

The fluorescence response of **D-P5**/Cd^{2+} complex in the presence of group I, II metal ions were investigated (Fig. 4). The emission intensity was not affected obviously by these metal ions even though their concentrations were 1000 times higher than Cd^{2+} concentration. These results indicated that the presence of alkali metal ions and alkaline earth metal ions at higher concentration had no influence on detection of Cd^{2+}.

The binding reversibility of D-P5 to Cd^{2+} was studied by adding excess ethylene glycol tetraacetic acid (EGTA) to the D-P5/Cd^{2+} system. The addition of excess EGTA reversed the spectrum to the Cd-free state, which demonstrates the reversibility of the signaling mechanism of the peptide (Fig. S5).

The determination of optimal conditions was achieved by comparing the interference of Pb^{2+}, Ag^{+}, Zn^{2+} to Cd^{2+} at different pH values. Figure 5A showed the interfere experimental of 1 equiv

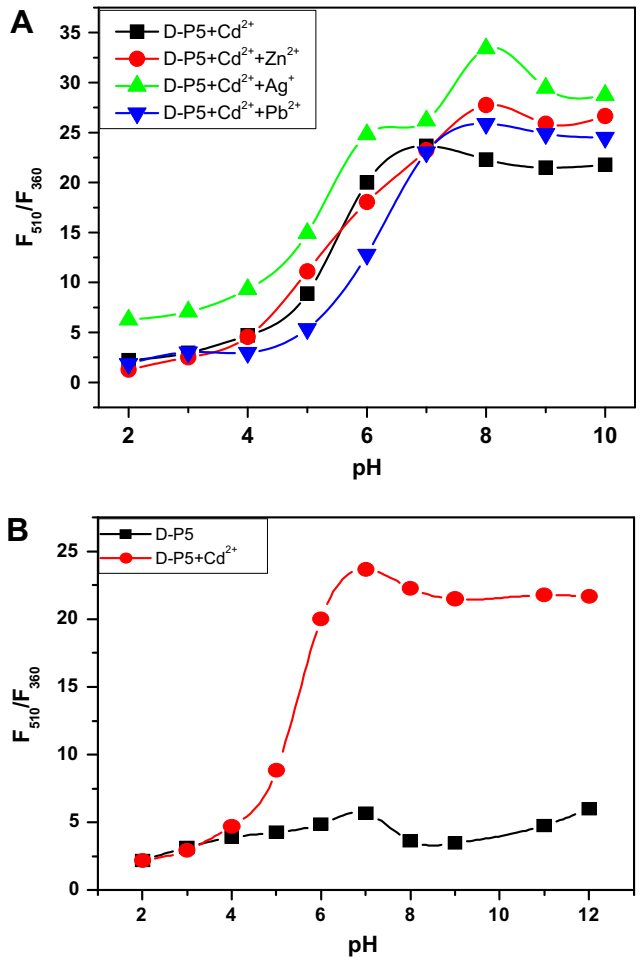

Figure 5. (A) The fluorescence ratio F_{510}/F_{360} of Zn^{2+}, Ag^{+}, Pb^{2+} to D-P5/Cd^{2+} system. (B) Fluorescence emission ratio of D-P5 and D-P5/Cd^{2+} (10 μM) in 50 mM HEPES solution at different pH with an excitation at 290 nm. The pH value was adjusted by $HClO_4$ and NaOH.

Zn^{2+}, Pd^{2+} and Ag^{+} to D-P5/Cd^{2+} system at different pH. We measured the florescence emission spectra at different pH. We found the pH value with minimal interference of these ions was pH 7. The emission intensity of D-P5/Cd^{2+} reached the maximum at pH

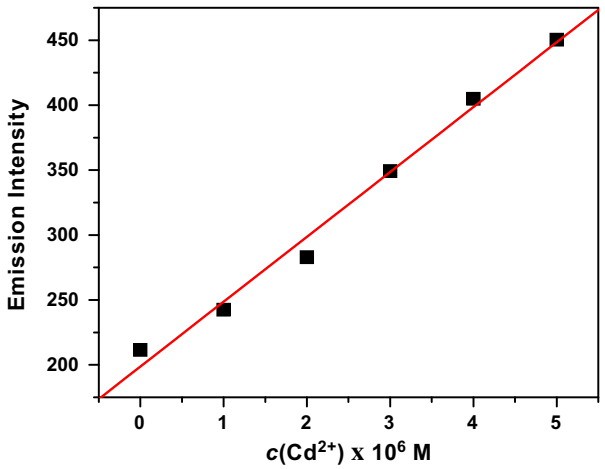

Figure 6. Linear relationship of emission intensity and concentration of Cd^{2+}.

7 and the saturation of the fluorescence emission intensity of D-P5 was achieved at 0.5 of $c(Cd^{2+})/c$(D-P5) in above condition. The optimal conditions with minimal interference was pH 7 and $c(Cd^{2+})/c$(D-P5) = 1:1.

In order to investigate the pH effect on the response of D-P5 to Cd^{2+}, pH titration was performed in 50 mM HEPES solution (Fig. 5B). Results showed that the F_{510}/F_{360} ratio was very low at pH <5 both in the presence or absence of Cd^{2+}. This result indicates that the addition of Cd^{2+} has no influence on the fluorescence emission of D-P5, probably due to the protonation of dimethylamino group ($pK_a \approx 4$) of dansyl fluorophore.[18] At pH $\geqslant$5, the F_{510}/F_{360} ratio of D-P5/Cd^{2+} increased with increasing pH, but the F_{510}/F_{360} ratio of D-P5 showed negligible change. This observation suggests that the interaction of D-P5 with Cd^{2+} occurs at pH >5, which enhances the FRET effect obviously. This might be due to the increasing of the negative charge of side groups of Cys residues.[11] The ratio of F_{510}/F_{360} reached the maximum at pH 7 and remained stable over the range of pH 7–12. This suggests that D-P5 detection for Cd^{2+} can be reliable in neutral and basic conditions.

We have studied the effects of counter ions in the same experimental condition. Due to the limit of solubility of different cadmium salt, we chose the sodium salt containing different anions as counter ion. The emission intensity at 510 nm was almost unchanging even after the concentration of the sodium salt was increased to 1000 times of D-P5 (Fig. S6).

We calculated the detection limit of Cd^{2+} on the basis of the linear relationships between the emission intensity and concentration of Cd^{2+}. Figure 6 showed a linear change of intensity as a function of the concentration of Cd^{2+}. The calculated detection limit was to be 33 μg/L.

In conclusion, a new fluorescent peptide chemsensor based on Dansyl fluorophore and tryptophan was synthesized by Fmoc solid-phase peptide synthesis. The sensor was designed to utilize FRET effect between Trp residue and dansyl group, and successfully exhibited a ratiometric response for Cd^{2+}. It showed high sensitivity and high selectivity toward Cd^{2+} and formed 2:1 complex with Cd^{2+} in solution. The sensor could be a potentially applicable for the detection of Cd^{2+} in neutral and basic conditions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.088.

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