A protein-supported fluorescent reagent for the highly-sensitive and selective detection of mercury ions in aqueous solution and live cells†

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A fluorescent sensor, dansyl-L-aspartic acid (1), coupled with BSA was used to specifically detect Hg^{2+} in a neutral aqueous solution as well as in live cells; the fluorescence emission spectrum underwent an obvious blue shift with an enhancement in fluorescence intensity, and these effects were evident as color changes in fluorescence imaging pictures.

The recent development of selective and sensitive fluorescent tools capable of detecting heavy- or transition-metal ions has attracted considerable attention due to the widespread use of these metals and their subsequent impact on the environment and nature.[1] Hg^{2+} is a highly toxic heavy metal ion and, even at very low concentrations, its toxicity has long been recognized as a problem in environmental and health systems.[2] For the selective detection of Hg^{2+}, several optical techniques have been developed, based on Hg^{2+}-induced changes in the UV-Vis or fluorescence spectra of the indicators.[3] However, the design of sensors that give fluorescent enhancement (FE) upon Hg^{2+} binding is a particular challenge because, like many other heavy metals, Hg^{2+} often causes fluorescence quenching *via* enhanced spin–orbit coupling associated with the heavy atom effect.[4] This limitation may be overcome by coupling of the fluorophore to a protein within its hydrophobic region as has been reported for the coupling between an environment-sensitive probe and protein, either in a covalent or non-covalent manner, which induced dramatic changes of fluorescent properties.[5] The complexation of a fluorophore in the hydrophobic area of a water-soluble protein has the added advantage of facilitating the probe's solubility in aqueous media, as low water solubility is another often encountered obstacle in the development of fluoroionophores.[1e,4a,6]

Herein, we report a fluorometric assay for Hg^{2+} as proof of principle for protein-supported fluorescence enhancement of sodium dansyl-L-aspartic acid (**1**) (Fig. 1) with a signification emission wavelength shift in a neutral aqueous solution. We chose the dansyl group as the fluorophore of **1** due to its characteristic photophysical properties.[7] Its emission properties are strongly dependent upon the nature of the environment; in particular, it exhibits a large blue shift on going from a polar to a nonpolar environment.[8] In addition, as we demonstrated earlier with a water-soluble Pb^{2+} probe,[1e] the incorporation of an amino acid in **1** mainly enhanced its water solubility, and in the present case, it may also enhance the electrostatic interaction of **1** with proteins. The molecular structure of **1** (Fig. 1), was confirmed by its spectroscopic data (ESI†). Bovine serum albumin (BSA), with a well characterized hydrophobic cavity,[9] was chosen as the support protein to change the polarity of the dansyl group environment. The addition of Hg^{2+} to an aqueous solution of **1** alone resulted in strong fluorescence quenching, while in the presence of BSA, the addition of Hg^{2+} induced a blue shift and enhanced the fluorescence emission, suggesting the potential for the highly-sensitive and selective detection of Hg^{2+} in aqueous solution and in live cells.

The addition of BSA to a solution of **1** changed the fluorescence spectrum (Fig. 2). **1** itself had a very weak emission in solution. The addition of 5 equiv. BSA to **1** solution led to a pronounced blue shift ($\Delta\lambda_{max}$ = 44 nm) and a significant increase in the intensity (about 10.7 times) of the fluorescence emission (Fig. S1†). These spectral changes were caused by a complexation, in which **1** is strongly bound to the hydrophobic region of the BSA through hydrophobic interaction.[9a,d] The fluorometric titration measurement shows a binding constant ($K_{\mathbf{1}/BSA}$) of $(6.35 \pm 0.03) \times 10^4\ M^{-1}$ (R^2 = 0.995) with a 1 : 1 stoichiometry between **1** and BSA (Fig. S1†),[10] indicating one BSA can occupy a sensor in its hydrophobic cavity.

The addition of Hg^{2+} to an aqueous solution containing the complex of **1** and BSA (**1**/BSA) induced additional spectral shifts toward shorter wavelengths and augmented the fluorescence emission. As shown in Fig. 2 (from (b) to (k)), **1**/BSA could easily detect Hg^{2+} at low concentration (Fig. 2(c)) and a detection limit of 0.5 μM can be estimated (Fig. 3 and S2†) as displaying changes in fluorescence properties upon the recognition of Hg^{2+} in aqueous solution (pH 6.7). During titration, the emission band was further blue-shifted by about 35 nm and the intensity was enhanced 1.5 times maximally. The changes in the enhanced ratio of fluorescence intensity $(I - I_0)/I_0$ (I_0 is the fluorescence intensity of **1**/BSA) at the maximal emission peak (λ_{max}) were measured upon the addition of 15 different metal ions (Fig. 3). The additions of Na^+, Mg^{2+},

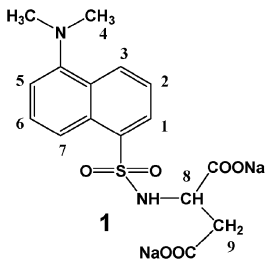

Fig. 1 Chemical structures of sodium dansyl-L-aspartic acid (**1**).

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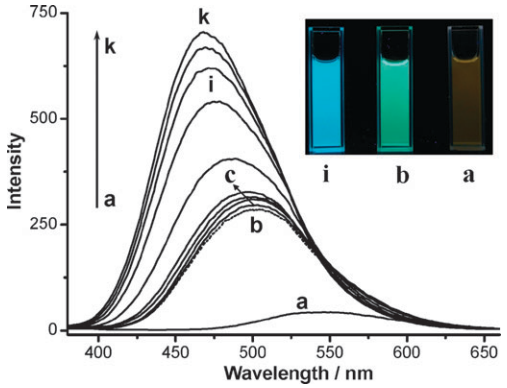

Fig. 2 Fluorescence emission spectra of (a) **1** (30.0 μM), (b) **1**/BSA (30.0/15.0 μM) and (c–k) **1**/BSA/Hg^{2+} with different concentrations of Hg^{2+} from 1.0 to 150.0 μM in 50.0 mM NaAc solution (pH 6.7). Inset shows fluorescence photographs of (a), (b) and (i) (here [Hg^{2+}] is 90.0 μM) under illumination with 365 nm light.

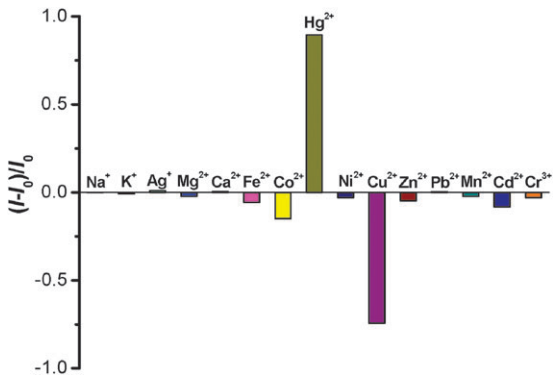

Fig. 3 $(I - I_0)/I_0$ ratios of **1**/BSA (30.0 μM/15.0 μM) in the presence of various metal ions (60.0 μM) in 50.0 mM NaAc solution (pH 6.7).

K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+} or Pb^{2+} did not have similar results as Hg^{2+}, *i.e.*, either they displayed no further blue-shift or there was no intensity enhancement other than a certain extent quenching of fluorescence of **1**/BSA (Fig. S3†). Therefore, **1**/BSA exhibited specific selectivity for Hg^{2+} over the other examined metal ions in aqueous solution.

The specific recognition for Hg^{2+} required the co-contributions of **1** and BSA. While the addition of Hg^{2+} enhanced fluorescence emission of the complex of **1**/BSA, strong fluorescence quenching occurred when Hg^{2+} was mixed with either BSA or **1** separately (Fig. S4 and S5†). The fluorometric titration measurements showed binding constants for BSA/Hg^{2+} and **1**/Hg^{2+} with 1 : 1 stoichiometry of $K_{BSA/Hg} = (5.47 \pm 0.70) \times 10^3\ M^{-1}$ (R^2 = 0.998) and $K_{\mathbf{1}/Hg} = (1.71 \pm 0.12) \times 10^5\ M^{-1}$ (R^2 = 0.991), respectively (Fig. S4 and S5†). Comparison of the binding constants show that $K_{\mathbf{1}/Hg} > K_{\mathbf{1}/BSA} > K_{BSA/Hg}$, revealed that the interaction of **1** and Hg^{2+} is evidently stronger than those of **1**/BSA and BSA/Hg^{2+} and, consequently, supply a possibility of Hg^{2+} detection after the formation of a complex between **1** and BSA.

The concentration of BSA affects the response of **1** to Hg^{2+} using the BSA platform. The equiv. ratio between **1** and BSA strongly influenced the response of **1**/BSA to Hg^{2+}, which confirmed that a ratio of 2 : 1 of **1**:BSA was best for Hg^{2+} detection (Fig. S6†). Meanwhile, a study of the effect of pH on the $(I - I_0)/I_0$ and $\Delta\lambda_{max}$ of **1**/BSA with the addition of Hg^{2+} indicated that a pH between 6.0–7.0 was the optimum condition (Fig. S7†). Therefore, in the current study, all the determinations of Hg^{2+} and other metal ions are carried out in the presence of 30.0 μM **1** and 15.0 μM BSA in 50.0 mM NaAc solution (pH 6.7). We also investigated the potential interference of competing metal ions on Hg^{2+} detection. The presence of a 1-fold molar excess of Cu^{2+} reduced the fluorescence intensity response for Hg^{2+} detection, but accentuated the blue-shift of fluorescent peak. No obvious interferences by other metal ions (Na^+, Ca^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Cr^{3+} *etc.*) were observed (Fig. S8†).

Upon substituting the naphthalenyl group for the dansyl moiety in **1**, we noted that the addition of Hg^{2+} to the new complex and BSA induced only detectable fluorescence quenching instead of the enhanced and blue-shifted response (Fig. S9†). When dansylated derivatives of glutamic acid, serine, phenylalanine or tryptophan were prepared, similar phenomenon as for the aspartyl variant **1** were achieved (data not shown). These results demonstrate that on the platform of BSA, the dansyl moiety of **1** plays a crucial role while aspartic acid is not an exclusive amino acid required for the specific recognition of Hg^{2+}.

NMR experiments were performed to explore the coordination mechanism between **1** or **1**/BSA and Hg^{2+}. Comparison of the ^{1}H NMR spectra of **1** before and after the addition of BSA (0.01 equiv.) and Hg^{2+} (from 0.5 to 1.5 equiv.) (Fig. 4 and S10†) revealed that all the protons shifted upfield upon BSA binding, while obvious shifts downfield were observed with gradual addition of Hg^{2+}. Chelation of Hg^{2+} with 5-dimethylamino and $-COO^-$ close to C(9) led to large chemical shifts of H(5) ($\Delta\delta_{H5}$ = 0.087 ppm), H(7) ($\Delta\delta_{H7}$ = 0.161 ppm) and H(9) ($\Delta\delta_{H9}$ = 0.064 and −0.057 ppm) in **1** (the detailed mechanistic investigations that would be required to probe the observed prompting effect of BSA are beyond the scope of the present study).

Finally, **1**/BSA was used for imaging in fetal bovine serum (Fig. S11†) and B16–F10 cells to confirm that our fluoroprobe can detect Hg^{2+} in live cells. To observe the entry of free **1** and Hg^{2+} into mammalian cells, B16–F10 cells were dyed with **1**

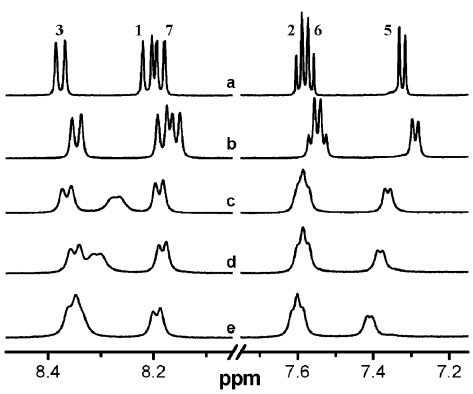

Fig. 4 Selected region of the ^{1}H NMR spectra of (a) **1** (10.0 mM), (b) **1**/BSA (1 : 0.01), (c) **1**/BSA/Hg^{2+} (1 : 0.01 : 0.5), (d) **1**/BSA/Hg^{2+} (1 : 0.01 : 1) and (e) **1**/BSA/Hg^{2+} (1 : 0.01 : 1.5) in D_2O.

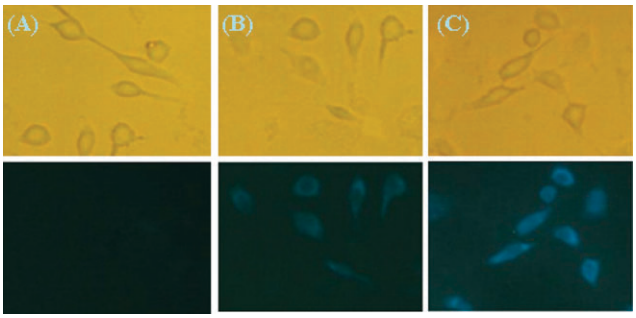

Fig. 5 Bright field (up) and fluorescence microphotographs (bottom) of $HgCl_2$ uptake by live B16–F10 cells: Images were taken by a fluorescent microscope. The excitation wavelength is at 350 nm. (A) Control cells with neither **1** nor $HgCl_2$; (B) 30 min after exposure to 30.0 μM **1**; (C) 30 min exposure to 30.0 μM **1** and further 30 min exposure to 100 μM $HgCl_2$. Conditions of the microscope were the same for (A–C).

(30.0 μM) prior to exposure to 100.0 μM $HgCl_2$. Fig. 5 shows a series of images recorded in the absence and presence of **1** and $HgCl_2$ by using the described microscope (ESI†). The cells became markedly different in brightness between Fig. 5A, B and C, and were distinguishable from the background as they took up the probe and the mercury.

In conclusion, we have established a strategy for a protein-supported Hg^{2+} probe, which was used to specifically detect Hg^{2+} in high sensitivity (0.5 μM) over other 14 metal ions in neutral aqueous solution and in live cells by monitoring the changes in the fluorescence emission wavelength and intensity. This is the first report of a protein-supported probe for the detection of heavy metal ions in live cells. The detection of Hg^{2+} by **1**/BSA is proof that specific properties of the support protein strongly influence the emission behavior of **1** both in aqueous solution and in live cells. Although we have used this system to demonstrate the detection of Hg^{2+} ion only, this approach holds potential application to other metal ions, DNA, or protein through the complexation of the appropriate fluorescent reagent and a compatible protein platform.

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