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Turn-on fluorescent probe with visible light excitation for labeling of hexahistidine tagged protein

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ABSTRACT

We report here the development of a novel fluorescein-based probe which shows selective fluorescence enhancement on binding to a hexahistidine-tagged protein. No fluorescence change was observed with untagged protein. This probe is excitable with visible light and is considered to be suitable for use in biological applications.

Fluorescence labeling of proteins is a powerful technique in protein research.[1] Many labeling techniques have been developed based on the reaction of fluorescent dyes bearing functional groups, such as succinimidyl ester or maleimide, that react with primary amines or thiols exposed on a protein surface. However, these techniques are typically nonspecific. The most commonly applied method for selective labeling of a target protein is to express the protein as a fusion with a fluorescent protein, such as green fluorescent protein (GFP).[2] Although this technique offers absolute specificity, there remain some limitations, such as significant perturbation of folding, trafficking, and function of proteins to which GFP is fused.[3] In attempts to overcome these limitations, alternative labeling techniques have been developed, including the introduction of a short peptide that is able to react/interact specifically with a designed fluorescent molecule.[4–6] This approach has several advantages, for example, reducing perturbation caused by attachment of the fluorophore, and offering the possibility of using a wide variety of functional molecules. However, most of the reported techniques show no significant change in fluorescence intensity upon the recognition of a peptide tag,[7] so detection requires fluorescence anisotropy measurement,[6d,e] or the use of the fluorescence resonance energy transfer technique[6a] with another fluorophore.

We have recently developed 'turn-on' fluorescent probes which show an increase in fluorescence in the presence of hexahistidine peptide.[8] Our strategy is summarized in Scheme 1. To control the fluorogenic property, we adopted an intramolecular fluorophore displacement strategy, as has been successfully applied to the fluorometric detection of nitric oxide,[9] pH,[10] and anions.[11] An intramolecular fluorescent ligand coordinates to a metal ion chelated by nitrilotriacetic acid (NTA) with a relatively weak affinity, and is displaced in the presence of the hexahistidine peptide sequence, resulting in enhanced fluorescence. The 'turn-on' nature is preferable in terms of improved signal-to-noise ratio and possible application for one-pot labeling without purification or washing-out of excess unreacted probe. The probes that we developed contain a coumarin moiety as the fluorophore, and this requires UV light excitation.[12] Although such probes could be useful for many in vitro protein labeling/detection experiments, some applications, including cell-imaging, are hampered by the need for UV excitation. UV light excitation is subject to interference by autofluorescence of biological molecules, such as pyridine and flavin nucleotides. We describe herein a new turn-on probe which is excitable by visible light. This probe is expected to be especially useful for the labeling of tagged proteins in biological applications.

We designed the fluorescein derivative shown in Figure 1, composed of metal-NTA complex as the hexahistidine tag recognition site, fluorescein as the fluorophore, and a linker. The hydroxyl group of fluorescein is expected to coordinate to the metal-NTA complex. A fluorescein derivative, calcein, is a known indicator of various metal ions, and shows a fluorescence decrease upon coordination with metal ion.[13]

The designed fluorescein derivative **4** without metal ion was synthesized according to Scheme 2. Commercially available fluorescein **1** was formylated via Reimer–Tieman reaction to give monoaldehyde **2** according to the reported procedure.[14] The NTA

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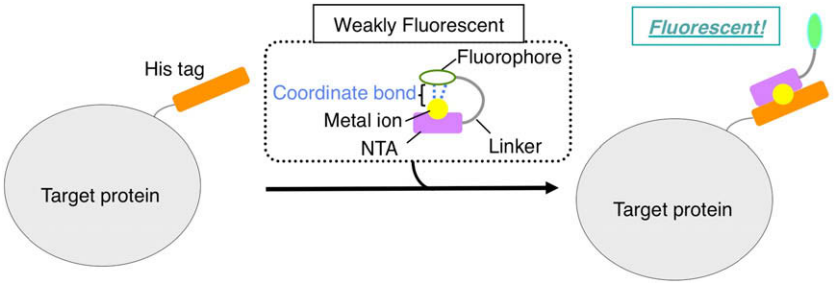


Scheme 1. Our strategy for protein labeling.

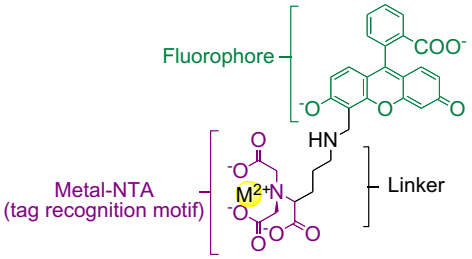


Figure 1. Design of fluorescent probe. M^{2+} = Ni^{2+}, Co^{2+}.

unit **3** was synthesized from *N*-δ-benzyloxycarbonyl-L-ornithine as reported.[8] The target compound **4** was obtained by reductive amination of **2** and **3**, followed by hydrolysis with lithium hydroxide.

As the metal ion, Ni^{2+} and Co^{2+} were examined. These metal ions are good candidates from the standpoint of both fluorescence quenching and hexahistidine tag recognition.[8] Ni^{2+}-NTA and Co^{2+}-NTA are well known to interact selectively with hexahistidine sequences. We investigated the fluorescence of **4** in the absence or presence of Ni^{2+} or Co^{2+}. Compound **4** itself is highly fluorescent in 50 mM Tris buffer (pH 7.4). Upon addition of Ni^{2+} or Co^{2+} ion, the fluorescence was decreased in a concentration-dependent manner, as shown in Figure 2a. A Job's plot analysis was performed to determine the complexation stoichiometry of **4** with Ni^{2+} or Co^{2+}. The plots showed a peak at 0.5, indicating the formation of a 1:1 complex in each case (Fig. 2b). This result is consistent with our hypothesis that fluorescein coordinates intramolecularly to the metal, which is chelated by NTA. The complex formation was also investigated by isothermal titration calorimetry (ITC), and the

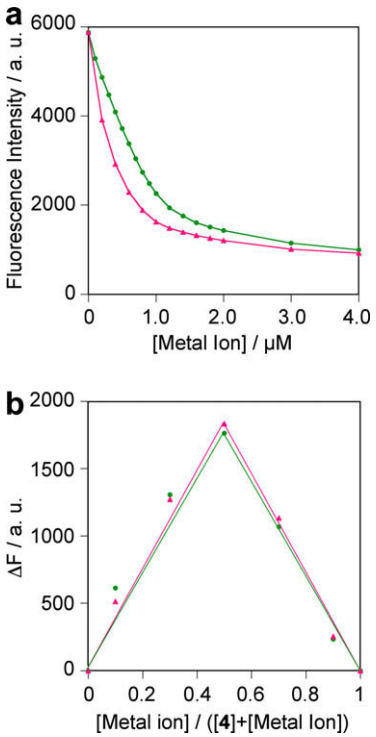
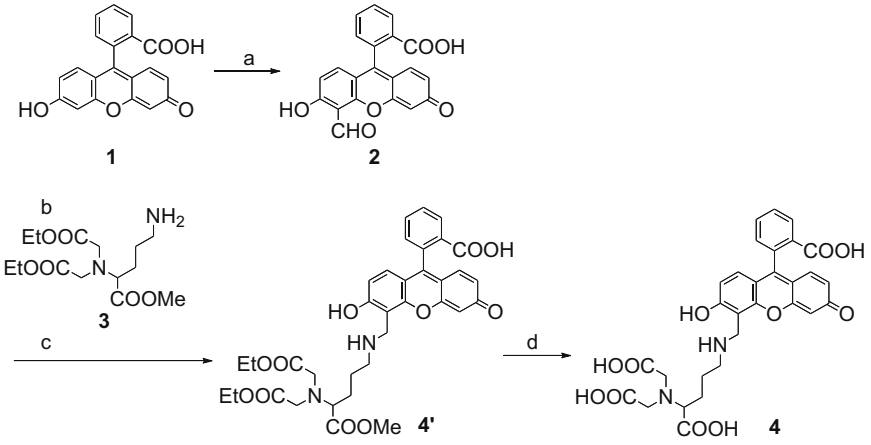


Figure 2. (a) Fluorescence response of **4** (1.0 μM) in the presence of metal ion. (b) Job's plot of fluorescence changes upon complexation of **4** with metal ion. [**4**] + [metal ion] = 1.0 μM. Solvent: 50 mM Tris buffer (pH 7.4). Closed circle (green): Co^{2+}, closed triangle (red): Ni^{2+}.



Scheme 2. Synthesis of metal-free target molecule. Reagents and conditions: (a) 50% NaOH, 15-crown-5, $CHCl_3$, 18%; (b) CH_3OH, $CHCl_3$; (c) $NaBH(OAc)_3$, dichloroethane, 62%; (d) LiOH, $MeOH/H_2O$, 49%.

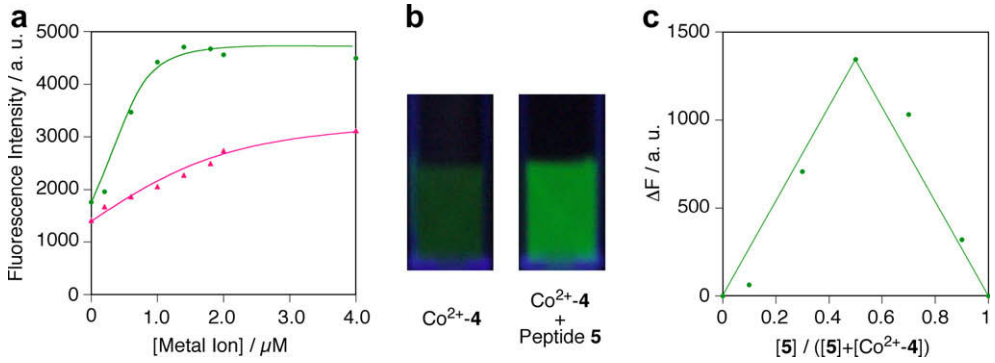


Figure 3. (a) Fluorescence response of 1.0 μM metal-**4** complexes (**4**: 1.0 μM, $CoCl_2$: 1.0 μM) in the presence of peptide **5** (H-$(His)_6$-Tyr-NH_2) in 50 mM Tris buffer (pH 7.4). Closed circle (green): Co^{2+}-**4**, closed triangle (red): Ni^{2+}-**4**. (b) Photographs of the fluorescence of the probe in 50 mM Tris buffer (pH 7.4). (c) Job's plot of the fluorescence change upon complexation of Co^{2+}-**4** complex with peptide **5** in 50 mM Tris buffer (pH 7.4) at 25 °C. [Co^{2+}-**4** complex] + [peptide **5**] = 1.0 μM. Co^{2+}-**4** complex was prepared by mixing equimolar amounts of **4** and $CoCl_2$. Excitation: 490 nm, emission: 510 nm.

binding constants of $2.3 \times 10^6\ M^{-1}$ for Ni^{2+} and $0.74 \times 10^6\ M^{-1}$ for Co^{2+} were obtained.

We investigated the fluorescence change of metal-**4** complexes upon addition of a model hexahistidine tag peptide **5** (H-$(His)_6$-Tyr-NH_2; Tyr was attached to allow convenient determination of concentration).[15] Solutions of Ni^{2+}-**4** and Co^{2+}-**4** complexes were prepared in situ by combining **4** with $NiCl_2$ or $CoCl_2$ in a 1:1 ratio in 50 mM Tris buffer (pH 7.4). Peptide was added to the Ni^{2+}-**4** and Co^{2+}-**4** solutions and the fluorescence spectra were measured. The fluorescence increase of metal-**4** complexes was dependent on the peptide concentration, as shown in Figure 3a. Co^{2+}-**4** complex showed a larger fluorescence change than Ni^{2+}-**4** complex, suggesting that the Co^{2+}-**4** complex is superior for our purpose. The intrinsic fluorescence of Co^{2+}-**4** complex was weak, whereas strong green fluorescence was observed in the presence of peptide **5**, as shown in Figure 3b. The final fluorescence recovery upon binding of Co^{2+}-**4** to the peptide **5** reached >80% of the fluorescence intensity of the corresponding metal-free dye **4**. To determine the stoichiometry of the Co^{2+}-**4** and hexahistidine peptide **5** complex in solution, Job's plot analysis of the fluorometric change was employed. Job's plot showed a maximum at the molar ratio of 0.5, indicating the formation of a 1:1 complex (Fig. 3c).

The fluorescence quantum yield of Co^{2+}-**4** complex is 0.11, and this was increased to 0.62 upon addition of an excess amount of peptide **5**; the increase in fluorescence quantum yield was 5.6-fold. The apparent dissociation constant K_d of Co^{2+}-**4** complex for peptide **5** was calculated to be 1.5×10^{-7} M, which is comparable with those of other Ni^{2+}-NTA or Co^{2+}-NTA based probes, including the probes that we previously developed.[6,8] A similar value was obtained by ITC; the binding constant K_a was calculated to be $1.1 \times 10^6\ M^{-1}$. As shown in Scheme 1, we hypothesize that the fluorescence enhancement of Co^{2+}-**4** complex is due to the formation of the ternary complex of Co^{2+}, **4**, and peptide **5**. However, similar fluorescence enhancement is expected when Co^{2+} is removed from Co^{2+}-**4** complex by peptide **5**. To confirm our hypothesis, we examined the labeling of immobilized peptides with Co^{2+}-**4** complex. We prepared H_6-angiotensin I (H-$(His)_6$-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and angiotensin I (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) bound on TentaGel S-NH_2 resin via stable amide bond linkage. The peptide-bound beads were treated with Co^{2+}-**4** complex and washed with buffer. Bright fluorescence was observed from the beads bearing H_6-angiotensin I, whereas little fluorescence was observed from the beads bearing angiotensin I (Fig. S1, Supplementary data). This result supports our hypothesis.

The interaction between Co^{2+}-**4** and a hexahistidine tag on a protein surface was evaluated using a soluble protein, RNase S. RNase S can be prepared by reconstruction of S-protein and S-peptide.[16] We synthesized S-peptide and hexahistidine-tagged S-peptide for the preparation of RNase S and hexahistidine-tagged RNase S, respectively. RNase S was prepared by mixing equimolar S-protein and synthesized S-peptides, and the successful reconstruction of RNase S was confirmed by measuring the enzymatic activity (see Supplementary data). The fluorescence change of Co^{2+}-**4** was observed upon addition of the RNases. As shown in Figure 4a, strong fluorescence enhancement was seen when hexahistidine-tagged RNase S was added. On the other hand, only a very small fluorescence change was observed upon addition of RNase S (Fig. 4b). These results suggest that our probe selectively recognizes hexahistidine-tagged protein.

In conclusion, we have developed a novel 'turn-on' fluorescent probe that binds selectively to the tag of a hexahistidine-tagged

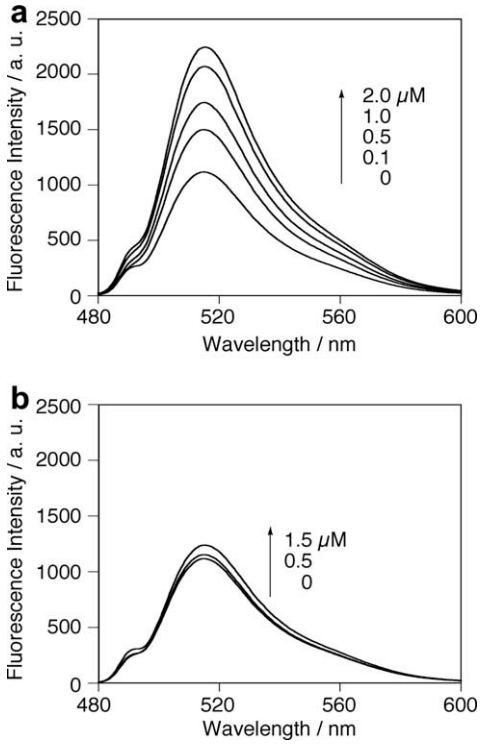


Figure 4. Emission spectra of Co^{2+}-**4** complex in the presence of (a) hexahistidine-tagged RNase S, (b) RNase S in 10 mM HEPES-buffer (pH 7.4) and 100 mM NaCl. Co^{2+}-**4** complex was prepared in situ by mixing **4** and $CoCl_2$ (**4**: 0.5 μM, $CoCl_2$: 0.5 μM). All spectra were obtained with excitation at 490 nm.

protein, with a substantial enhancement of its fluorescence. The new probe contains fluorescein as the fluorophore, which is excitable by visible light. This probe showed weak intrinsic fluorescence, but addition of hexahistidine-tagged peptide in neutral aqueous buffer resulted in up to 5.6-fold increase in the fluorescence quantum yield. The new probe is useful for the labeling of hexahistidine tag on a protein surface, as well as hexahistidine peptide. Our results indicate that this probe would be suitable for detecting tagged proteins in biological applications, such as cell imaging.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.084.

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