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Construction of a 'turn-on' fluorescent probe system for His-tagged proteins

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ABSTRACT

Hexahistidine ($(His)_6$) tags are used to purify genetically engineered proteins. Herein, we describe the construction of a 'turn-on' fluorescent probe system that consists of the fluorescence quencher, Dabcyl, conjugated to $(His)_6$, and fluorescent tetramethylrhodamine conjugated to nitrilotriacetic acid, which, in the presence of Ni^{2+}, can bind $(His)_6$. The system is turned off when Dabcyl-$(His)_6$ is bound to the fluorescent nitrilotriacetic acid derivative. The binding strength of this system was assessed using electrospray ionization mass spectrometry, fluorescence correlation spectroscopy, and fluorescence intensity distribution analysis-polarization. Although there was no significant enhancement in fluorescence after addition of an equimolar amount of ubiquitin, the fluorescence increased from 14% to 40% of its initial intensity when an equimolar amount of $(His)_6$-ubiquitin was added. Therefore, this system should be able to specifically recognize $(His)_6$-proteins with good resolution and has the additional advantage that a washing step is not required to remove fluorescent probe, that is, not bound to the $(His)_6$-protein.

Fluorescence imaging is an excellent way to visualize biomolecules under physiological conditions because it is sensitive and has great temporal/spatial resolution.[1,2] When covalently bound to a genetically engineered protein, a fluorescent protein, for example, the green fluorescent protein, can serve as a marker for the genetically engineered protein, because its fluorescence can be specifically monitored and it is a stable label.[3,4] However, the fluorescent protein may negatively affect the dynamics of the genetically engineered protein as a result of its large molecular weight or affect the functions (e.g., localization, mobility and transport pathway) of the target protein because the folding of the genetically engineered protein is altered by the attached fluorescent protein.[5] Conversely, when a target protein is labeled with a small chemical probe, the aforementioned disadvantages can be circumvented. Small chemical probes have also been used to tag genetically engineered proteins.[6–12] Hexahistidine ($(His)_6$) has been widely used as an affinity tag for purification of genetically engineered proteins.[13] Fluorescent molecules that recognize proteins tagged with $(His)_6$ are convenient probes and have been used in various applications, for example, live cell imaging[10,11] and analyzes of protein structure.[14]

Recently, Higuchi and co-workers described a 'turn-on' fluorescent probe system that target His-tagged proteins via binding to an intramolecular complex formed between a fluorophore and a receptor for $(His)_6$.[15] This kind of 'turn-on' system has the advantage that a washing step that removes probes that are not bound to a His-tagged protein is not necessary, which simplifies the labeling procedure. However, for this type of 'turn-on' fluorescent probe system, the type of fluorophore that can be used is restricted to make an intramolecular complex between the fluorophore and the receptor for $(His)_6$. To date, coumarin and fluorescein derivatives have been used as the fluorophores in this system.[15,16] The selection of the fluorophore is important for, among other techniques, multicolor imaging, fluorescence resonance energy transfer analysis, and single molecular imaging.

Herein, we describe a new type of a 'turn-on' fluorescent probe system that includes a quencher conjugated to $(His)_6$, that inhibits the fluorescence of the 'turn-on' probe by binding to it in the absence of a competing $(His)_6$-protein (Scheme 1). Quenchers have been used during the development of fluorogenic nucleic acid hybridization probes, for example, molecular beacon[17] and strand-displacement probes.[18] In our approach, a quencher is utilized to overcome the restriction of fluorophore selection because the intramolecular complex to quench the fluorescence between the fluorophore and the receptor for $(His)_6$ is not required. The general strategy, exemplified by Scheme 1, should allow us to choose among different combinations of fluorophores and quenchers and ligands and receptors. As diagramed, the fluorophore is conjugated to a receptor, and the quencher is conjugated to the ligand. If the ligand is also conjugated to a protein, the system is 'turned on' when the quencher-conjugated ligand is displaced from the receptor by the ligand-conjugated protein. As proof-of-concept experiments, we developed and tested the following system. For the

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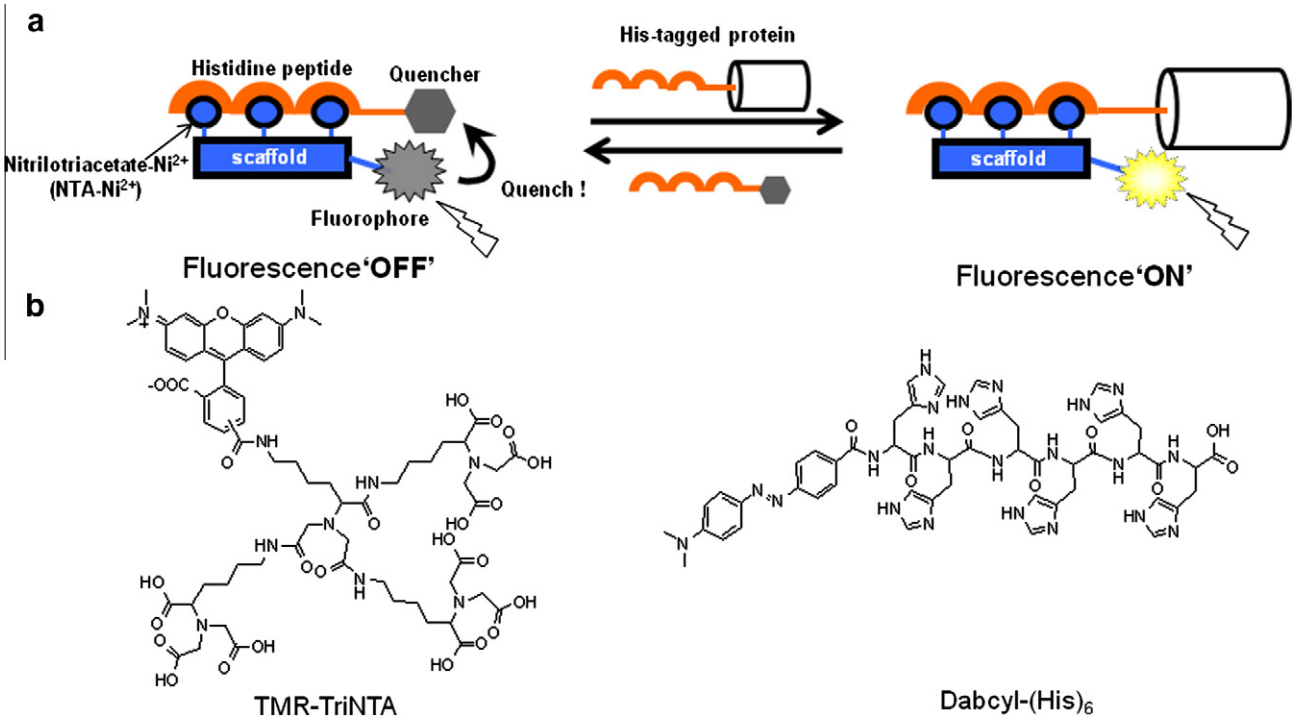

Scheme 1. (a) Conceptual scheme of a 'turn-on' fluorescent probe system that uses the ability of a $(His)_6$-protein to displace a $(His)_6$-quencher from a fluorescent probe. (b) Structures of the fluorescent probe (TMR-TriNTA) and the $(His)_6$-quencher (Dabcyl-$(His)_6$) used in the study reported herein.

ligand, a $(His)_6$ was coupled to the quencher Dabcyl (Dabcyl-$(His)_6$; Scheme 1b). For the receptor, fluorescent tetramethylrhodamine was conjugated to tris-nitrilotriacetic acid (TMR-TriNTA; Scheme 1b), which can form a complex with histidine in the presence of Ni^{2+}. The system was tested by adding either ubiquitin or $(His)_6$-ubiquitin to a solution of Dabcyl-$(His)_6$, TMR-TriNTA, and Ni^{2+}. In summary, our immediate goal was to develop a system that could be used to identify a $(His)_6$-protein by constructing a fluorescent probe, that is, turned on only when bound to the $(His)_6$-protein.

The synthetic procedure for TMR-TriNTA is summarized below and greater detail is shown in Supplementary data. NH_2-tri-NTA(t-Bu)$_3$ was synthesized as described.[19] 5(6)-Carboxytetramethylrhodamine *N*-succinimidyl ester was conjugated to the amino group of NH_2-tri-NTA(t-Bu)$_3$, after which the *tert*-butyl groups were removed by treatment with TFA to give TMR-TriNTA.

The intermolecular interactions among TMR-TriNTA, Ni^{2+}, and $(His)_6$ were assessed using electrospray ionization mass spectrometry (ESI-MS), which can detect noncovalent complexes that involve intermolecular hydrogen and coordination bonds because the soft ionization used for ESI does not disrupt such bonds.[20–22] A solution of CH_3CN/H_2O (1:1, v/v) that contained 5 μM TMR-TriNTA, 15 μM $NiCl_2$, and 5 μM $(His)_6$ was subjected to ESI-MS. The flow rate, capillary temperature, and spray voltage were 10 μl/min, 250 °C, and 4.5 kV, respectively. We added CH_3CN to the aqueous sample solution because we could not measure the sample dissolved in pure water. The resulting ESI-MS spectrum displayed peaks that could be ascribed to the $[TMR\text{-}TriNTA + 3Ni^{2+} + (His)_6 - 9H^+]^{3-}$ (805 m/z) and $[TMR\text{-}TriNTA + 3Ni^{2+} + (His)_6 - 8H^+]^{2-}$ (1207 m/z) complexes (Fig. S1). These complexes were not detected when $NiCl_2$ was omitted (data not shown).

To characterize the strength of the TMR-TriNTA/$3Ni^{2+}$/$(His)_6$ complex, its dissociation constant (K_D) was determined using fluorescence correlation spectroscopy (FCS) and fluorescence intensity distribution analysis-polarization (FIDA-PO) in conjunction with a single-molecule fluorescence detection system (MF20, Olympus Optical Co.). Single molecule imaging, for example, FCS, is a most useful application for the investigation of the function of a protein labeled with a fluorescent group under physiological conditions. The diffusion time and the degree of polarization, which are related to the apparent molecular size of a fluorophore, can be obtained from FCS and FIDA-PO, respectively. The TMR-TriNTA/$3Ni^{2+}$ complex was purified by anion exchange chromatography (HiTrap, GE Healthcare) as described.[12] The changes in diffusion time and degree of polarization of the TMR-TriNTA/$3Ni^{2+}$ complex were measured before and after addition of ubiquitin or $(His)_6$-ubiquitin (Fig. 1). The TMR emission intensity was stable without any antifade reagents. When present at a final concentration >30 nM, $(His)_6$-ubiquitin increased the diffusion time and the degree of polarization of TMR-TriNTA/$3Ni^{2+}$, which indicated that TMR-TriNTA/$3Ni^{2+}$ and $(His)_6$-ubiquitin formed a complex via the $(His)_6$-tag (K_D = 210 nM). In the presence of ubiquitin no change in the diffusion time or the degree of polarization was seen. The K_D of TMR-TriNTA/$3Ni^{2+}$ for native histidine sequences was estimated to be higher than the K_D of $(His)_6$-ubiquitin and closely resemble the K_D of NTA/Ni^{2+} for two or three histidines (K_D is about 10–100 μM).[23] In this reason, the TMR-TriNTA/$3Ni^{2+}$ should recognize His-tag sequence more preferentially than native histidine sequences.

The fluorescence spectrum of TMR-TriNTA/$3Ni^{2+}$, before and after addition of an equimolar amount of Dabcyl-$(His)_6$, was recorded to determine the extent of fluorescence quenching. When an equimolar amount of Dabcyl-$(His)_6$ was added to a solution of 5 μM TMR-TriNTA/$3Ni^{2+}$, 20 mM Tris–HCl (pH 7.4), the fluorescence intensity of the solution decreased to 8% of its initial value (Fig. 2a). The fluorescence intensity returned to a level of 44% (5.4-fold increase) after an equimolar amount of $(His)_6$ was added (Fig. 2b). This fluorescence enhancement indicated that Dabcyl-$(His)_6$ had been partially displaced from TMR-TriNTA/$3Ni^{2+}$ and that an equilibrium had been established between the TMR-TriNTA/$3Ni^{2+}$/Dabcyl-$(His)_6$ and TMR-TriNTA/$3Ni^{2+}$/$(His)_6$ complexes. Moreover, a fluorescence titration experiment with $(His)_6$ was performed to characterize the apparent equilibrium dissociation

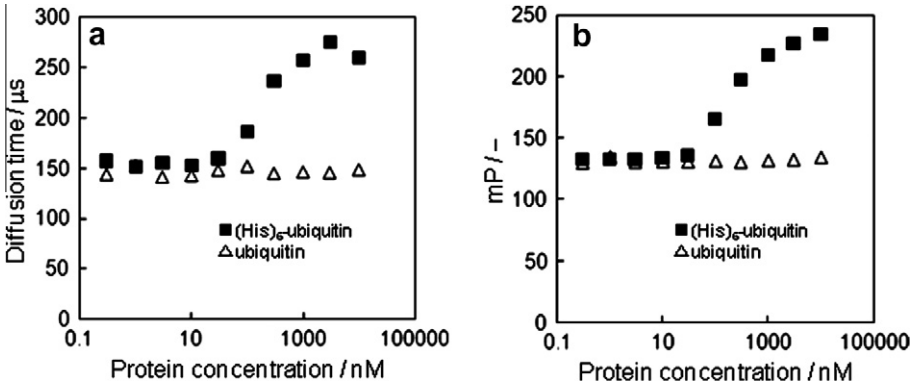

Figure 1. Binding behavior of TMR-TriNTA/3Ni^{2+} and $(His)_6$-ubiquitin or ubiquitin monitored using (a) fluorescence correlation spectroscopy and (b) fluorescence intensity distribution analysis-polarization, which were recorded using a single-molecule fluorescence detection system. Fluorescence polarization values are given as millipolarization (mP).

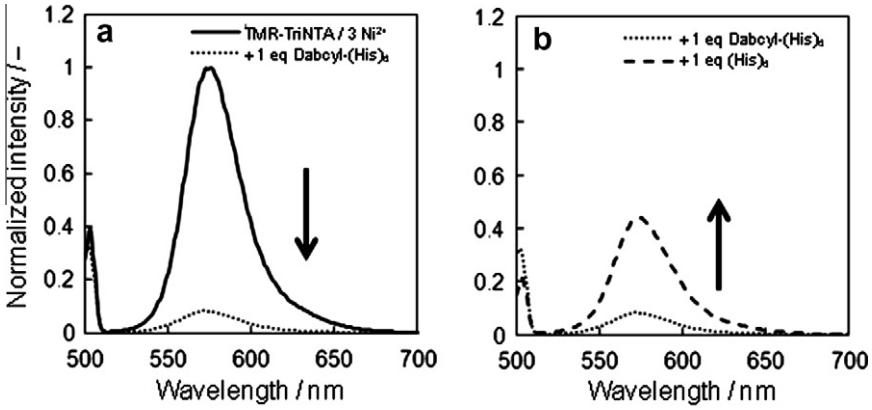

Figure 2. Fluorescent spectra of 5 μM TMR-TriNTA/3Ni^{2+} in Tris–HCl (pH 7.4) (λ_{ex} = 500 nm). (a) Before and after addition of an equimolar amount of Dabcyl-$(His)_6$, and (b) before and after addition of an equimolar amount of $(His)_6$ to the Dabcyl-$(His)_6$-containing solution of Figure 2(a).

constant (K_{app}). The fluorescence intensity was efficiently increased by addition of $(His)_6$ (f.c. 0–50 μM) to the solution of 5 μM TMR-TriNTA/3Ni^{2+}/Dabcyl-$(His)_6$. The fluorescence of the TMR was easily detected with naked eyes under UV light (365 nm) excitation (Fig. S2). The K_{app} was approximately 12 μM by fitting the data to a nonlinear least square.

To confirm that Dabcyl-$(His)_6$ had been displaced by $(His)_6$, ESI-MS spectra of solutions containing 5 μM TMR-TriNTA, 15 μM $NiCl_2$ and 5 μM Dabcyl-$(His)_6$ without (Fig. S3a) or with (Fig. S3c) 5 μM $(His)_6$ were acquired under the conditions described above. [TMR-TriNTA + 3Ni^{2+} + Dabcyl-$(His)_6$ − 8H$^+$]$^{2-}$, [TMR-TriNTA + 3Ni^{2+} + Dabcyl-$(His)_6$ − 7H$^+$]$^{3-}$, and [TMR-TriNTA + 3Ni^{2+} + Dabcyl-$(His)_6$ − 6H$^+$]$^{4-}$ were detected in the spectrum shown in Figure S3a, and, when [TMR-TriNTA + 3Ni^{2+} + Dabcyl-$(His)_6$ − 7H$^+$]$^{3-}$ (889 m/z) was subjected to collision-activated dissociation (5.0 m/z isolation band width), peaks that could be ascribed to Dabcyl-$(His)_6$

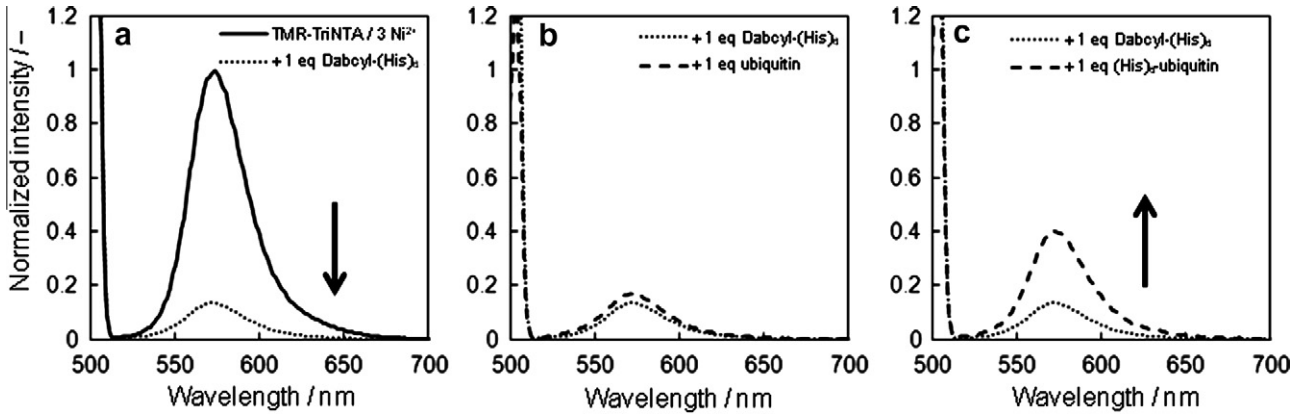

Figure 3. Fluorescent spectra of 1 μM TMR-TriNTA/3Ni^{2+} in Tris–HCl (pH 7.4) (λ_{ex} = 500 nm). (a) Before and after addition of an equimolar amount of Dabcyl-$(His)_6$, (b) before and after addition of an equimolar amount of ubiquitin to the Dabcyl-$(His)_6$-containing solution of Figure 3(a), and (c) before and after addition of an equimolar amount of $(His)_6$-ubiquitin to the Dabcyl-$(His)_6$ and ubiquitin-containing solution of Figure 3(b).

and TMR-TriNTA were found (Fig. S3b). After addition of $(His)_6$ to the solution of TMR-TriNTA/Dabcyl-$(His)_6$, the resulting ESI-MS spectrum contained peaks that were ascribed to [TMR-TriNTA + $3Ni^{2+}$ + $(His)_6$ − $8H^+$]$^{2-}$, [TMR-TriNTA + $3Ni^{2+}$ + $(His)_6$ − $7H^+$]$^{3-}$, and [TMR-TriNTA + $3Ni^{2+}$ + $(His)_6$ − $6H^+$]$^{4-}$ (Fig. S3c). Peaks ascribed to $(His)_6$ and TMR-TriNTA were found when [TMR-TriNTA + $3Ni^{2+}$ + $(His)_6$ − $7H^+$]$^{3-}$ (805 m/z) was subjected to collision-activated dissociation (5.0 m/z isolation band width; Fig. S3d).

Finally, we measured the fluorescence intensity of a 1 μM TMR-TriNTA/$3Ni^{2+}$, 20 mM Tris–HCl (pH 7.4) solution before and after sequential additions of an equimolar amount of Dabcyl-$(His)_6$, ubiquitin, and $(His)_6$-ubiquitin. The addition of Dabcyl-$(His)_6$ decreased the fluorescence of the TMR-TriNTA solution to 14% of its initial intensity (Fig. 3a). When ubiquitin was added to this mixture, there was no significant change in the fluorescent intensity during the 3-h period that the fluorescence was monitored (Fig. 3b). Conversely, after $(His)_6$-ubiquitin was added to the solution, the fluorescence increased to 40% of the initial intensity (Fig. 3c). Therefore, Dabcyl-$(His)_6$ was displaced from its complex with TMR-TriNTA/$3Ni^{2+}$ by only $(His)_6$-ubiquitin, which resulted in an enhancement in the fluorescent intensity of TMR-TriNTA/$3Ni^{2+}$.

In summary, we constructed a 'turn-on' fluorescent probe system for which the fluorescent receptor, TMR-TriNTA/$3Ni^{2+}$, is 'turned on' when the bound quencher, Dabcyl-$(His)_6$, is replaced by a $(His)_6$-protein. In near future, selectivity in the displacement between Dabcyl-$(His)_6$ and His-tagged protein will be evaluated in cell lysate. This system should be applicable to cell membrane permeability, toxicity, and non-specific adsorption studies, as well intracellular protein imaging. By optimizing suitable combinations of receptor and ligand, similar systems could also be used for single molecule imaging and multicolor imaging in conjunction with fluorescence resonance energy transfer for the analysis of living cells.

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Supplementary data

Supplementary data associated (detailed experimental procedures) with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.011.

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