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Design of a binuclear Ni(II)–iminodiacetic acid (IDA) complex for selective recognition and covalent labeling of His-tag fused proteins

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ABSTRACT

Selective protein labeling with a small molecular probe is a versatile method for elucidating protein functions under live-cell conditions. In this Letter, we report the design of the binuclear Ni(II)–iminodiacetic acid (IDA) complex for selective recognition and covalent labeling of His-tag-fused proteins. We found that the Ni(II)–IDA complex **1**-2Ni(II) binds to the His6-tag (HHHHHH) with a strong binding affinity (K_d = 24 nM), the value of which is 16-fold higher than the conventional Ni(II)–NTA complex (K_d = 390 nM). The strong binding affinity of the Ni(II)–IDA complex was successfully used in the covalent labeling and fluorescence bioimaging of a His-tag fused GPCR (G-protein coupled receptor) located on the surface of living cells.

Selective protein labeling with a synthetic molecular probe is an important research tool to facilitate detection and functional analysis of proteins. For this purpose, a variety of protein labeling methods have been developed in the last few decades to visualize the target protein in a real time manner under live-cell conditions.[1] Among them, the use of a complementary recognition pair between a short peptide-tag and a small molecular probe represents a viable strategy for selective protein labeling.[2] This method can be used to selectively label a recombinant tag-fused protein with various functional molecules at appropriate timing. This method also benefits from the small molecular sizes of both the peptide-tag and probe (<3 kDa), and thus less likely to perturb protein functions unlike the protein-based labeling methods involving GFPs (27 kDa).

The oligo-histidine tag (His-tag) is a representative epitope tag, which has been used widely in the purification or immobilization of recombinant proteins.[3] Owing to its selective binding property with a Ni(II) complex of nitriloacetic acid (NTA), several groups including us have reported the application of this tag-probe pair to the labeling of His-tag-fused proteins for fluorescence bioimaging under live-cell conditions.[4,5] However, the rather weak binding affinity of the Ni(II)–NTA complex to the His-tag (K_d = 1–10 μM) results in the requirement of excess amounts of probe and/or the use of a multinuclear Ni(II) complex (n >2) for bioimaging studies,[4d–f] which often causes non-selective labeling and a high background fluorescence signal. In addition, the synthesis of the multinuclear Ni(II) complex demands a cumbersome multistep synthetic procedure, which largely diminishes the availability of this pair for protein analysis. In this letter, we report the new design of the binuclear Ni(II)–iminodiacetic acid (IDA) complex for selective protein labeling of His-tag-fused proteins. The fluorescence binding assay revealed that the binuclear Ni(II)–IDA complex **1**-2Ni(II) binds to the His6-tag (HHHHHH) with a strong binding affinity (K_d = 24 nM), the value of which is 16-fold higher than the conventional Ni(II)–NTA complex (K_d = 390 nM). Taking advantage of the strong binding and facile structural modification of **1**-2Ni(II), the fluorescent Ni(II)–IDA derivative **5**-2Ni(II) was successfully applied to the covalent labeling and imaging of a His-tag fused GPCR (G-protein coupled receptor) located on the surface of living cells, thereby demonstrating the use of the Ni(II)–IDA probe in protein analysis.

Our design strategy of the Ni(II) complex to obtain a strong binding affinity for the His-tag was primarily based on increasing the Lewis acidity of the Ni(II) ion. We thus employed the tridentate iminodiacetate (IDA) as a ligand for the Ni(II) ion instead of the conventional tetradentate NTA ligand (Fig. 1). In the probe synthesis, introduction of the IDA units into a simple aminobenzene scaffold[6] gave the ligands **1**–**3**, each of which has a different linker unit between the scaffold and the IDA units. The ligands were then

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treated with 2 equiv of $NiCl_2$ to afford the binuclear Ni(II)–IDA complexes **1**–**3**. The Ni(II)–NTA complex **4** was also prepared as a control compound. The detail synthetic procedure is described in Supporting information.

The binding affinities of the Ni(II)-complexes with the His-tag were initially evaluated by a fluorescence quenching titration.[7] When the Ni(II)–IDA probe **1**-2Ni(II) was added to an aqueous buffer solution of the His6 peptide appended with a fluorescent 7-hydroxycoumarin unit (hc-His6), its fluorescence decreased to ~40% of its original intensity owing to the paramagnetic quenching effect of the Ni(II) ions (Fig. 2a). The change in fluorescence intensity at 448 nm was analyzed by curve fitting to afford the apparent dissociation constant K_d = 24 nM (Fig. 2b). The fluorescence Job's plot between **1**-2Ni(II) and hc-His6 clearly indicated that their binding stoichiometry is 1:1 (Fig. S1). The dissociation constants of the series of the Ni(II) complexes **1**–**4** for the hc-His6 peptide are summarized in Table 1. Interestingly, all IDA-type probes **1**-2Ni(II)–**3**-2Ni(II) showed the stronger binding affinities than the Ni(II)–NTA probe **4**-2Ni(II) (K_d = 390 nM, Fig. S2). The binding affinity of the IDA-type probes was found to be dependent on the length of the linker units. Among them, **1**-2Ni(II) showed the strongest binding affinity (K_d = 24 nM) for the hc-His6 peptide, which is ca. 16-fold stronger than that of **4**-2Ni(II). These results demonstrate the validity of our design strategy of the Ni(II) complex with a strong binding affinity towards a His-tag.

The Ni(II)–IDA complex was next applied to covalently label the protein. For this purpose, we prepared a MBP (maltose binding protein) protein tethered with a reactive His6-tag (CH6-tag: CHHHHHH), which contains a nucleophilic cysteine residue to form a covalent bond with a bound Ni(II) complex.[4a] The covalent labeling of MBP-CH6 was examined with the fluorescent BODIPY derivative **5**-2Ni(II), which has an α-chloroacetyl group reactive to the Cys residue of CH6-tag. The fluorescent quantum yield (Φ) of **5**-2Ni(II) was determined to be 0.1, a sufficiently high value for the protein labeling experiment (Table S1). Figure 3 shows the time-trace of the covalent labeling reaction by in-gel fluorescence analysis. The labeling reaction rapidly proceeded and was almost complete within 20 min under neutral aqueous conditions (50 mM HEPES, 100 mM NaCl, pH 7.2). The labeled protein was also identified by MALDI-TOF mass analysis, in which the new peak (m/z = 45,150) corresponding to the MBP-CH6 labeled with **5**-2Ni(II) was observed (Fig. S3). The kinetic analysis, by varying

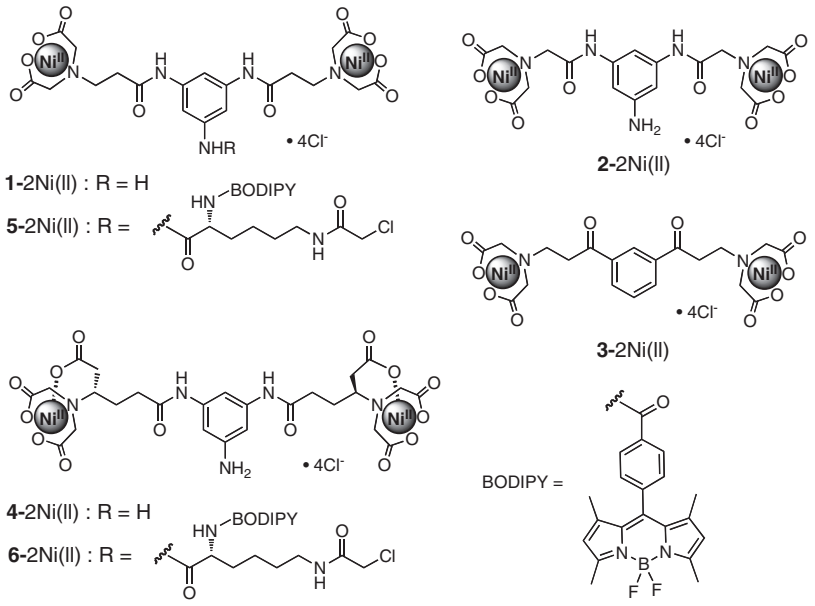

Figure 1. Structures of the Ni(II) complexes.

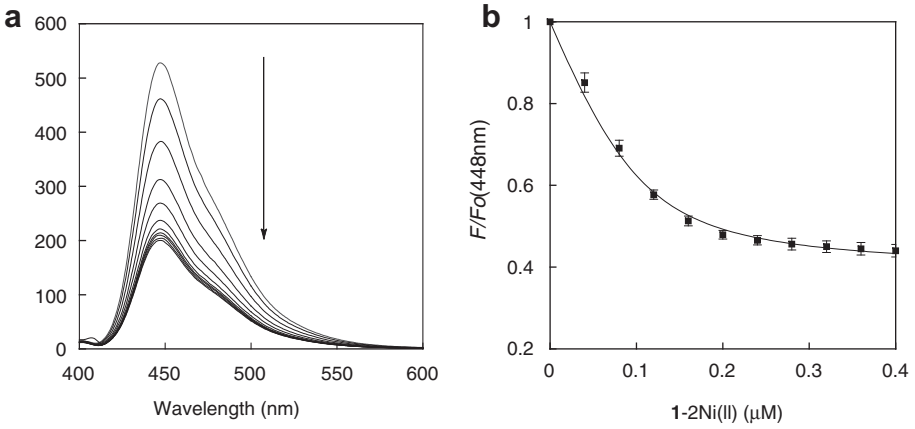

Figure 2. Fluorescence titration of **1**-2Ni(II) to the hydroxy coumarin-appended His-6 peptide (hc-His6). (a) Fluorescence spectral changes of hc-His6 upon addition of **1**-2Ni(II). Conditions: [hc-His6] = 0.1 μM, [**1**-2Ni(II)] = 0–0.24 μM, 50 mM HEPES, 100 mM NaCl, pH = 7.2. (b) Plot of the fluorescence intensity change at 448 nm. The data were obtained from the experiment performed in triplicate.

Table 1
Summary of the dissociation constants (K_d) of the Ni(II)-complexes with the hc-CH6 peptide

	1-2Ni(II)	**2**-2Ni(II)	**3**-2Ni(II)	**4**-2Ni(II)
K_d (nM)	24	97	123	390

the concentration of **5**-2Ni(II), revealed that the second-order rate constant of the labeling reaction between MBP-CH6 and **5**-2Ni(II) is 270 M^{-1} s^{-1} (Fig. S4). This value is comparable to the biorthogonal reactions for protein labeling reported to date.[8] Conversely, none of the fluorescent band was detected when using the Ni(II)-free ligand **5** (Fig. 3), clearly indicating that the labeling reaction between MBP-CH6 and **5**-2Ni(II) is driven by the tag-probe interaction. The labeling reaction was further conducted with MBP-CH6 in an *Escherichia coli* crude lysate (Fig. S5). A single fluorescence band corresponding to the labeled MBP-CH6 was detected by in-gel fluorescence analysis, indicative of the high labeling selectivity of the Ni(II)–IDA probe for a His-tag-fused protein.

The present protein labeling method was further applied to the fluorescent bioimaging of GPCR expressing on the surface of living cells. In this experiment, the bradykinin receptor type2 (B2R) protein tethered with a CH6 tag (CHHHHHH) at the explasmic N-terminus was transiently expressed in HEK293 cells. When the cells expressing the tag-fused B2R were treated with **5**-2Ni(II) (0.5 μM in HBS) and then repeatedly washed with a high concentration of imidazole solution (50 mM in HBS, three times), the clear fluorescence owing to BODIPY was undetectable on the cell surface (Fig. S6). This result indicates that **5**-2Ni(II) did not form a covalent bond with the CH6 tag of B2R and thus be removed readily from the cell surface by the imidazole washing. However, when the cells were first treated with the reducing agent tris-(carboxyethyl)phosphine (TCEP) to activate the cysteine residue of the CH6 tag,[9] followed by labeling with **5**-2Ni(II), the clear fluorescence of the BODIPY was detectable on cell surfaces even after repeatedly applying the imidazole washing step (Fig. 4a). It is noteworthy that this image overlapped well with that of a B2R antagonist peptide appended with a rhodamine fluorophore. This result suggests that **5**-2Ni(II) selectively forms a covalent bond with the cysteine residue of the CH6 tag to allow the selective visualization of the tag-fused B2R protein. The trypan blue assay revealed that **5**-2Ni(II) scarcely affects the cell viability under the labeling conditions (Fig. S9). In the control labeling experiment with the Ni(II)-free ligand **5**, negligible fluorescence of BODIPY was detected on cell surfaces (Fig. 4e), suggesting that the strong binding between **5**-2Ni(II) and the His-tag was crucial for the covalent labeling of the tag-fused B2R protein. The labeling of the tag-fused B2R was also conducted with the Ni(II)–NTA probe **6**-2Ni(II) under the same

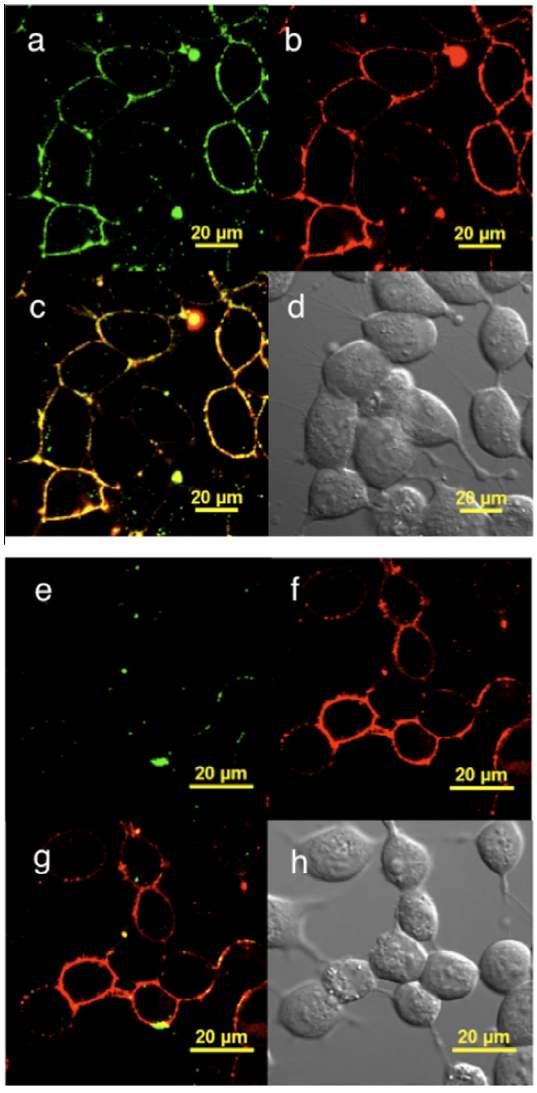

Figure 4. Covalent labeling of the CH6-tag-fused B2R protein on the surface of HEK293 cells. (upper) Fluorescence visualization of the B2R by labeling with **5**-Ni(II) (a) and the rhodamine-appended B2R antagonist (b), (c) is the overlay image of (a) and (b), and (d) is the transmission image. (lower) Fluorescence visualization of B2R by labeling with the ligand **5** (e) and the rhodamine-appended B2R antagonist (f). (g) is the overlay image of (e) and (f), and (h) is the transmission image.

labeling conditions (Fig. S7). In this case, the fluorescence due to BODIPY was apparently weak and did not provide a clear fluorescent image of the cell surfaces, indicative of the lower labeling

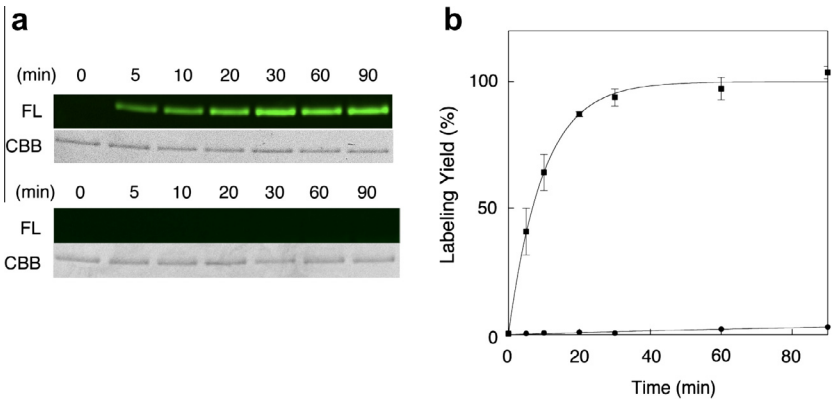

Figure 3. In-gel fluorescence analysis of the covalent labeling of MBP appended with the CH6-tag. (a) Fluorescence (FL) and CBB detection of CH6-MBP labeled with either **5**-2Ni(II) (upper) or the Ni(II)-free ligand **5** (lower). (b) Plot of the fluorescence band intensity of CH6-MBP labeled with either **5**-2Ni(II) (■) or **5** (●).

efficiency of **6**-2Ni(II) compared to **5**-2Ni(II). Although the use of a higher concentration (5 μM) of **6**-2Ni(II) was effective for improving the labeling efficiency, the high background fluorescence owing to non-selective binding or internalization of the probe inside cells was apparently problematic for the selective visualization of the labeled B2R (Fig. S8). These results indicate that the strong binding affinity of the Ni(II)–IDA probe **5**-2Ni(II) gives a clear advantage to the fluorescence imaging of His-tag-fused proteins over the conventional Ni(II)–NTA probe.

In conclusion, we have developed the Ni(II)–IDA complex as a new probe for the selective labeling of a His-tag-fused protein. The improved strong binding affinity of the Ni(II)–IDA probe compared with the conventional Ni(II)–NTA probe was fully exploited to covalently label a modified His-tag (CH6-tag) fused protein under in vitro and in cell conditions. We envision the further application of the present tag-probe pair to functional analyses of proteins under various biological conditions. Our research is ongoing along this line of research.

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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.2014.04.096.

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