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Design of a multinuclear Zn(II) complex as a new molecular probe for fluorescence imaging of His-tag fused proteins†

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A Zn(II) complex (Zn(II)–Ida) was designed as the new fluorescent probe for His-tag fused proteins. Thanks to the tight binding ability to histidine-rich sequences and bright fluorescence property of the Cy5-appended Zn(II)–Ida probes, selective and clear fluorescent imaging of the His-tag fused G-protein coupled receptors on live cell surfaces was carried out.

The complementary recognition pair of an oligo-histidine tag (His-tag) with a Ni(II)–nitrilotriacetic acid complex (Ni(II)–NTA), invented by researchers of F. Hoffmann-La Roche,[1] is now widely used as a valuable tool for purification, immobilization, and detection of proteins[2] (Fig. 1a). Owing to the highly selective binding ability, this pair has recently been applied to protein bioimaging in living cells. For example, Vogel *et al.* reported the first bioimaging study of His-tag fused proteins on cell surfaces using the fluorophore-appended Ni(II)–NTA probe.[3] Piehler *et al.* also reported the multinuclear Ni(II)–NTA probes for the fluorescence labeling of a His-tag fused protein on live cell surfaces.[4] Although these works demonstrated the utility of this pair as a tool for bioimaging, these labeling methods often suffered from the dimness of the Ni(II)–NTA probe due to the strong fluorescence quenching by Ni(II) ions, limiting their application in bioimaging study. Another problem of Ni(II)–NTA probes is the intrinsic toxicity of Ni(II) ions as a human carcinogen,[5] which may cause a deleterious effect on living cells and *in vivo*. Therefore, there is a strong need to develop an alternative probe that can circumvent these drawbacks to expand the versatility of the tag–probe pair in biological research.[6] To meet these requirements, Hauser and Tsien recently reported a fluorescent zinc complex HisZiFiT as a strong binding probe for His-tag.[6c] In this manuscript, we describe the design of a Zn(II) complex (Zn(II)–Ida) as the new fluorescent probe for His-tag fused proteins. The selective binding ability to histidine-rich sequences and bright fluorescence properties of Zn(II)–Ida probes tethered with a Cy5 fluorophore allowed us to clearly visualize the His-tag fused G-protein coupled receptors (GPCRs) on live cell surfaces.

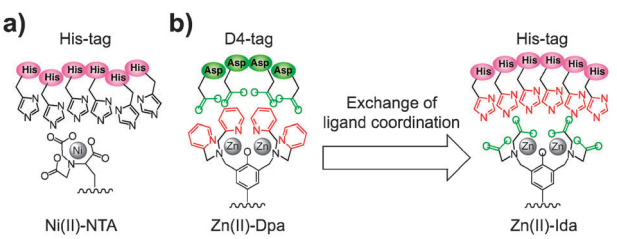

Fig. 1 (a) Conventional tag–probe pair of His-tag/Ni(II)–NTA. (b) Molecular design of the new probe with a binding affinity to His-tag.

We have recently reported that the binuclear Zn(II)–Dpa complex comprised of two sets of 2,2′-dipicolylamine (Dpa) serves as a molecular probe for selective binding to proteins tethered with a tetra-aspartate tag (D4-tag; DDDD).[7] While the Zn(II)–Dpa complex showed a strong binding affinity to D4-tag peptide (Boc-DDDD-NH_2) (K_d = ~1.5 μM) under the neutral aqueous conditions (50 mM HEPES, pH 7.2), the binding affinity to a His6-tag peptide (HHHHHH) was negligible (K_d > 1 mM). Our design strategy of the molecular probe for His-tag is based on exchange of the coordination ligand (Fig. 1b). We here assumed that the replacement of the nitrogen-containing pyridine rings of Dpa in the Zn(II)–Dpa complex with carboxylates may give rise to a zinc complex exhibiting a high binding affinity to the His-tag which instead possesses the multiple nitrogen-containing imidazole rings in lieu of the carboxylates of D4-tag. We thus designed the new binuclear Zn(II) complex (Zn(II)–Ida) possessing two sets of iminodiacetic acid (Ida) and synthesized its derivatives (Fig. 2).

The binding affinity of Zn(II)–Ida **1** to His6-tag peptide (Ac-WAHHHHHH-NH_2) was evaluated by isothermal titration calorimetry (ITC) under the neutral aqueous conditions (50 mM HEPES, 100 mM NaCl, pH 7.2). Fig. 3a indicates that their interaction was an enthalpy-driven exothermic process (0 > ΔH). The resultant binding isotherm was fitted with a curve derived from the 1 : 1 binding algorithm to yield a binding constant of (3.2 ± 0.2) × 10^3 M^{-1} (n = 1.4).[8] This value is almost 20-fold lower than that of Ni(II)–NTA (K_a = 7.1 × 10^4 M^{-1}) reported in the literature,[9] though the binding isotherm was not apparently 1 : 1 (n = 0.35). On the other hand, our ITC experiment revealed that the interaction between the Zn(II) complex of nitrilotriacetic acid (Zn(II)–NTA) **2** and His6 peptide was apparently weaker than that of Zn(II)–Ida (10^3 M^{-1} > K_a,

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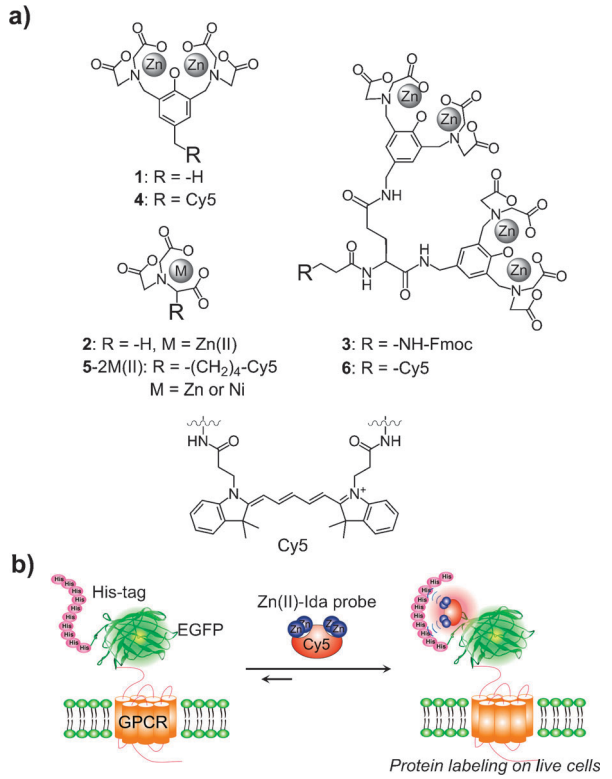

Fig. 2 (a) Molecular structures of the metal complexes and the fluorescent derivatives used in the present study. (b) Selective labeling of a His-tag fused GPCR protein on live cell surfaces with the Cy5-appended fluorescent Zn(II)–Ida probes.

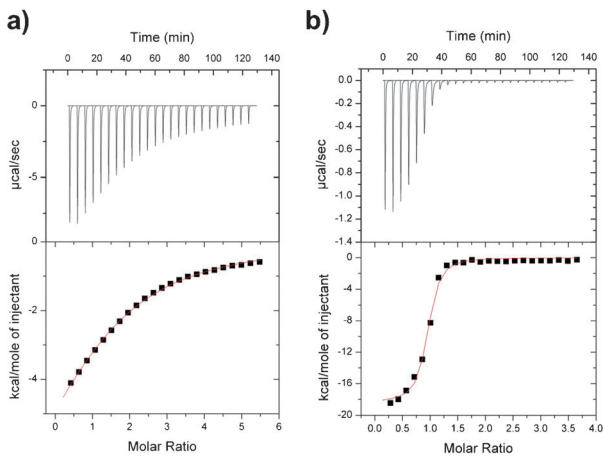

Fig. 3 ITC titration curve (upper) and processed data (lower) in the titration of (a) His6 peptide (200 μM) with **1** (6 mM, 10 μL × 24 injections), or (b) His10 peptide (10 μM) with **3** (0.2 mM, 10 μL × 24 injections). Measurement conditions: 50 mM HEPES (pH 7.2), 100 mM NaCl, 25 °C.

data not shown). Fortunately, the rather low binding affinity of **1** to His-tag was dramatically improved by increasing the number of the Zn(II)–Ida units in the probe. As shown in Fig. 3b and Table 1, the binding affinity of the bis-Zn(II)–Ida complex **3** to His10 peptide (Ac-WAHHHHHHHHHH-NH_2) was evaluated to be $(7.7 \pm 1.1) \times 10^6$ M^{-1} (n = 0.91) by ITC measurement, the value of which is *ca.* 2000-fold larger than that of **1** to His6 peptide.

We next evaluated the affinity of the multinuclear Zn(II)–Ida probe to a tag-fused protein in a test tube experiment.

Table 1 Summary of the ITC titration experiments between various Zn(II) complexes and oligo-His peptides[a,b]

	His6 peptide		His10 peptide
	1	**2**	**3**
n	1.40 ± 0.07		0.91 ± 0.01
K_{app}	(3.23 ± 0.18) × 10^3	$<10^3$	(7.74 ± 1.33) × 10^6
ΔH	−9.93 ± 0.61	—[c]	−18.37 ± 0.32
$T\Delta S$	−5.13		−8.97

[a] n = stoichiometry, K_{app} = binding constant (M^{-1}), ΔH = enthalpy (kcal mol^{-1}), $T\Delta S$ = entropy (kcal mol^{-1}). [b] Measurement conditions: 50 mM HEPES (pH 7.2), 100 mM NaCl, 25 °C. [c] Heat formation was scarcely detected (data not shown).

The cyanine dye is a suitable platform for the design of a multi-functionalized fluorescent probe. Thus, the probes **4** and **6** were newly designed, which have two and four sets of Zn(II)–Ida units as the binding sites at both ends of the Cy5 fluorophore, respectively.[10] The fluorescent titration experiment revealed that the binding affinity of **4** to a His10-tag fused EGFP (enhanced green fluorescent protein) was 1.3×10^6 M^{-1}, which was almost comparable to the case of **3** to His10 peptide (Fig. S1, ESI†). **6** showed a remarkably strong binding affinity (8.1×10^8 M^{-1}), which is more than 600-fold larger than that of **4** (Fig. S2†). These results encouraged us to examine the fluorescence bioimaging under live cell conditions. We initially employed bradykinin receptor type2 (B2R) as a target protein expressing on cell surfaces, which possesses a His10-tag and an EGFP domain at the exoplasmic *N*-terminus (His10-EGFP-B2R). When HEK 293 cells transiently expressing His10-EGFP-B2R were incubated with a low concentration of **4** (0.5 μM), the fluorescence image due to Cy5 was observed on cell surfaces by confocal laser scanning microscopy (CLSM) (Fig. 4a). This fluorescence image was well overlapped with that of EGFP fused to the B2R, indicative of co-localization of the probe with the B2R receptor. In the control experiment, the negligible fluorescence of the probe was observed on the cells expressing B2R lacking the His10-tag (Fig. 4b). These results indicate that **4** selectively binds to the His10-tag site so as to enable fluorescence visualization of the B2R on cell surfaces. The labeling with **4** is also applicable to the visualization of another GPCR, muscarinic acetylcholine receptor tethered with a His10 tag and EGFP at the exoplasmic *N*-terminus (His10-EGFP-m1AchR). As shown in Fig. 4c, clear fluorescence due to **4** was observed on cell surfaces, which co-localized well with that of EGFP. In contrast, the Cy5 fluorescence was scarcely observed in the case of m1AchR lacking His10-tag (Fig. 4d).

To demonstrate the advantage of the Zn(II)–Ida complex **4**, we carefully compared the fluorescence bioimaging data of B2R using other metal complexes. The fluorescence of the Ni(II)–NTA probe **5**–2Ni(II) was almost co-localized with that of the EGFP on the cell surfaces as shown in Fig. 4e. However, the fluorescence intensity was apparently weaker than that of **4**. The quantitative analysis revealed that the fluorescence intensity of **4** relative to the EGFP fluorescence on the same cell surface was 0.87 ± 0.16 (N > 10), which is 3-fold greater than the case of **5**–2Ni(II) (0.31 ± 0.07 (N > 10)). The dim fluorescence image of **5**–2Ni(II) is reasonably ascribed to its lower fluorescence quantum yield (Φ = 0.048) compared to that of **4** (Φ = 0.24). In the case of the Zn(II)–NTA type probe

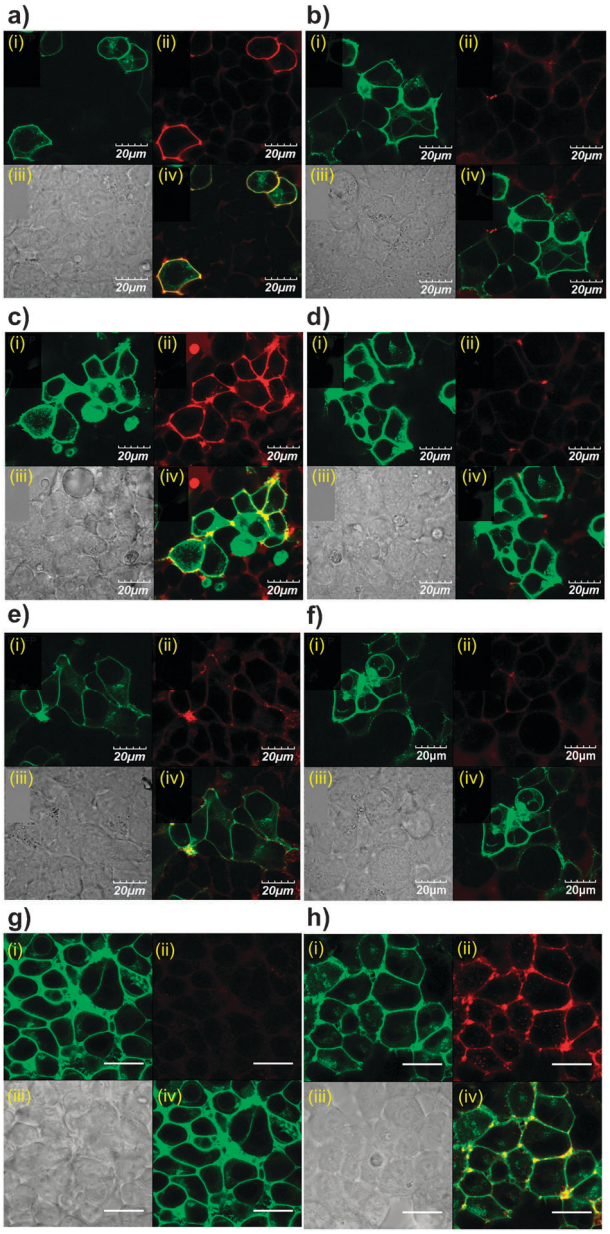

Fig. 4 Fluorescence visualization of the His-tag fused GPCR proteins on live cell surfaces. HEK 293 cells expressing the GPCRs (B2R or m1AchR) were cultivated on a poly-Lys coated glass base dish. The cells were treated with (a, b, c, d, g) 0.5 μM of **4**, (e) 0.5 μM of **5**–2Ni(II), (f) 0.5 μM of **5**–2Zn(II) or (h) 0.1 μM of **6** in HBS buffer for 10 min at rt. The cells were observed by CLSM without washing (a–f) or after single wash with HBS buffer (g & h). In each labeling experiment, the fluorescence images were obtained using two different channels corresponding to (i) EGFP as a protein expression marker and (ii) Cy5. The transmission image is shown in (iii), and the overlay image of (i) and (ii) is shown in (iv). Scale bars, 20 μm.

5–2Zn(II), on the other hand, the fluorescence due to Cy5 was scarcely observed on cell surfaces (Fig. 4f), despite the fact that **5**–2Zn(II) has a comparable fluorescence quantum yield (Φ = 0.26) to that of **4**. This result was probably because of the lower binding affinity of Zn(II)–NTA **2** to His-tag than that of **1** (Table 1).

One limitation of Zn(II)–Ida probe **4** was that the fluorescence imaging is needed to be conducted in the presence of excess of **4** (0.5 μM). Due to the rather low affinity of **4** toward His10-tag, the fluorescence image of **4** bound to the GPCRs easily disappeared when the cells were washed with HBS buffer (Fig. 4g). Fortunately, this was overcome by probe **6**. We found that **6** provided a clear and lasting fluorescence image of His10-EGFP-B2R on live cell surfaces even after removal of the excess of **6** by the single wash with HBS buffer (Fig. 4h). This result clearly demonstrates the validity of our design strategy of the His-tag binding probe based on multivalent coordination chemistry.

In conclusion, we successfully developed Zn(II)–Ida compounds as a new molecular probe for His-tag based on the two strategies involving exchange of the ligand coordination and multiple coordination chemistry. We further demonstrated that Zn(II)–Ida is a versatile alternative to the conventional Ni(II)–NTA in the fluorescent bioimaging of the His-tag fused proteins, especially by harnessing the less fluorescence quenching property of the Zn(II)–Ida than that of Ni(II)–NTA. We believe that, due to its simple structure and ease of functionalization, Zn(II)–Ida would become a molecular scaffold widely applicable in purification, handling, and detection of His-tag proteins in many biological research. To this end, we are going to improve their binding affinity through optimization of the His-tag sequence. Our research is now on going along this way.

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