Optimized Reaction Pair of the CysHis Tag and Ni(II)-NTA Probe for Highly Selective Chemical Labeling of Membrane Proteins

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Abstract

Chemical labeling of proteins with synthetic molecular probes offers the possibility to probe the functions of proteins of interest in living cells. However, the methods for covalently labeling targeted proteins using complementary peptide tag-probe pairs are still limited, irrespective of the versatility of such pairs in biological research. Herein, we report the new CysHis tag-Ni(II) probe pair for the specific covalent labeling of proteins. A broad-range evaluation of the reactivity profiles of the probe and the CysHis peptide tag afforded a tag-probe pair with an optimized and high labeling selectivity and reactivity. In particular, the labeling specificity of this pair was notably improved compared to the previously reported one. This pair was successfully utilized for the fluorescence imaging of membrane proteins on the surfaces of living cells, demonstrating its potential utility in biological research.

Keywords: Chemical protein labeling | Cysteine conjugation | Peptide tag

1. Introduction

Methods for selectively labeling proteins with a synthetic molecular probe is an important research tool that facilitates the functional analysis of proteins in biological systems. Among various methods, chemical labeling of a short peptide attached to the protein of interest has attracted considerable attention. The pioneering work of Tsien and his co-workers has led to the development of a method employing a genetically encoded short peptide tag (CysCysXXCysCys) and a biarsenical probe.[1] Since then, several peptide tag-based approaches have been devised for specific covalent labeling of proteins, and used in biological studies involving fluorescence imaging of proteins, cell functional analysis, and the design of antibody-drug conjugates.[2–5] Compared with the enzyme-mediated protein labeling methods such as Halo tag and SNAP tag systems,[6] these chemical labeling methods benefit from the small molecular size of the tag-probe pair, which is unlikely to disturb protein functions, flexible probe design independent from the substrate specificity of enzymes, and high tolerability under various labeling conditions.

The oligo-histidine tag (His tag) is a representative epitope tag that has been widely used for the purification of recombinant proteins.[7] By exploiting its specific interaction with the Ni(II) complex, we and others have reported methods for the covalent labeling of His tag-fused proteins with a synthetic probe.[8,9] We have recently demonstrated that His tag peptide containing a cysteine residue (CysHis tag) underwent a rapid reaction with Ni(II) complexes (Figure 1). This chemistry was
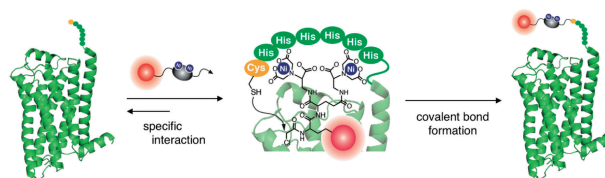

Figure 1. Schematic illustration of the covalent labeling of a His tag-fused protein with a Ni(II) complex probe.

successfully applied to the labeling of CysHis tag-fused proteins inside living cells. However, the overly reactive α-chloroacetamide probe often induced non-specific labeling, hampering its wide use in fluorescence analysis of proteins. Despite this problem, we have not fully optimized the reaction kinetics and labeling selectivity of this tag-probe pair, both of which are crucially important to achieve highly specific labeling of targeted tag-fused proteins in complicated biological contexts. This situation spurred our interest in finding a new CysHis tag-probe pair that would promote use of peptide tag-based chemical labeling in biological research. Here, we report the development of the new pair of CysHis tag-Ni(II) complex probe, which enables highly selective covalent labeling of proteins. A broad-range evaluation of the reactivity profiles of the metal complex probes and the CysHis peptides tag afforded an optimized tag-probe pair with a desired labeling specificity and reactivity. The utility of this pair was demonstrated in the fluorescence imaging of membrane proteins on the surfaces of living cells.

2. Experimental

Covalent Labeling of Tag-Fused MBP Protein. A solution of tag-fused MBP (1 μM) was mixed with Ni(II)-NTA probe (10 μM) in 50 mM HEPES, 100 mM NaCl, 20 μM TCEP, pH 7.2 (degassed under reduced pressure and charged with nitrogen before use), and the solution was incubated in a plastic tube at 37 °C. Aliquot of the solution (5 μL) was sampled at the appropriate times and mixed with 2 μL imidazole solution (600 mM in water) to quench the reaction. The solution was mixed with 2 μL 5x sampling buffer and kept at 4 °C. The collected samples were heated at 95 °C for 2 min and applied to SDS-PAGE. In-gel fluorescence experiment was performed with LAS-4000 lumino image analyzer (FUJIFILM) by EPI mode (520 nm excitation, 575DF20 filter). The first-order constant k (min^{-1}) and half reaction time ($t_{1/2}$, min) were calculated by nonlinear least-square curve-fitting analysis.

Evaluation of Binding Constant. In a quartz cell, a solution of the oligo-His peptide appended with 7-hydroxycoumarin (hc-His10 or hc-His6)[10] (0.2 or 1 μM) in 50 mM HEPES, 100 mM NaCl (pH 7.2) was titrated with the metal complex of **7** or **8** at 25 °C. The fluorescence spectra were measured using a PerkinElmer LS55 spectrofluorophotometer (λ_{ex} = 380 nm). The plot of the fluorescence intensity at 450 nm was analyzed by nonlinear least-square curve-fitting analysis to evaluate the apparent binding constant (K_a, M^{-1}).

Evaluation of Non-Specific Labeling Activity of Probe in *E. coli* Lysate. A solution of H5CH5 tag-fused MBP (0.25 μM) was mixed with **6**-2Ni(II) or **12**-2Ni(II) (0.5–10 μM) in *E. coli* lysate diluted with 50 mM HEPES, 100 mM NaCl, 20 μM TCEP, pH 7.2 (degassed under reduced pressure and charged with nitrogen before use). The mixture was incubated in a plastic tube at 37 °C. Aliquot of the solution (5 μL) was sampled at 1 h, and mixed with 2 μL imidazole solution (600 mM in water) to quench the reaction. The solution was mixed with 2 μL 5x sampling buffer and kept at 4 °C. The collected samples were heated at 95 °C for 2 min and applied to SDS-PAGE. In-gel fluorescence analysis was performed with LAS-4000 lumino image analyzer (FUJIFILM) by EPI mode (520 nm excitation, 575DF20 filter).

Cell Culture and B2R Expression in HEK293 Cells. HEK293 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL). Cells were maintained at 37 °C in humidified atmosphere of 5% CO$_2$ in air. Subculture was performed every 3–4 days from subconfluent (<80%) cultures using trypsin-EDTA solution. Transfection of the expression vector for B2R was carried out in a 35 mm glass-bottomed dish (Iwaki) using Lipofectamine LTX (Invitrogen) according to the general procedure. The cells were subjected to labeling experiment after 48 h of the transfection.

Evaluation of Non-Specific Labeling Activity of Probe on Non-Transfection Cell Surface. In a glass bottom dish, HEK293 cells ($\sim$1 × 10^6) were incubated in non-serum DMEM containing 2-deoxy-D-glucose (10 mM) and sodium azide (6 mM) for 30 min at 37 °C in a CO$_2$ incubator. After removal of DMEM, cells were treated with TCEP (1 mM) for 10 min at rt in 1 mL HEPES-buffered saline (HBS, containing 107 mM NaCl, 6 mM KCl, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 11.5 mM glucose, 20 mM HEPES, adjusted to pH 7.4 with NaOH) containing 2-deoxy-D-glucose (10 mM), sodium azide (6 mM). TCEP solution was removed and then cells were treated with **6**-2Ni(II) or **12**-2Ni(II) (2 μM, final concentration) for 10, 30 or 60 min in HBS containing 2-deoxy-D-glucose (10 mM) and sodium azide (6 mM) at 37 °C. After washing three times with HBS containing 50 mM imidazole (1 mL), the cells were fixed by treatment with PBS (100 μL) containing 4% paraformaldehyde, 0.2% glutaraldehyde, and 50 mM imidazole for 10 min at rt. The fixed cells were washed three times with HBS containing 0.1% TritonX-100 and 50 mM imidazole (1 mL) for 10 min at rt. Cells were immersed in HBS (100 μL) and the fluorescence on the cell surfaces was detected by confocal laser scanning microscopy (Leica TCS SP8) equipped with a HyD detector using a high laser power (4%) for Oregon Green 488 (λ_{ex} = 488 nm). The fluorescence image was analyzed to obtain the fluorescence intensity per area on the cell surface.

Fluorescence Imaging of Tag-Fused B2R. In a glass bottom dish, HEK293 cells ($\sim$1 × 10^6) transiently expressing H5CH5-tag-fused B2R were incubated in non-serum DMEM containing 2-deoxy-D-glucose (10 mM) and sodium azide (6 mM) for 30–60 min at 37 °C in a CO$_2$ incubator. After removal of DMEM, cells were treated with TCEP (1 mM) for 10 min at rt in 1 mL HBS containing 2-deoxy-D-glucose (10 mM), sodium azide (6 mM). TCEP solution was removed and then cells were treated with **12**-2Ni(II) (2 μM, final concentration) for 30 or 60 min in HBS containing 2-deoxy-D-glucose (10 mM) and sodium azide (6 mM) at 37 °C. Cells were washed three times with HBS containing 50 mM imidazole (1 mL) to remove unreacted **12**-2Ni(II). HBS containing Cy5-appended B2R antagonist peptide (0.5 μM) was added and cells were analyzed by confocal laser scanning microscopy (Leica TCS SP8) equipped with a HyD detector for Oregon Green 488 (λ_{ex} = 488 nm, λ_{em} = 490–555 nm, 1% laser power) and Cy5 (λ_{ex} = 639 nm, λ_{em} > 640 nm).

3. Results and Discussion

Structural Optimization of the Tag-Probe Pair. We initially designed the binuclear Ni(II) complex **1**-2Ni(II) as a
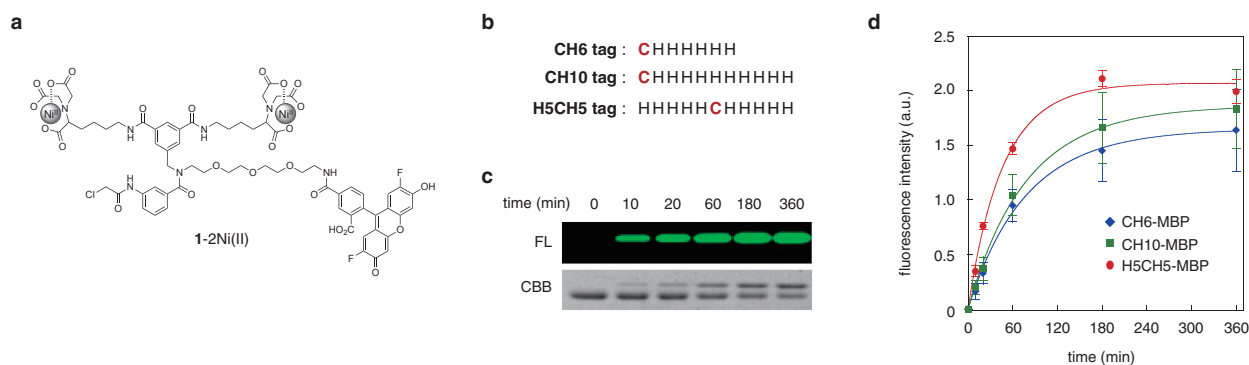
Figure 2. Fluorescent labeling of CysHis tag-fused proteins with **1**-2Ni(II). (a) Structure of **1**-2Ni(II). (b) Sequences of CysHis tags. (c) In-gel fluorescence analysis of the labeling reaction of H5CH5 tag-fused MBP with **1**-2Ni(II). (d) Time trace plot of the labeling reaction of the CysHis tag-fused proteins with **1**-2Ni(II) (mean ± s.d., three independent experiments). Labeling conditions: [tag-fused MBP] = 1 μM, [**1**-2Ni(II)] = 10 μM, 50 mM HEPES, 100 mM NaCl, pH 7.2, 37 °C.

Table 1. Summary of the kinetics parameters of the labeling reaction of CysHis tag-fused MBPs with **1**-2Ni(II).[a]

CysHis tag	k (min^{-1})	$t_{1/2}$ (min)
CH6	1.32×10^{-3}	54.1
CH10	1.33×10^{-3}	53.7
H5CH5	2.21×10^{-3}	32.3

[a]Values were obtained by nonlinear least-square curve fitting analysis of the averaged data obtained from three independent experiments.

reactive probe for the CysHis tag (Figure 2a and 2b). Probe **1**-2Ni(II) was constructed based on the isophthalic acid scaffold, which possessed two sets of Ni(II)-nitriloacetic acid (NTA) as the binding units. At the 5-position of the scaffold, the probe also has α-chloroacetamide (CA) as a reactive warhead for the CysHis tag and Oregon Green 488 as the fluorescent signal unit. The synthetic procedure of ligand **1** is described in detail in the Supporting Information.

The reactivity of **1**-2Ni(II) was evaluated by using maltose-binding proteins (MBPs) fused with different CysHis tags such as CH6, CH10, and H5CH5 under neutral aqueous conditions (50 mM HEPES, 100 mM NaCl, pH 7.2, 37 °C). In-gel fluorescence analysis showed that the fluorescence band of the MBPs labeled with **1**-2Ni(II) gradually increased in a time-dependent manner (Figure 2c and 2d), the analysis of which by the first-order reaction kinetics afforded the half reaction time ($t_{1/2}$, min). The data revealed that H5CH5-MBP exhibited faster reaction kinetics ($t_{1/2}$ = 32.3 min) than CH6-MBP ($t_{1/2}$ = 54.1 min) and CH10-MBP ($t_{1/2}$ = 53.7 min) (Table 1). The labeling reaction of H5CH5-MBP with **1**-2Ni(II) did not proceed in the presence of a large excess of imidazole (100 mM), clearly suggesting that this reaction was driven by the tag-probe interaction (Figure S1). Furthermore, His10 tag-fused MBP was scarcely labeled with **1**-2Ni(II), indicating that the labeling reaction occurred at the cysteine residue of the CysHis tag (Figure S2).

To achieve a higher labeling rate of the CysHis tag-fused MBP, we next synthesized a series of Ni(II)-NTA probes with the modified structure of **1**-2Ni(II) (Table 2). Each probe has alkyl chains with different lengths in both the Ni(II)-NTA-
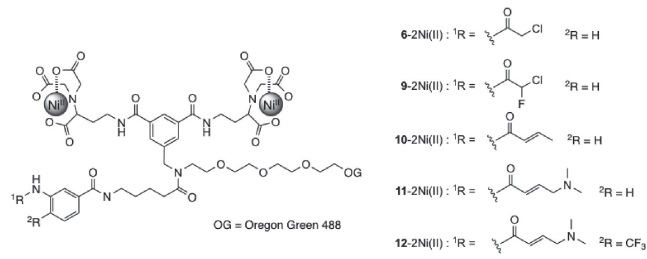
Table 2. Summary of the kinetics parameters of the labeling reaction of H5CH5 tag-fused MBP with Ni(II)-NTA probes.[a]

		Linker		
		1-2Ni(II)	**2**-2Ni(II)	**3**-2Ni(II)
n = 2	k (min^{-1})	2.21×10^{-3}	4.27×10^{-3}	1.27×10^{-3}
	$t_{1/2}$ (min)	32.3	16.7	56.0
		4-2Ni(II)	**5**-2Ni(II)	**6**-2Ni(II)
n = 1	k (min^{-1})	2.09×10^{-3}	6.08×10^{-3}	10.54×10^{-3}
	$t_{1/2}$ (min)	34.1	10.5	6.8

binding unit and the CA reaction site. Their reaction kinetics with H5CH5-MBP were evaluated by in-gel fluorescence analysis (Figure S3), and their half-reaction times are summarized in Table 2. We found that the reactivity of the probes varied largely depending on their structures. Among them, **6**-2Ni(II) exhibited the highest reaction rate ($t_{1/2}$ = 6.8 min), which was 8-fold faster than that of **3**-2Ni(II) ($t_{1/2}$ = 56.0 min) with the lowest reaction rate. These results clearly suggest the importance of fine-tuning of the probe structure for the rapid labeling. We assumed that such structural modifications optimized the special orientations of the reacting groups in tag-probe binding complexes, to achieve rapid labeling. We further confirmed that **6**-2Ni(II) reacted with H5CH5-MBP faster than it did with CH6-MBP and CH10-MBP, as observed in the case of **1**-2Ni(II). (Figure S4).

For further optimization of the probe structure, we evaluated the reactivity of the Co(II) and Zn(II) complexes of ligand **6**

Table 3. Summary of the kinetics parameters of the labeling reaction of H5CH5 tag-fused MBP with Ni(II)-NTA probes bearing various reactive groups.[a]

6-2Ni(II) : ¹R = [structure] Cl   ²R = H
9-2Ni(II) : ¹R = [structure] Cl, F   ²R = H
10-2Ni(II) : ¹R = [structure]   ²R = H
11-2Ni(II) : ¹R = [structure]   ²R = H
12-2Ni(II) : ¹R = [structure]   ²R = CF₃

OG = Oregon Green 488

	6-2Ni(II)	9-2Ni(II)	10-2Ni(II)	11-2Ni(II)	12-2Ni(II)
k (min^{-1})	10.54 x 10^{-3}	0.08 x 10^{-3}	0.04 x 10^{-3}	0.85 x 10^{-3}	4.26 x 10^{-3}
$t_{1/2}$ (min)	6.8	>360	>360	83.6	16.7

[a]Values were obtained by nonlinear least-square curve fitting analysis of the averaged data obtained from three independent experiments.

towards H5CH5-MBP. In-gel fluorescence analysis revealed that the reactivities of **6**-2Co(II) ($t_{1/2} = 22.5$ min) and **6**-2Zn(II) ($t_{1/2} = 130$ min) were apparently lower than that of **6**-2Ni(II) ($t_{1/2} = 6.8$ min) (Figure S5). To gain insights into the difference in the reaction kinetics depending on the metal ions used, we evaluated the binding affinity between the His tag peptide and the metal ion complexes of the NTA probes by the fluorescence titration method (Table S1). The results revealed that the binding affinity of the NTA complexes **8**-2Co(II) and **8**-2Zn(II) for the His10 peptide ($K_d = 0.89$ and $0.40\,\mu$M, respectively) is over 10-fold weaker than that of **8**-2Ni(II) ($K_d = 0.04\,\mu$M). These results imply that the strong binding affinity of the Ni(II)-NTA complex can largely contribute to the efficient labeling reaction with H5CH5-MBP. The fluorescence titration also revealed that the His10 peptide showed stronger binding affinities for the Ni(II)-NTA complexes **7**-2Ni(II) and **8**-2Ni(II) than the His6 peptide. This result is consistent with the previous results reported for other types of binuclear Ni(II)-NTA probes.[11]

Tuning of the Reactivity Profiles of Probes. To achieve high labeling selectivity for the targeted tag-fused protein, we synthesized a series of Ni(II)-NTA probes with different reacting groups, and evaluated their reactivity by in-gel fluorescence analysis (Table 3, Figure S6). The reactivity of probe **9**-2Ni(II) bearing α-chlorofluoroacetamide (CFA)[12] towards H5CH5-MBP was significantly lower than that of **6**-2Ni(II). This result agrees well with the fact that CFA has a much weaker reactivity than CA. Michael acceptors are an important class of reactive groups for a cysteine thiol and are widely used for protein modification. Considering the broad tunability of their reactivity, we prepared the Ni(II) complexes **10**-2Ni(II) to **12**-2Ni(II) bearing different Michael acceptor groups, and evaluated their reactivity. The labeling reaction of the crotonyl amide-type probe **10**-2Ni(II) with H5CH5-MBP was very slow ($t_{1/2} > 6$ h), while that of probe **11**-2Ni(II) with γ-dimethyl-aminocrotonate (DMAC) showed a moderate reactivity ($t_{1/2} = 83.6$ min). The reactivity of **11**-2Ni(II) was improved when an electron-drawing trifluoromethyl group was introduced on the DMAC reactive unit; the half reaction time ($t_{1/2}$) of **12**-2Ni(II)
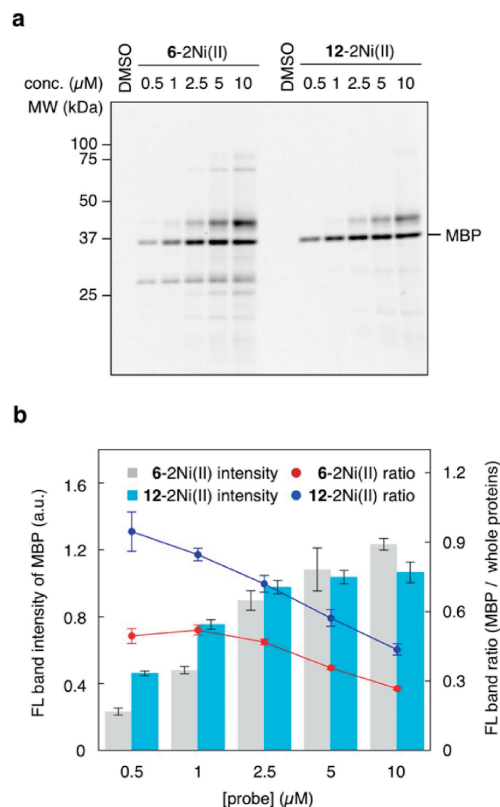

Figure 3. Evaluation of the non-specific labeling activity of **6**-2Ni(II) and **12**-2Ni(II) in *E. coli* lysate. (a) In-gel fluorescence analysis of the labeling profiles of the probes. (b) Comparison of the labeling efficiency and selectivity of the probes for labeling H5CH5-MBP (mean ± s.d., three independent experiments). Labeling conditions: [H5CH5-MBP] = 0.25 μM, [probe] = 0.5–10 μM, 50 mM HEPES, 100 mM NaCl, pH 7.2, 1 h, 37 °C.

was 16.7 min, which was only slightly longer than that of the CA probe **6**-2Ni(II) ($t_{1/2} = 6.8$ min).

Next, we evaluated the off-target reactivity of the CA probe **6**-2Ni(II) and DMAC probe **12**-2Ni(II) (Figure 3). When the labeling reaction was conducted in *E. coli* lysate solution containing various proteins (Figure S7), off-target proteins other than H5CH5-MBP were detectable in the in-gel fluorescence analysis. The quantitative band intensity analysis revealed that **12**-2Ni(II) exhibited a higher target specificity towards H5CH5-MBP than **6**-2Ni(II). Interestingly, the labeling efficiency of **12**-2Ni(II) is significantly higher than that of **6**-2Ni(II) when the labeling reaction was conducted using less than 1 μM of the probe. This might be ascribed to the decrease in the actual amount of **6**-2Ni(II) available for labeling with H5CH5-MBP at the low concentration range, due to its off-target reaction with other biomolecules in the lysate.

Fluorescence Imaging of Cell Surface Proteins. The above-mentioned data suggest that the H5CH5 tag and **12**-2Ni(II) is the optimized tag-probe pair that combines sufficiently high reactivity and target selectivity. The second-order rate constant between H5CH5-MBP and **12**-2Ni(II) was determined to be $1.4 \times 10^3\,M^{-1}s^{-1}$ by the detailed reaction kinetics study (Figure S8). This value is lower than those of the
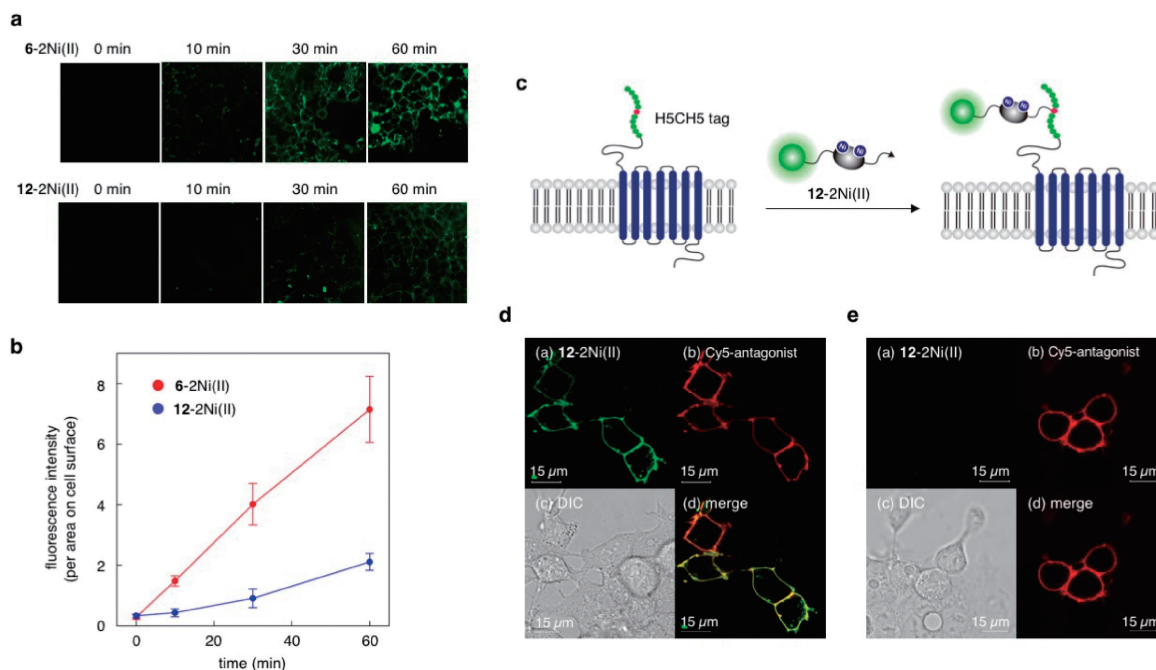
Figure 4. Evaluation of the non-specific labeling activity of **6**-2Ni(II) and **12**-2Ni(II) on the surface of HEK293 cells that did not express the CysHis tag-fused protein. (a) Fluorescence imaging of the HEK293 cells upon treatment with **6**-2Ni(II) or **12**-2Ni(II) at each time point. (b) Time trace plot of the fluorescence intensity change of the HEK293 cells (per unit area on the cell surface) upon treatment with **6**-2Ni(II) or **12**-2Ni(II) (mean ± s.d., n = 5). Labeling conditions: [probe] = 2 μM, 50 mM HEPES, 100 mM NaCl, HEPES, pH 7.2. (c) Schematic illustration of chemical labeling of H5CH5 tag-fused B2R with **12**-2Ni(II). (d, e) Fluorescence imaging of HEK293 cells expressing B2R tagged with H5CH5 tag (d), or DCP3 tag (e) upon the labeling with **12**-2Ni(II). Labeling conditions: [**12**-2Ni(II)] = 2 μM, HEPES-buffered saline, pH 7.4.

enzyme-mediated protein labeling methods such as the Halo tag and SNAP tag labeling systems,[13] but sufficiently large for labeling membrane proteins on the cell surface. Prior to the fluorescence imaging of cell surface proteins, we evaluated the non-specific labeling activity of the reactive probes in HEK293 cells that did not express the tag-fused protein (Figure 4a and 4b). The cells were treated with the DMAC probe **12**-2Ni(II) or CA probe **6**-2Ni(II) (4 μM, 37 °C), and the fluorescence of the cell surface was measured by confocal microscopy after formaldehyde fixation. The quantitative fluorescence analysis revealed that the fluorescence of **12**-2Ni(II) associated with non-specific reaction was much weaker than that of the highly reactive **6**-2Ni(II). In contrast, **6**-2Ni(II) induced a noticeable time-dependent increase of the fluorescence signal on the cell surface. These data indicate that the lower off-target activity of **12**-2Ni(II) on the cell surface compared to that of **6**-2Ni(II). Next, we used the tag-probe pair for the covalent labeling of GPCR proteins on the cell surface (Figure 4c). In this experiment, bradykinin receptor type2 (B2R) was tagged with the H5CH5 tag (located at the extracellular N-terminal region of B2R) and transiently expressed in HEK293 cells. When the cells were pre-treated with tris(2-carboxyethyl)phosphine (TCEP) to activate the cysteine residue of the tag, and then incubated with 2 μM of **12**-2Ni(II), a bright fluorescence signal was observed on the cell surface (Figure 4d). The obtained fluorescence image overlapped well with that of the fluorescent B2R antagonist peptide, indicative of selective covalent labeling of the tag-fused B2R with **12**-2Ni(II). The fluorescence of **12**-2Ni(II) was not observed on the cell surface

when the labeling reaction was conducted with the transiently expressed B2R tagged with an aspartate-rich cysteine containing a DCP3 tag (Figure 4e),[14] indicative of the specific labeling of the CysHis tag of the B2R with **12**-2Ni(II). Cell viability assay revealed that neither **12**-2Ni(II) nor TCEP show any toxic effect on the cells under the labeling conditions (Figure S9).

4. Conclusion

In conclusion, we have developed the new CysHis tag (H5CH5 tag)/Ni(II)-NTA probe pair for the selective covalent labeling of target proteins. The current research revealed that the labeling selectivity and reactivity can be largely changed by designing the sequence of the peptide tag and the structure of the probe. In particular, we found that fine-tuning the reactive group of the probe was crucial to gain high target protein selectivity while maintaining sufficient reactivity for the tag-fused protein. By exploiting this high target selectivity, further application of the optimized tag-probe pair to label other proteins, including other membrane-bound receptors and intracellular proteins, is expected. We also envision that the optimized tag-probe pair would not only be useful for fluorescence imaging, but also for high-resolution electron microscopy imaging of single proteins. Our research on this topic is ongoing.

Supporting Information

This material is available on https://doi.org/10.1246/bcsj.20190034.

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