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FLAG-tag selective covalent protein labeling via a binding-induced acyl-transfer reaction

Hiroshi Nonaka [a], Sho-hei Fujishima [a], Sho-hei Uchinomiya [a], Akio Ojida [a,b], Itaru Hamachi [a,c,*]

[a] Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Nishikyo-ku, Kyoto 615-8510, Japan
[b] JST, PRESTO (Life Phenomena and Measurement Analysis), Sanbancho, Chiyodaku, Tokyo 102-0075, Japan
[c] CREST (Creation of Next-generation Nanosystems through Process Integration), Sanbancho, Chiyodaku, Tokyo 102-0075, Japan

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ABSTRACT

A FLAG tag selective protein labeling method is newly developed in this study. Coupling of the selective binding between synthetic Ni-complex probe and FLAG tag with the acyl transfer reaction enables the site-selective covalent modification of FLAG peptide and FLAG-tag fused protein.

Specific covalent labeling of a protein with a small molecular probe is a powerful technique for elucidation of protein functions. Among various labeling methods, the modification using a short peptide tag incorporated into a recombinant protein is a recently developing useful technique.[1] In recent years, several enzymatic[2] or non-enzymatic[3] methods have been reported for the covalent labeling of tag-fused proteins. It is still strongly desirable to expand the repertory of the labeling techniques, which is available for multi-color protein labeling or in a complementary way with other labeling techniques.[4]

FLAG tag (DYKDDDDK), a genetically encodable short peptide tag, has been widely used in protein research, especially in Western blotting and affinity purification of recombinant proteins.[5] Such analyses only relies on the FLAG tag specific antibody (FLAG-antibody). However, non-specific binding and the large size of expensive FLAG-antibody often limit the utility of FLAG tag. The small molecular probe that is able to specifically label FLAG tag is expected to largely expand the utility of FLAG tag in protein research. We recently proposed that the binding-induced chemical reaction for a peptide tag is a powerful strategy for selective protein labeling.[3a,b] Here we extend this chemical strategy for the covalent labeling of the FLAG tag system. The selective binding of the probe to a FLAG tag facilitated a chemical reaction in the binding complex, allowing the covalent modification of FLAG tag fused proteins with synthetic markers. This covalent labeling system may partially serve as an alternative to FLAG-antibodies.

Figure 1 shows our strategy for the selective covalent modification of a FLAG tag fused protein. We recently found that the binuclear Ni(II) complex of DpaTyr **1**-2Ni(II) binds with an oligo-aspartate tag (DDD or DDDD) with a strong affinity (K_{app} >10^5 M^{-1}) under neutral aqueous conditions.[6] Containing a tetra-aspartate sequence in FLAG tag (DYKDDDDK) made us expect that Ni(II)–DpaTyr could interact with FLAG tag. We further envisioned that the proximity of the tag and the probe in the binding complex enhances a nucleophilic reaction of amino acid residues of FLAG tag towards a Ni(II)–DpaTyr probe having an electrophilic counterpart. Among various electrophiles, thioester is selected because of its biocompatibility and moderate reactivity under biological conditions.[7] Thus, we designed Ni(II)–DpaTyr probe appended with a thioester group (**2**-2Ni(II)), which can potentially undergo an acyl-transfer reaction with some amino acid residues (e.g., Lys) of FLAG tag to form the covalent bond of the tag-fused protein. The reactive thioester-type of probe **2**-2Ni(II) possessing a coumarin fluorophore was synthesized as shown in Scheme 1.

The labeling reaction was initially examined using **2**-2Ni(II) and a FLAG peptide (Ac-DYKDDDDK-NH_2) under slightly basic conditions (50 mM HEPES, pH 8.0). MALDI-TOF mass analysis (negative mode) showed a new peak at 1353 (*m/z*), which corresponds to the FLAG tag peptide labeled with one coumarin unit of the probe, together with a small peak observed at 1653 (*m/z*), which was assignable as the FLAG peptide modified with two coumarin units (Fig. 2).

* Corresponding author.
E-mail address: ihamachi@sbchem.kyoto-u.ac.jp (I. Hamachi).

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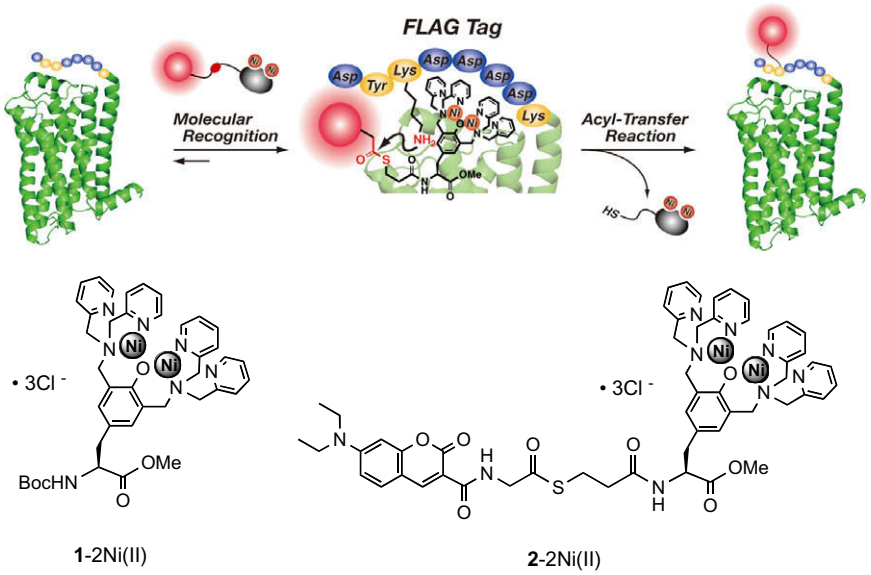
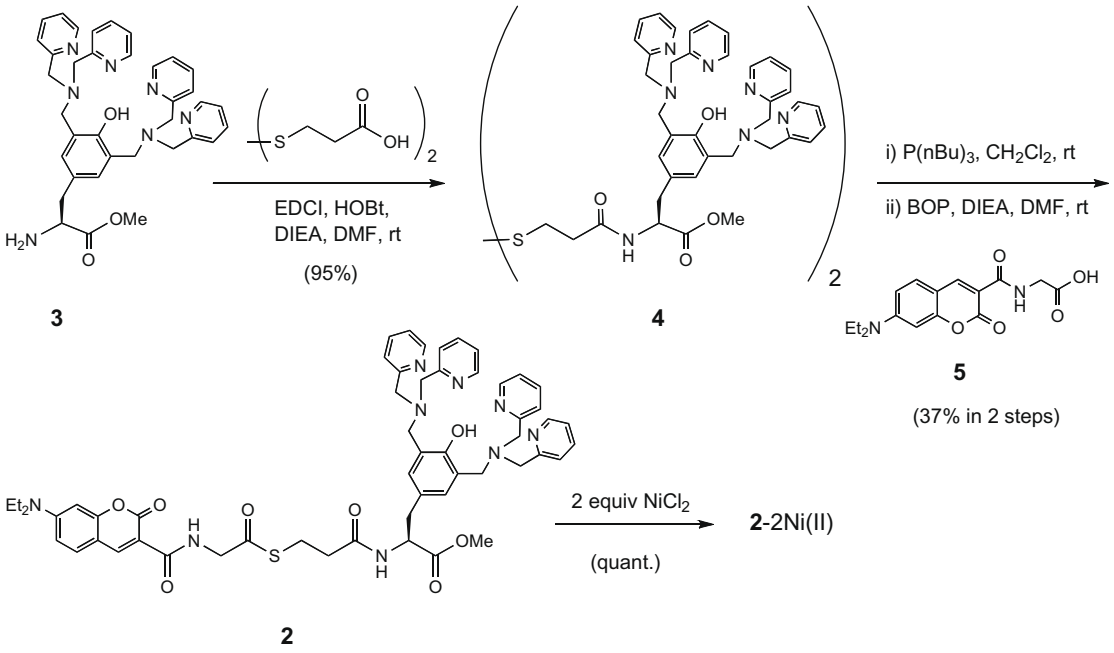


Figure 1. (Upper) Selective covalent labeling of a FLAG tag fused protein via an acyl-transfer reaction. (Lower) Structure of the Ni(II)-DpaTyr probes.



Scheme 1. Synthesis of **2**-2Ni(II).

In the HPLC analysis of the reaction mixture (Fig. 3a), three new peaks (peak 1, 2, and 3 in Fig. 3a) appeared, all of which were assignable by MALDI-TOF mass analysis (positive mode) as the FLAG tag peptides labeled with one coumarin unit, which had a calculated mass number of 1355 (*m/z*). The sequence analysis by the PSD mode of tandem mass identified that these labeled peptides were modified with one coumarin unit at Tyr2 (peak 3), Lys3 (peak 2), or Lys8 (peak 1) (Fig. S1), as a result of the acyl-transfer reaction (for the labeling ratio, 1:4:2 = Tyr2: Lys3: Lys8 in Fig. 3b).

The present acyl-transfer type of labeling was next examined using ubiquitin protein (Ubi) fused with FLAG tag at its N-terminal (FLAG-Ubi). The reaction was performed with 5 μM of FLAG-Ubi in the presence of 40 μM of **2**-2Ni(II) in aqueous solution (50 mM HEPES buffer, 100 mM NaCl, pH 8.0). In-gel fluorescence analysis showed the gradual increase of the fluorescent band intensity over 8 h, indicative of the progress of the acyl transfer reaction in a time-dependent manner (Fig. 4a). The reaction was also monitored by MALDI-TOF mass analysis, in which a new peak corresponding to FLAG-Ubi (10,054, *m/z*) labeled with one coumarin unit of **2**-2Ni(II) was observed at 10,357 (*m/z*), along with a small peak at 10,659 (*m/z*) corresponding to FLAG-Ubi labeled with two coumarin units (Fig. S2). The time-trace plot of the labeling reaction based on the in-gel fluorescence analysis indicated that ca. 30% of FLAG-Ubi is labeled for 8 h (Fig. 4b). In a good contrast, ubiquitin lacking FLAG tag (control-Ubi) was negligibly labeled even after 8 h (Fig. 4b, Fig. S2), demonstrating the binding-induced selective labeling of FLAG-Ubi. The labeling site was determined by the selective cleavage of the FLAG-tag part from FLAG-Ubi at the single Met16 site by the treatment of CNBr.[8] In-gel fluorescence analysis

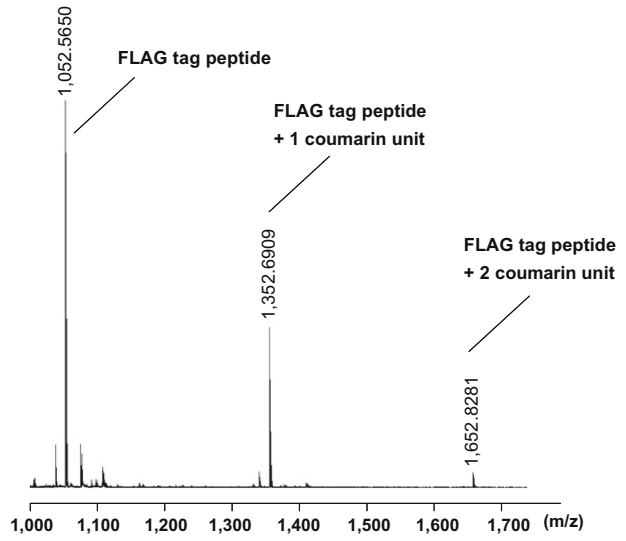


Figure 2. MALDI-TOF mass analysis of the acyl-transfer reaction of **2**-2Ni(II) with FLAG tag peptide. Reaction conditions; 20 μM of FLAG peptide, 40 μM of **2**-2Ni(II), 50 mM HEPES, 100 mM NaCl, pH 8.0, 37 °C, 4 h.

showed that the fluorescence due to the coumarin unit was not detectable in the cleaved ubiquitin part (Fig. S3). The MALDI-TOF mass analysis further confirmed that the main mass peak detected at 8487 (*m/z*) corresponded to the cleaved Ubi without any coumarin units, whereas the FLAG tag labeled with one coumarin unit was indentified in the lower mass range at 1908 (*m/z*) as another main peak (Fig. S4). These results clearly indicate that the labeling reaction takes place site-selectively at the FLAG tag. To confirm the protein selectivity of this covalent labeling method, the labeling experiment was then carried out under the conditions of a protein mixture including carbonic anhydrase (CA), avidin (AV), ribonuclease A (RNase), cytochrome C (CyC), and FLAG-Ubi (Fig. 5). In-gel fluorescence analysis showed only one fluorescent band from FLAG-Ubi, demonstrating that the acyl transfer reaction selectively proceeded in FLAG tag-fused Ubi.

In conclusion, we succeeded in the selective covalent labeling of FLAG tag and tag-fused protein on the basis of the binding-induced acyl-transfer reaction. In addition, we showed a potential utility of the present labeling method under the biologically complicated conditions. Further improvement of this method in the labeling rate and yield would be anticipated to provide a useful molecular tool as an alternative to the conventional FLAG-antibody. We envision further applications of this acyl-transfer based labeling

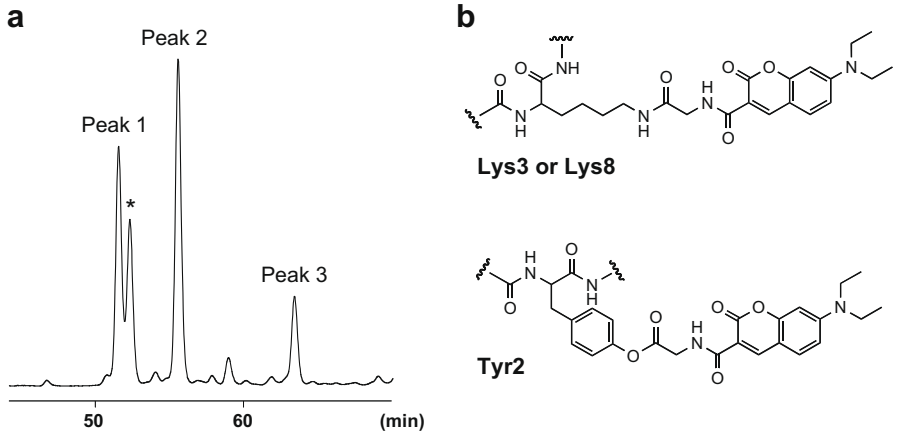


Figure 3. (a) HPLC analysis of the acyl-transfer reaction of **2**-2Ni(II) with FLAG tag peptide. Reaction conditions; 20 μM of FLAG peptide, 40 μM of **2**-2Ni(II), 50 mM HEPES, 100 mM NaCl, pH 8.0, 37 °C, 4 hr. The peaks were detected by UV absorbance change at 430 nm. The peak marked with ✠ was identified as the hydrolysis fragment of **2**-2Ni(II). (c) Structures of the covalent adducts of FLAG tag peptide formed by the acyl-transfer reaction at Lys3, Ly8, or Tyr2 with **2**-2Ni(II).

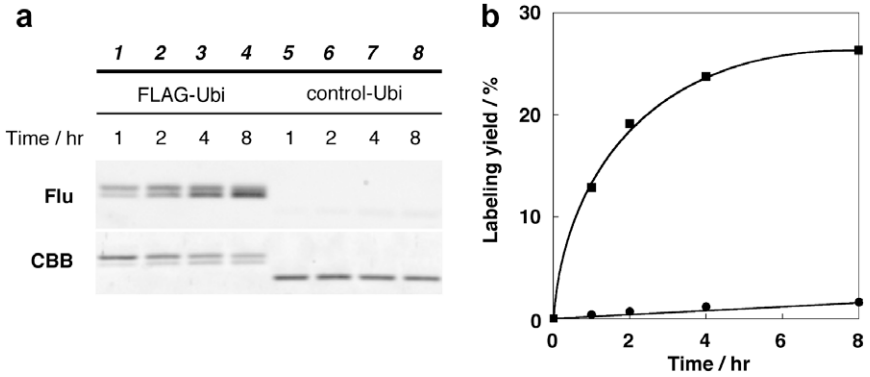


Figure 4. (a) SDS–PAGE analysis of the labeling reaction of **2**-2Ni(II) with FLAG tag fused ubiquitin (FLAG-Ubi, lanes 1–4) and control-Ubi lacking a FLAG tag (lanes 5–8). Reaction conditions; 5 μM of FLAG-Ubi or control-Ubi, 40 μM of **2**-2Ni(II), 50 mM HEPES, 100 mM NaCl, pH 8.0, 25 °C. (b) Time-trace plot of the labeling reaction of **2**-2Ni(II) with FLAG-Ubi (■) and control-Ubi (●) based on in-gel fluorescence analysis. The labeling yield (%) is defined as the labeled coumarin unit per total amount of the ubiquitin, and calculated based on the fluorescence band intensity of each sample relative to the authentic sample.

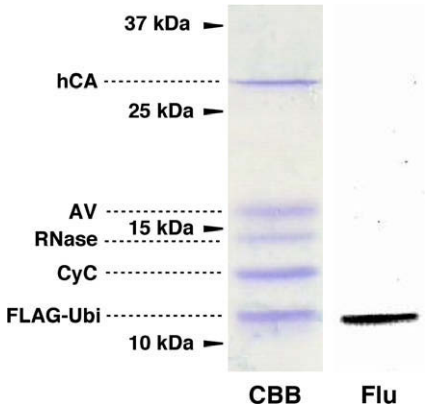


Figure 5. Selective covalent labeling of FLAG-Ubi with **2**-2Ni(II) among the protein mixture. hCA = human carbonic anhydrase, AV = avidin, RNase = ribonuclease A, CyC = cytochrome C. Reaction conditions; 0.05 μg/μL of each protein, 20 μM of **2**-2Ni(II), 50 mM HEPES, 100 mM NaCl, pH 8.0, 25 °C, 4 h.

method for wider range of protein researches in living cells and in vitro.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.122.

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