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Site-Selective and Nondestructive Protein Labeling through Azaelectrocyclization-Induced Cascade Reactions

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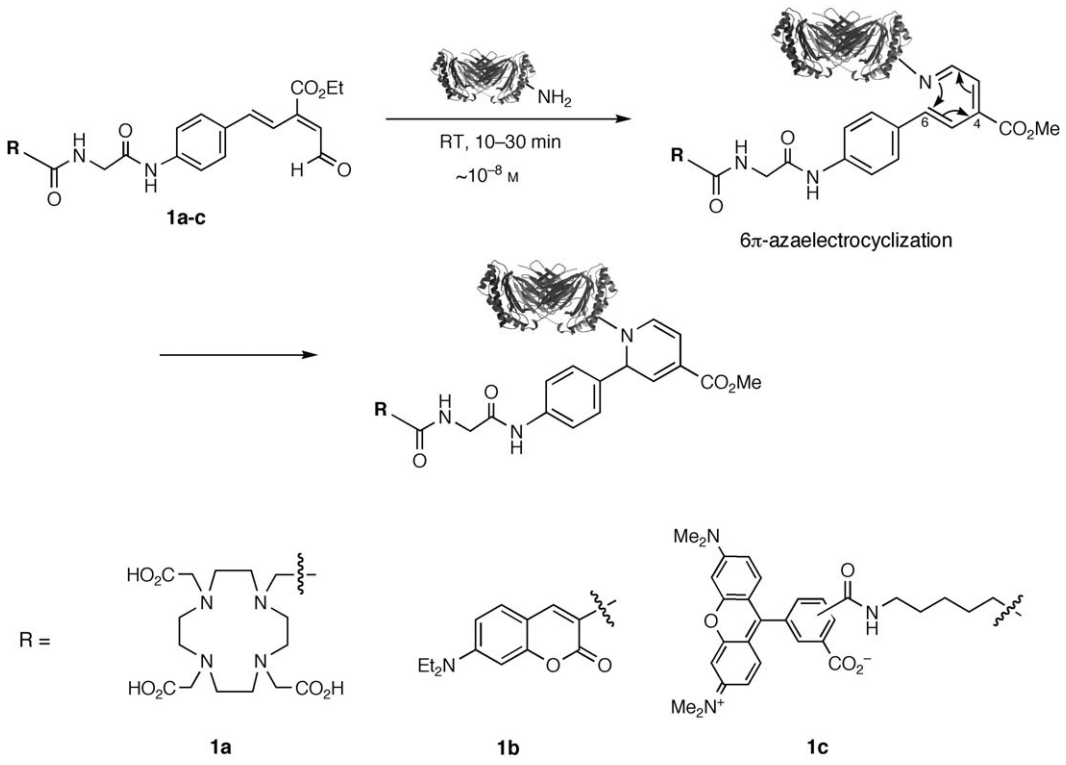
Chemical methods for protein labeling have garnered significant attention in the field of molecular imaging because of the easy operation, general applicability, as well as the smaller size of the chemical labels relative to genetically encoded protein-based labels, such as green fluorescent protein (GFP).[1] For chemistry-based labeling, it is essential to be able to label minuscule amounts of proteins at low concentrations under mild conditions, and to preserve the function of the unmodified protein. Therefore, the undesirable modification of key amino acids should be avoided, and the number of the labels introduced into proteins should also be limited to retain the native conformation and the protein–protein associations of the unmodified, biologically active forms. Recently, a variety of new chemical methods[2] which can be combined with biological techniques have been actively investigated, for example, the Cu^{I}-mediated Meldal/Sharpless click reaction with azide genetically introduced at desired positions within proteins.[3] Cell-friendly versions of the Cu^{I}-free click reaction using the strained acetylenes,[4] as well as the Staudinger reaction,[5] have also been developed by Bertozzi and co-workers; these reactions have been used successfully in applications of fluorescence imaging on cell surfaces. Entirely chemical methods have been thoroughly investigated by Hamachi and co-workers;[6] a single molecule containing fluorescent labels, anchoring functional groups and protein ligands was developed to selectively label the nearby ligand-binding sites. After cleavage of the ligands from the labeled proteins, those with the recovered activity have been used as sensitive reporter molecules for fluorescent



Scheme 1. Previously established method of labeling lysine by 6π-azaelectrocyclization.[8]

imaging, for example, visualization of glycolysis rates inside cells.[6a–d] Many efficient variants of this strategy, which involves so-called post-affinity labeling modification (P-ALM),[6e] have also been developed by the same group.[6f,g]

We have recently developed new lysine-based labeling of biomolecules (Scheme 1), which is based on rapid 6π-azaelectrocyclization.[7,8] We have used this method to efficiently and

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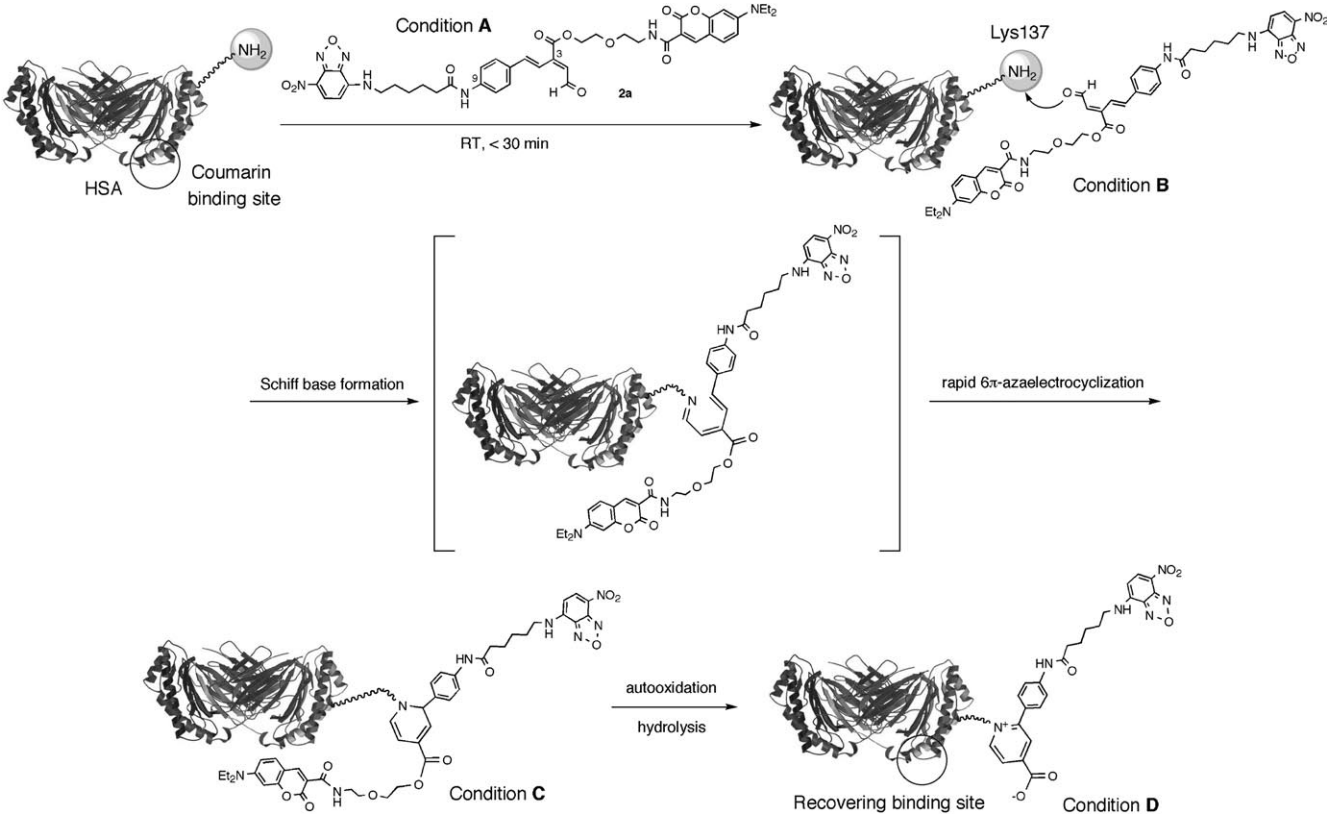
selectively introduce both DOTA (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid) as a metal chelating agent (either for MRI, PET, or other radiopharmaceutical purposes) and fluorescent groups to lysine residues by reaction with unsaturated aldehyde probes **1a–c** at very low concentrations ($\sim 10^{-8}$ M) within a short time (10-30 min) at room temperature.[8] Furthermore, the DOTA-labeled somatostatin and glycoproteins were subsequently radiometallated with ^{68}Ga and their receptor-mediated accumulation was successfully visualized by PET.

The method precisely controls introduction of DOTA or fluorescence labels onto lysines of target proteins. Labels can be introduced at a level of one or two molecules per protein, and this level can be controlled by adjusting the reaction concentrations so that the activity of the biomolecules can be retained. The efficiency of our rapid azaelectrocyclization protocol depends on the steric accessibility of the primary amino groups. Whereas the reactions with the internal lysines of the protein tertiary structure and the N-terminal amine (secondary amine) are very slow (>5 h at 24 °C),[7e] the lysines at the protein surfaces react rapidly (10–30 min at 24 °C); therefore, labeling occurs preferentially at these positions.[7b] For example, the antigen recognition activity of the anti-GFP antibody retains 90% of that of the intact mAb because of the preferential labeling of the sterically accessible lysines of the Fc moiety.[8a] However, when lysines that are critical for receptor binding are situated at a target protein's most accessible site a significant decrease in activity might result. In this paper, we report site-selective and nondestructive protein labeling by azaelectrocyclization. Site-selective modification of the target protein was achieved by directing reactive groups to a specific site using a small-molecule ligand of the protein. Labeling then took place via Schiff base formation and subsequent electrocyclization. The ligand was then cleaved from the conjugated protein by auto-oxidation of the 1,2-dihydropyridine and hydrolysis of the ester linkage connecting the ligand.

We chose human serum albumin (HSA, Mw = 66000)[9] as a target protein; not only is HSA readily available but it contains as many as 59 lysines, making it a very challenging target for testing selective labeling by the azaelectrocyclization protocol. The labeling mechanism using probe **2a** is outlined in Scheme 2. The fluorescent reporter group NBD (7-nitrobenz-2-oxa-1,3-diazole) was introduced at position C9 of the probe, whereas the HSA ligand, 7-diethylaminocoumarin, was attached at the C3 position via an ester linkage. 7-diethylaminocoumarin is known to strongly bind to HSA through either the subdomain II-A drug binding site 1 (amino acid residues 197–297) or subdomain IB (amino acid residues 107–196).[10] Of the many other coumarin candidates, the diethylamino derivative was selected because of its favorable fluorescence properties, and the excellent overlap of the emission spectrum of the diethylaminocoumarin (ex: 438 nm, em: 479 nm) with the excitation spectrum of the NBD fluorophore (λ_{ex}: 485 nm, λ_{em}: 549 nm) might enable the analysis of the whole labeling process in Scheme 2 by FRET.

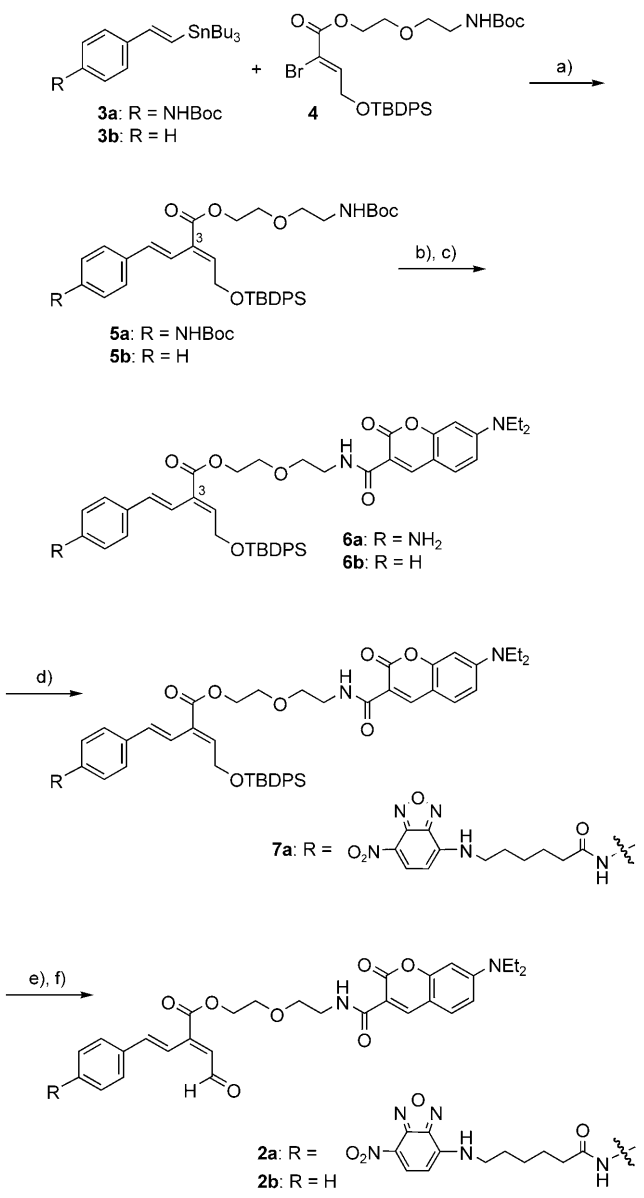
The incubation of HSA with probe **2a** might lead to the selective labeling of lysine(s) situated close to the ligand-binding



Scheme 2. Site-selective and nondestructive labeling of HSA by 6π-azaelectrocyclization.

site through Schiff base formation followed by rapid azaelectrocyclization (Scheme 2). Furthermore, we envisioned that the electrocyclization product, 1,2-dihydropyridine derivative (condition C), could be readily aromatized to the pyridinium ion by auto-oxidation, which could in turn accelerate the hydrolysis of the ester linkage at the C3 position to produce the zwitterion (condition D). This cascade process of oxidation-hydrolysis would, at the same time, recover the ligand-binding site in the NBD-labeled protein.

The synthesis of **2a** was carried out according to the previously established route for **1a**–**c** (Scheme 3).[8a] The TBDPS-protected (*Z*)-vinyl bromide **4** and (*E*)-stannane **3a** were heated to 115 °C in the presence of $Pd_2(dba)_3$, P(2-furyl)$_3$, and LiCl in DMF to provide the Stille coupling product **5a** at 42% yield. Two Boc-protecting groups in **5a** were removed by treatment with 20% TFA in CH_2Cl_2 to give the unstable diamine, which was immediately reacted with the succinimidyl ester of 7-diethylaminocoumarin-3-carboxylic acid to selectively introduce the ligand at the C3 position of **6a** at 64% yield over two steps. The remaining anilino nitrogen of **6a** was treated with NBD-X in the presence of HBTU and triethylamine in DMF to provide **7a** at 39% yield. Removal of the TBDPS group in **7a** and the subsequent oxidation were performed very rapidly because of the instability of these compounds,[8a] and the success of these transformations as well as the efficiency with which the final product **2a** labeled HSA (vide infra) was evaluated by MS analysis. Thus, TBDPS-deprotection was achieved in quantitative yield by the reaction with TBAF buffered with acetic acid in THF. Finally, the allylic alcohol was oxidized by PDC in CH_2Cl_2 for 30 min to afford the desired aldehyde probe **2a**, which was immediately used for protein labeling after rapid chromatography on silica gel. By applying the same route to **2a**, the analogue **2b** which lacks the NBD reporter group, was also synthesized as shown in Scheme 3. The analogue **2b** was used to facilitate the identification of the selectively labeled lysines by MALDI-TOF-MS analyses (vide infra).



Scheme 3. Synthesis of fluorescent labeling probes: a) $Pd_2(dba)_3$, P(2-furyl)$_3$, LiCl, DMF, 115 °C, 42% for **5a** and 72% for **5b**; b) 20% TFA, CH_2Cl_2, 0 °C; c) 7-diethylamino-3-carboxylic acid-OSu, CH_2Cl_2, room temperature, 64% for **6a** and 77% for **6b** (two steps); d) NBD-X, HBTU, Et_3N, DMF, room temperature, 39%; e) TBAF, AcOH, THF, 0 °C, quant. from **7a** and 42% from **6b**; f) PDC, CH_2Cl_2, room temperature, 70% for **2a** and 70% for **2b**.

To identify the lysine(s) selectively labeled by probes **2a** and **2b**, the probes were incubated with HSA in 0.1 M phosphate buffer at 40 °C for 30 min (final concentrations; HSA: 4.2×10^{-6} M, probes: 4.2×10^{-5} M). After the reaction was quenched by the addition of 1% AcOH, the excess probes were removed by quick size-partitioning gel-filtration. Based on the fluorescent spectra of the labeled HSAs, approximately 0.7–1 molecule of the probe was conjugated to each molecule of HSA (Supporting Information and Figure 2). The labeled HSAs were then subjected to lysyl endopeptidase (Lys-C) digestion after the cleavage of S–S bonds followed by the protection of the resulting -SH groups by 4-vinylpyridine treatment.[7b] The mixture of the Lys-C digests was directly analyzed by MALDI-TOF-MS[11] without further purification of each peptide. As the HSA labeled by the NBD analogue **2a** seemed unstable and decomposed during the S–S bond cleavage or Lys-C digestion, MS analysis focused on the digests of **2b**-labeled HSA. The peptide maps of labeled HSA and intact HSA, as well as the amino acid sequence of HSA (focused on T133–T162) are shown in Figure 1.

Digestions of intact HSA by Lys-C, which cleaves the C-terminal bond of lysine residues, detected 80% of the peptides[11] (from the total list of theoretical digestion products). We could observe a molecular ion (a) at $[M+H]^+ = 3115.5$ in the labeled digests, as the only peak that was not observed in the intact HSA digests.[12] This molecular ion is derived when Lys137 is labeled by **2b** (K137–K158 plus probe, Figure 1). Consistent with our earlier observations,[7b] the molecular weight of the probe adduct on Lys137 is well-matched to that of the zwitterion structure as shown in Figure 1, which justifies applying the cascade-type labeling chemistry envisioned in Scheme 2. Namely, the azaelectrocyclization product (1,2-dihydropyridine, Scheme 2) might be readily transformed into the correspond-

ing zwitterion by oxidation and hydrolysis of the ester during the enzyme digestion and/or MS analysis.[7b]

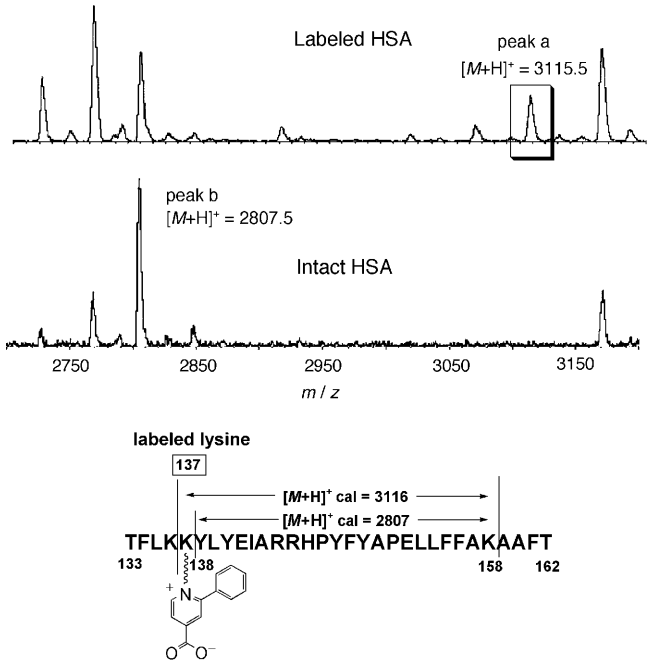
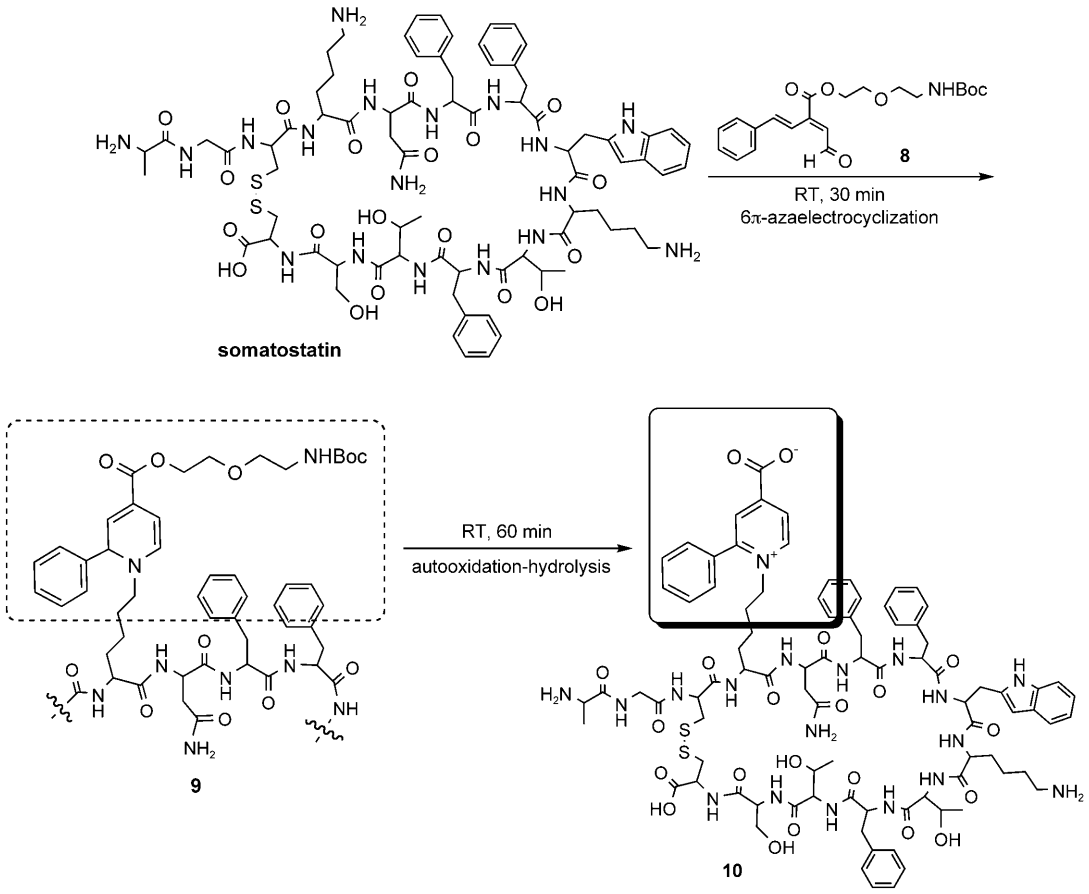


Figure 1. Amino acid sequence of HSA (T133-T162) and MALDI-TOF-MS digest maps of intact and labeled HSA by **2b**.

On the other hand, the intensity of the molecular ion at 2807.5 (peak b) is significantly reduced in the labeled HSA. This ion peak corresponds to amino acid residues Y138–K158. Thus, the results also support labeling at Lys137, that is, the enzymatic digestion was not possible because of the modification at Lys137. Based on these MALDI-TOF-MS analyses, we concluded that Lys137, which is in subdomain IB (amino acid sequences of 107–196), was preferentially labeled by the probe. The control labeling experiment using **8** (compound structure is shown in Scheme 4, vide infra), which did not contain the HSA ligand, did not show any selectivity for Lys-labeling as analyzed by MALDI-TOF-MS (Supporting Information). Thus, we conclude that the ligand-directed labeling of the target lysine was achieved.

As noted previously, we have designed probe **2a** to effect FRET between NBD and coumarin fluorophores. We then analyzed the whole labeling process in Scheme 2 (that is, the binding of **2a** to HSA[13] as well as the release of the coumarin ligand from the labeled protein) by fluorescence spectra. For all the fluorescence spectra in Figure 2, the absorbance maximum of the 7-diethylaminocoumarin fluorophore (donor) at 420 nm was excited to effect efficient FRET to NBD (acceptor).

Whereas the fluorescence spectrum of **2a** in the buffer solution (4.2×10^{-6} M) demonstrated efficient FRET between the two fluorophores (Figure 2A, spectrum a and condition A in Scheme 2), the fluorescence of coumarin significantly increased when the probe[13] was treated with an equimolar amount of



Scheme 4. Azaelectrocyclization with lysine of somatostatin. Reaction conditions: 2.9×10^{-3} M for somatostatin, 100 mM for **8** in H_2O.

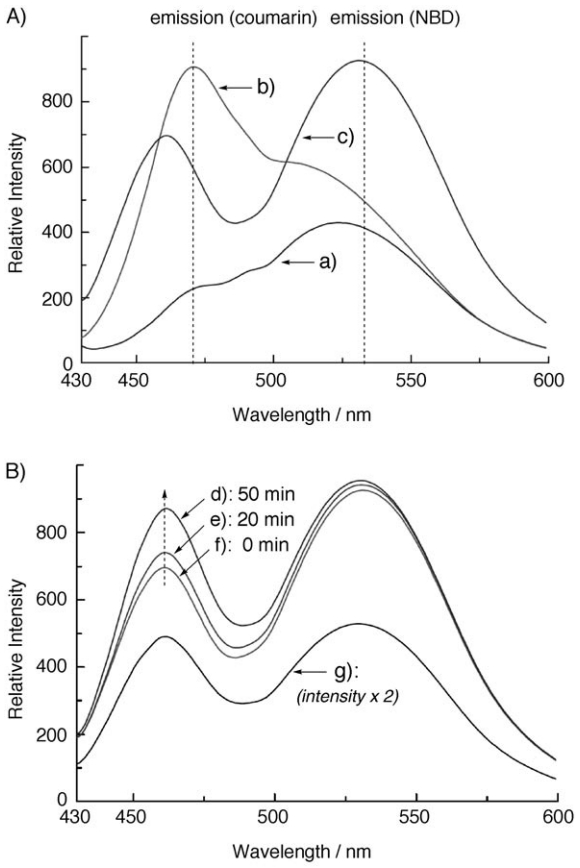


Figure 2. Fluorescence analysis of the labeling process in Scheme 2 (excitation at 420 nm). A) Labeling of HSA by **2a** through conditions A to C (conc: 4.2×10^{-6} M); a) Probe **2a** (condition A). b) Binding of **2a**[13] to HAS (condition B). c) 1,2-Dihydropyridine formation with Lys137 (condition C). B) d)–f) Time-course fluorescence analysis during conversion from dihydropyridine (condition C) to pyridinium zwitterion (condition D; conc: 4.2×10^{-6} M). g) Addition of 7-diethylaminocoumarin-3-carboxylic acid to labeled HSA by **2a** (after cleavage of coumarin ligand, conc: 1.0×10^{-6} M).

HSA (spectrum b and condition B in Scheme 2). The result is in good agreement with previous reports that the intensity of 7-alkylaminocoumarin fluorescence increases when bound to subdomains II-A or IB of HSA.[10] After 30 min, the spectrum gave another, different FRET (spectrum c) which corresponds to the formation of the electrocyclization product on Lys137 (condition C in Scheme 2). When the solution was kept at room temperature, the coumarin fluorescence gradually increased and it reached a maximum 50 min after dihydropyridine formation, as shown in Figure 2B, spectra d–f.

When the resulting solution was subjected to size-partitioning gel-filtration, the coumarin fluorescence disappeared from the NBD-labeled HAS. We reasoned that the ester linkage at C3, which connects the ligand to the probe, could be cleaved (even when the solution is kept under the neutral conditions in Figure 2) through an auto-oxidation–hydrolysis cascade (see mechanism in Scheme 2). No fluorescent signals, even of NBD, were observed as coumarin absorbance at 420 nm was excited for this experiment. Although many protocols for ligand-directed labeling of biomolecules have been reported, the removal of the ligands from the labeled samples is always a problem.[1,2] Even if the labeling could be performed under mild conditions, the conditions for the removal of the ligands (such as the hydrolytic cleavage of the ester linkages by increasing the solution pH) significantly limit the applicability of these methods. If the present labeling protocol using azaelectrocyclization proceeds to zwitterion formation in a cascade fashion, the labeled proteins may be used directly for molecular imaging immediately following incubation with the probes.

Unfortunately, we could not obtain a clear conclusion from the MALDI-TOF-MS of the labeled HSA; therefore, the critical process of zwitterion formation was examined by using a model peptide with a lower molecular weight. Thus, we labeled somatostatin (Mw = 1639)[8] with a simpler analogue of **2a** (**8**) under identical labeling conditions to those used for HSA (Scheme 4). After the electrocyclization product **9** (ESI-MS: calcd for $[M+H+NH_4]^{2+}$ 1013.4, found 1013.2) was kept at 24 °C for 1 h under neutral conditions, a single molecular ion of the zwitterion **10** could be detected (ESI-MS: calcd $[M+2H]^{2+}$ for 910.4, found 910.2). Thus, we have demonstrated cascade-type labeling of lysine under neutral conditions, as in Scheme 2.

Finally, 7-diethylaminocoumarin-3-carboxylic acid was added to the NBD-labeled HSA obtained above. The resulting labeled HSA–coumarin complex showed a fluorescence spectrum similar to that observed for (d) in Figure 2B (spectrum g, conc: 1.0×10^{-6} M, fluorescence intensity magnified twice for clarity); thus, we conclude that nondestructive labeling was achieved.

In summary, we have developed a selective and nondestructive method for protein labeling using an azaelectrocyclization-induced cascade reaction. HSA was used as a model protein target, and one of 59 lysines in subdomain IB was selectively labeled by designing probe **2a** that was loaded with the high affinity ligand of HSA. Furthermore, because of the auto-oxidation susceptibility of the electrocyclization products, the ligand on the labeled protein could automatically be released under both neutral and physiological conditions. The results described herein may be applicable for the selective labeling of target proteins in cell lysates, as well as at specific positions on cell surfaces. These studies are now in progress in our laboratory.

Experimental Section

Labeling of HSA by the probe **2a**: A solution of **2a** in 1,4-dioxane (53 μg, 5.0×10^{-3} M, 20 μL) was added to a solution of HSA in 0.1 M phosphate buffer (680 μg, 5.1×10^{-5} M, 200 μL, pH 7.4), and the mixture was incubated for 30 min at 40 °C. The final concentrations of HSA and **2a** were 4.2×10^{-6} M and 4.2×10^{-5} M, respectively. The reaction was quenched with 1% acetic acid (300 μL) and the resulting mixture was immediately passed through a NAP-5 column (Pharmacia LKB Biotechnology) equilibrated with 1% acetic acid (total elution volume of 1.0 mL). After the eluted protein was lyophilized, the amount of the label introduced to the protein was calculated based on the fluorescence spectra, as previously described[8a] (excitation of 7-diethylaminocoumarin excitation at 420 nm).

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Keywords: domino reactions · electrocyclic reactions · fluorescent probes · lysine · protein modifications

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[12] The smaller peaks other than $[M+H]^+$ 3115.5, observed only in labeled digests (Figure 1), are not particular to the labeled digests. These smaller peaks were also observed in intact digests when the laser power of MALDI was further increased.

[13] Determination of the precise fluorescence measurements during binding of the probe **2a** to HSA (condition B in Scheme 2) was not possible due to very rapid azaelectrocyclization. FRET of condition B was therefore analyzed using the corresponding alcohol derivative of **2a**.

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