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**COMMUNICATION**

# Target-selective fluorescent "switch-on" protein labeling by 6π-azaelectrocyclization†

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**Application of azaelectrocyclization and FRET techniques to lysine groups enabled the selective and sensitive detection of a target protein from a mixture, with high fluorescence contrast.**

Chemical methods for labeling proteins have garnered much attention in the fields of molecular imaging due to their straightforward procedures, general applicability, and the small size of chemical labels, at least relative to the genetically-encoded protein-based labels, such as Green Fluorescent Protein (GFP).[1] Recently, a variety of new chemical methods[2] that can be combined with biological techniques have been actively investigated.[3] For example, the Cu(I)-mediated Sharpless–Meldal click reaction employs an azide moiety that may be genetically introduced at desired positions within a protein. Bio-friendly (Cu(I)-free) versions of the click reaction using strained acetylenes[4] or the Staudinger reaction[5] have been developed by Bertozzi and co-workers. These reactions have been successfully applied to fluorescence imaging on cell surfaces. Entirely chemical methods that use protein-specific ligands to direct labeling using probe molecules, involving post-affinity labeling modifications (P-ALM), have been thoroughly investigated by Hamachi and co-workers.[6] After cleavage of the ligands from the labeled proteins, the recovered activity was found to provide a sensitive reporter for fluorescent imaging. Recently, a ligand-directed tosyl (LDT) strategy, which selectively labeled target proteins without removing the ligand, was reported by the same group. The labeling reaction simultaneously released the protein ligand from the labeled protein.[7] Successful application of this protocol involves the fluorescence labeling of carbonic anhydrase in human blood cells, lactose-binding congerin in mucus tissues of conger eel skin, and the biotin-labeling of endogenous FKBP12 in Jurkat cells.[7a] *In vivo* fluorescence labeling of the carbonic anhydrase was also achieved by intravenous injection of the probe into living mice.[7a]

We have independently been investigating a lysine-based protocol for labeling peptides, proteins, and living cell surfaces, based on rapid 6π-azaelectrocyclization.[8,9] This method was used to efficiently and selectively introduce both DOTA (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid) and fluorescent groups onto lysine residues by reaction with unsaturated aldehyde probes (such as probe **1** shown in Scheme 1) at very low concentrations (~$10^{-8}$ M) within a short period of time (10–30 min) at room temperature.[9] Under such mild reaction conditions, the labels could be introduced onto the more "exposed" surface lysines at a level of one or two molecules per protein. The native activity of the biomolecules and/or cell surfaces, therefore, was retained. Using the electrocyclization protocol, the receptor-mediated accumulation, circulatory residence, and trafficking of glycoproteins and/or lymphocytes were successfully visualized by non-invasive PET and fluorescence imaging. Site-selective non-destructive modification of the target proteins was also achieved by directing reactive groups to a specific site using a small-molecule ligand of the protein (see Scheme 1).[10] Labeling took place *via* Schiff base formation and subsequent electrocyclization. The ligand was then automatically cleaved from the conjugated protein *via* autooxidation of the 1,2-dihydropyridine and hydrolysis of the ester linkage connected to the ligand.

The ligand-directed approach described above may be applied to the selective labeling of target proteins in cell lysates, on cell surfaces, or even in living animals. Usually, selective labeling of a target protein in a mixture of biomolecules, *e.g.*, by fluorescence reporter groups, yields poor fluorescence contrast between the labeled protein and the unreacted probes. Sensitive detection of the targeted protein is quite difficult unless the labeled proteins are purified, the label-treated cells are washed, or the unreacted probes are preferentially excreted from the serum during labeling in living animals. The development of labeling probes that are fluorescently silent by default ("caged" fluorescence) but are "switched on" when they selectively react with the target proteins would go a long way to removing the unlabeled background fluorescence signal.

Kikuchi and co-workers recently reported an elegant "switch-on" method using a designed fluorescence quenching substrate in which the quencher on the label was specifically released by a β-lactamase, $^{E166}$NTEM, fused to the proteins and/or expressed on the cell surfaces.[11] Hamachi and co-workers also developed a "switch-on" probe that utilized their LDT strategy.[7b] In this communication, we describe a unique strategy based on the electrocyclization technique, in which fluorescence quenching systems may be applied to an unsaturated aldehyde probe **1** (Scheme 1), which is only unlocked when an azaelectrocyclization-induced cascade reaction proceeds on a target lysine. This new strategy enabled sensitive detection of a target protein in an equimolar mixture containing seven non-targeted proteins and

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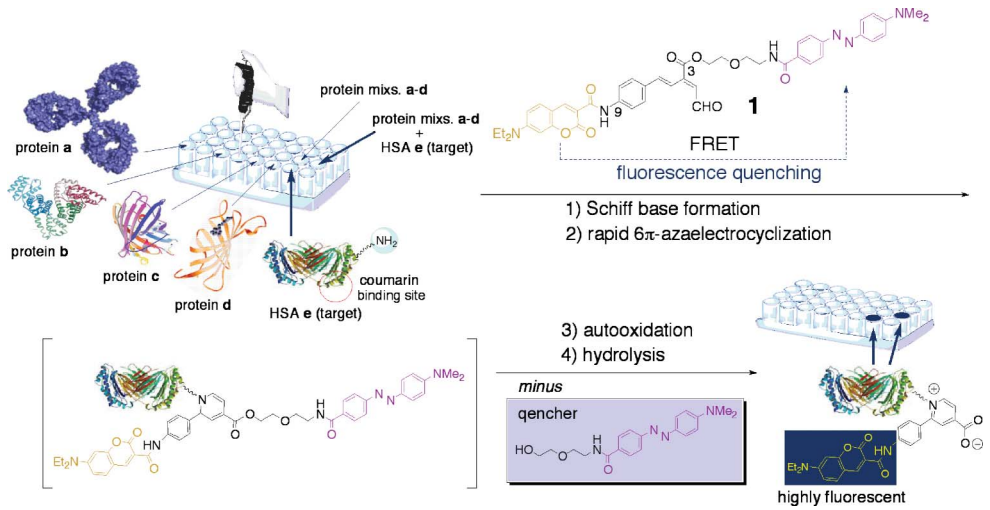


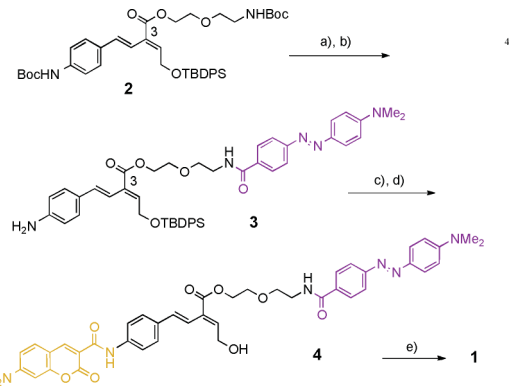

**Scheme 1** Target-selective and fluorescence "switch-on" mechanism of protein labeling by 6π-azaelectrocyclization.

peptides by achieving high fluorescence contrast from the labeled proteins without the need for isolation from the mixture.

Our "switch-on" labeling mechanism was hinted at by our earlier non-destructive labeling of human serum albumin (HSA, MW = 66 000)[10] and was investigated here using this model protein (Scheme 1). We have designed an unsaturated aldehyde **1** as the ligand-directed label-activated "switch-on" fluorescence probe. 7-Diethylaminocoumarin was introduced at the C9 position of the probe and the 4-[4-(dimethylamino)phenylazo]benzoic acid derivative (DABCYL) fluorescence quencher was attached at the C3 position *via* an ester linkage. The structural arrangement, shown in **1**, achieved efficient quenching of the coumarin fluorescence by DABCYL so that the probe **1** itself was non-fluorescent. 7-Diethylaminocoumarin is not only a "caged" fluorescence reporter group, but it also functions as a ligand of HSA:[12] it strongly binds HSA through the subdomain IB (amino acid residues 107–196).[13] We showed that loading of this ligand on the unsaturated aldehyde successfully directed the selective labeling of Lys137, among the 59 HSA lysines present, through azaelectrocyclization.[10]

Thus, incubation of HSA with probe **1** was hypothesized to favor the selective labeling of lysines situated close to the ligand-binding site, *i.e.*, Lys137, through Schiff base formation followed by rapid azaelectrocyclization. We envisioned that the 1,2-dihydropyridine derivative would produce zwitterions through autooxidation and hydrolysis. The cascade process would then release the DABCYL fluorescence quencher, thereby selectively recovering the coumarin fluorescence within the target protein.

The synthesis of **1** was carried out from the Boc-protected diamine **2**, which was previously prepared by Stille coupling as a key step (Scheme 2 and Supplementary Information †).[9a,10] Two Boc protecting groups in **2** 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 4-[4-(dimethylamino)phenylazo]benzoic acid to selectively introduce the fluorescence quencher at the C3 position of **2** in 62% yield over two steps. The remaining anilino nitrogen of **3** was treated with 7-diethylaminocoumarin-3-carboxylic acid in the presence of HATU and triethylamine in DMF to provide a coupling product in 76% yield. TBDPS deprotection was then achieved in 87% yield by




**Scheme 2** Synthesis of the "switch-on" labeling probe: a) 20% TFA, $CH_2Cl_2$, 0 °C; b) 4-[4-(dimethylamino)phenylazo]benzoic acid-OSu, $CH_2Cl_2$, rt, 62% (2 steps); c) 7-diethylamino-3-carboxylic acid, HATU, $Et_3N$, DMF, rt, 76%; d) TBAF, AcOH, THF, 0 °C, 87%; e) IBX-polystyrene, $CH_2Cl_2$, rt, quant.

reaction with TBAF buffered with acetic acid in THF. Finally, the allylic alcohol was quantitatively oxidized by 2-iodoxybenzoic acid (IBX) immobilized on polystyrene in $CH_2Cl_2$ for 2 h to afford the desired aldehyde probe **1**. The unstable aldehyde **1**, the structure of which was rapidly confirmed by the characteristic $^1$H NMR and MS, was immediately used for fluorescence and/or labeling experiments. As expected, fluorescence analysis of **1** (concentration of **1**: $1.0 \times 10^{-7}$ M) detected no coumarin fluorescence signal at 480 nm (excitation at 420 nm) due to efficient FRET (quenching) between the two dyes proximal to **1** (spectra similar to that shown in Fig. 1a). The labeling-induced "switch-on" strategy was thereby demonstrated using the HSA protein.

The probe **1** was first incubated with HSA in 0.1 M phosphate buffer at 25 °C (incubation concentrations, HSA: $1.0 \times 10^{-8}$ M, probe: $1.0 \times 10^{-7}$ M), and the resulting solution was directly analyzed by fluorescence spectroscopy. The coumarin fluorescence rapidly increased from 20 min to 2 days, and it reached the maximum in 5 days. The results indicate the release of the DABCYL quencher from the coumarin-labeled protein through the electrocyclization-initiated reaction on the HSA lysines (Fig. 1b). The formation of the zwitterion product was also confirmed by

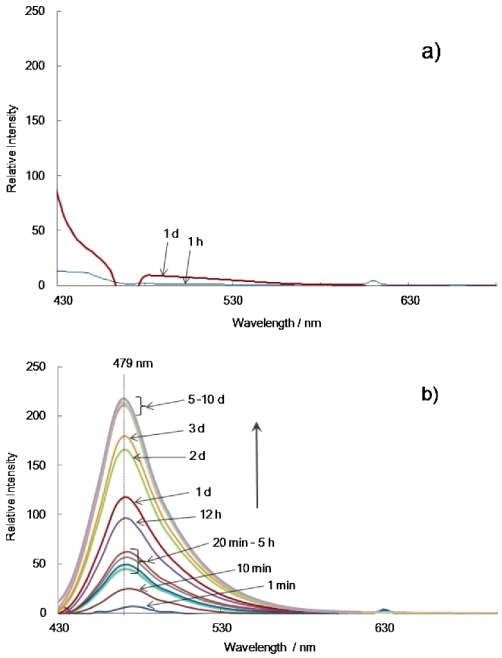



**Fig. 1** Fluorescence spectra of HSA solutions after treatment with (a) alcohol **4** (aldehyde precursor) as a control and (b) probe **1**. Both incubation and fluorescence analysis were performed at $1.0 \times 10^{-8}$ M for HSA, and at $1.0 \times 10^{-7}$ M for probes in PBS at room temperature (excitation at 420 nm).

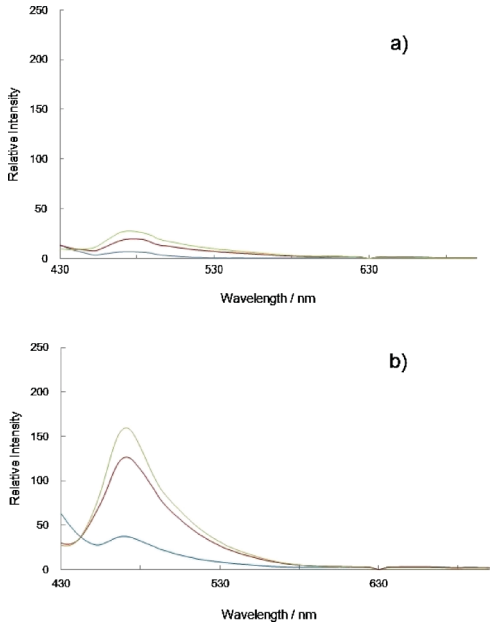



**Fig. 2** Fluorescence spectra of a solution containing several proteins and peptides after treatment with **1**. Incubation and fluorescence analysis were performed under conditions identical to those used for Fig. 1. (a) Protein and peptide mixtures consisted of phospholipase $A_2$, TGF-β3, orosomucoid, anti-Tau antibody, somatostatin, neurotensin, and ACTH. (b) Protein and peptide mixtures containing HSA. Blue: 1 h; red: 1 d; green; 2 d after incubation.

the MALDI-TOF-MS analysis (Supplementary Information†); since the 1,2-dihydropyridine can be very rapidly oxidized and hydrolyzed during the ionization by FAB, CI, ESI, and MALDI, the time-course analysis of the 1,2-dyhydropyridine formation, oxidation, and hydrolysis process was not possible by MS, as we have also experienced previously.[8b] In clear contrast, fluorescence was not enhanced for the alcohol precursor **4** upon incubation with HSA under identical conditions (Fig. 1a). All concentrations, even below $10^{-9}$ M HSA, were sensitively detected by the "switch-on" probe **1** (see the Supplementary Information †).

The promising fluorescence spectra drove examination of the labeling selectivity for the HSA protein of the ligand-directed probe **1** in the context of other proteins and/or peptides. Bovine pancreatic phospholipase $A_2$ (14 kDa), TGF-β3 (25 kDa), orosomucoid (44 kDa), and anti-Tau antibody (150 kDa) were tested as non-target proteins, and somatostatin (1.6 kDa), neurotensin (1.7 kDa), and ACTH (2.9 kDa) were tested as non-target peptides (Supplementary Information †). A $1.0 \times 10^{-8}$ M concentration (in PBS) of each protein or peptide was incubated in the presence of a 10 × concentration of the probe **1**. Gratifyingly, no detectable coumarin fluorescence was observed at 480 nm over 2 days. Fluorescence enhancement was not observed, even at $1.0 \times 10^{-6}$ M peptide or protein concentrations (Supplementary Information †). The data clearly showed preferential labeling of HSA by the probe **1** under mild reaction conditions. Selective labeling was efficiently achieved *via* the strong coumarin-protein interactions.

Last, a mixture of these seven proteins and peptides (each $1.0 \times 10^{-8}$ M in PBS) was treated with the probe **1** ($1.0 \times 10^{-7}$ M) in the absence or presence of HSA at the same concentration as the other biomolecules (Fig. 2). Coumarin fluorescence at 480 nm was not observed in the absence of HSA (Fig. 2a), whereas the fluorescence increased significantly in a time-dependent manner in the presence of the target protein (Fig. 2b). The degree of fluorescence enhancement was similar to that observed in the HSA/probe spectra shown in Fig. 1b, indicating selective reaction with HSA. To demonstrate that the fluorescence enhancement arose from the preferential labeling of HSA lysines, the mixed solution was directly analyzed by reverse-phase and size-partitioning gel-filtration HPLC with fluorescence detection. To confirm this analysis, MALDI-TOF-MS was also used for a few proteins (Supplementary Information †). Coumarin fluorescence was detected only at the elution time of the HSA, indicating ligand-directed selective labeling of this protein. Overall, the ligand-directed "switch-on" probe **1** labeled and sensitively detected a target protein in a solution containing seven non-target protein/peptides, all present at $10^{-8}$ M.

In summary, we developed a label-initiated "switch-on" probe for detecting target proteins using a fluorescence quenching system. This strategy selectively and sensitively detected a target protein by making use of the unique reactivity of an unsaturated aldehyde probe **1** on the target lysine. The target lysine was selectively labeled with a fluorescently "caged" probe **1** that could be loaded onto a high-affinity ligand of the target protein. The electrocyclization products were susceptible to autooxidation, so the fluorescence quencher present on the labeled protein was automatically released under both neutral and physiological conditions. Although many "fluorogenic" protocols in combination with various biological techniques directed to sensitive fluorescence labeling have been reported,[11] purely chemical methods that employ novel reactivity are quite rare.[7b] The results described here are applicable to the efficient labeling of target proteins in cell

lysates as well as on cell surfaces without the need for isolation and/or washing procedures. Alternatively, direct application to living animals permits the use of the labeled proteins and/or cells for molecular imaging immediately following injection of the probe. These studies are currently in progress in our laboratory.

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