Synthesis of Novel Luminescent Substrates and Their Incorporation into a Protein Only at a Terminal Site via a Transglutaminase-catalyzed Enzymatic Reaction

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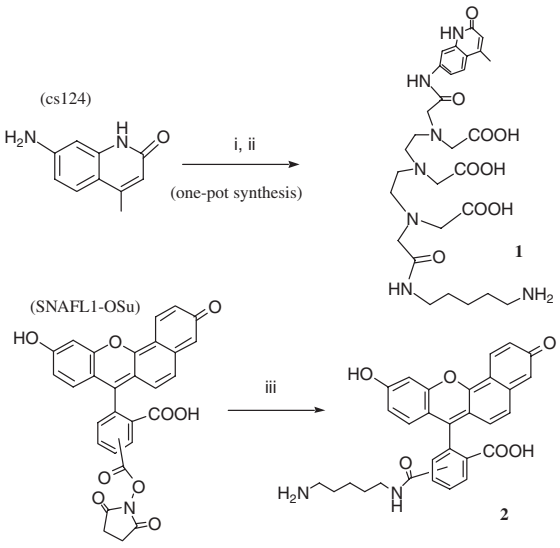
Luminescent lanthanide chelator and pH-sensitive fluorescent dyes were synthesized and conjugated only to a terminal site of a protein through mild enzymatic reaction.

N- or C-terminal-specific modification is an effective method for labelling proteins while retaining their activity.[1] Transglutaminase (TGase) is known to catalyze acyl transfer reactions between the γ-carboxyamide group of glutamine (Gln) residues and various molecules that contain certain lengths of primary alkyl amines. By applying this mild enzymatic reaction, we can obtain active proteins labelled only at a Gln site in a terminally appended TGase substrate peptide sequence (Pro–Lys–Pro–Gln–Gln–Phe–Met; TG1 sequence).[2,3] Compared to conventional chemical labelling and/or ribosome-mediated specific labelling,[4] the TGase-mediated labelling method is well suited for the introduction of bulky fluorescent groups into active proteins.[3] Because the recognition of the transglutaminase for the amine-substrate is fairly loose, different types of commercial amine-containing fluorophores, such as dansyl cadaverine[2] and fluorescein cadaverine,[3] have successfully been incorporated into a specific site of proteins.

These facts motivated us to synthesize some novel luminophores that can be recognized by TGase. First, a luminescent lanthanide chelate was chosen as the luminophore since it is known to exhibit distinct luminescence properties when irradiated with light.[5] The structure and synthesis[6] of the cadaverine-linked lanthanide chelator **1** is shown in Scheme 1.[7] In the presence of TGase, the HPLC-purified **1** was mixed with a target protein (glutathione S-transferase; GST) that has the terminally appended TG1 sequence.[3] Introduction of **1** into a terminal site of the target protein was confirmed by sodium dodecyl sulfate/poly-



Scheme 1. Reagents and conditions: (i) diethylenetriaminepentaacetic dianhydride, TEA, DMF, rt; (ii) cadaverine, rt, 70%; (iii) cadaverine, TEA, DMF, rt, 55%.

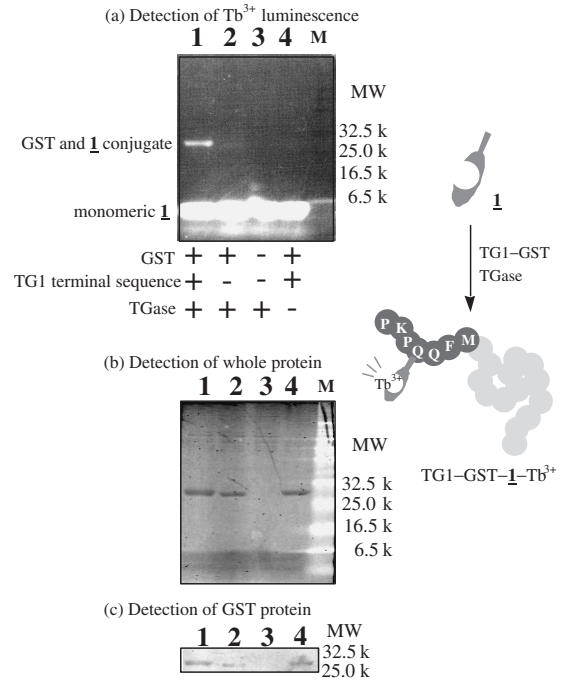


Figure 1. SDS-PAGE analysis of terminal-labelled protein. The proteins were applied to 15% SDS-PAGE and the gel was stained with Tb^{3+}. (a) The stained protein band was visualized with a UV transilluminator. (b) Whole proteins were visualized using the SYPRO(R) Orange (Molecular Probes, Inc.) protein gel staining system. The proteins were then transferred to PVDF membranes, followed by incubation with an anti-GST antibody and alkaliphosphatase-labelled *anti*-goat IgG. (c) The bands were visualized with Western Blue substrate (Promega). Each lane contains the following: lanes 1 and 4 contain TG1-GST and lane 2 contains wild-type GST. All lanes contain lanthanide chelator **1**. Lanes 1–3 contain transglutaminase (TGase). Lane M contains a prestained molecular weight marker.

acrylamide gel electrophoresis (SDS-PAGE), followed by staining with Tb^{3+}-containing PIPES/NaCl buffer. As shown in Figure 1, a characteristic green-white luminescence band was observed only in the presence of both TG1-GST and TGase (lane 1). Both SYPRO(R) Orange staining of the gel (Figure 1b) and Western blotting using an anti-GST antibody and alkaline phosphatase-labelled anti-Goat IgG (Figure 1c) indicated that this luminescent band was identified as a chimeric GST molecule. Once the chimeric GST and **1** conjugate was complexed with Tb^{3+} before electrophoresis, the very same green-white luminescence band was observed with the boiling pretreatment for SDS-PAGE without further staining with the Tb^{3+} solution after electrophoresis. This means that a certain amount of the GST–**1**-Tb^{3+} ternary complex still remains even after the harsh incubation conditions at 95 °C for 5 min in SDS-sample buffer (50 mM Tris-HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 12% glycerol, and 0.01% bromophenol blue).

The formation of the GST-**1**-Tb^{3+} ternary complex was further confirmed by 15% non-denaturing native PAGE analysis. As shown in Figure 2, the mobility of wild-type GST, TG1-GST, and TG1-GST-**1** (lanes 1, 2, and 4, respectively) were almost the same. However, once TG1-GST-**1** was complexed with Tb^{3+}, its electrophoretic mobility was quite different; over 90% of the GST band was shifted to a slower mobility (lane 3). This band shift seems to have mainly occurred from the cationic Tb^{3+}-neutralized electric charge of carboxylic anions on lanthanide chelator **1**. At this stage, we conclude that almost all of the TG1-GST was modified with **1**-Tb^{3+}. Earlier studies suggested that the amine specificity of TGase probably required the positioning of a suitable hydrophobic substituent at an optimal distance from the primary amino group of the alkylamine side chain.[8] It was surprising that lanthanide chelator **1** was successfully recognized by TGase, even though its hydrophilic substituent (i.e., diethylenetriaminepentaacetic acid; DTPA) is attached to one side of the cadaverine residue.

GST is a metabolizing enzyme that catalyzes the conjugation of glutathione to electrophilic substrates. To evaluate the effects of GST-**1**-Tb^{3+} ternary complex formation on the enzymatic activity, we performed the enzymatic activity assay as described in our previous paper.[3] More than 70% of the Tb^{3+} ternary complex protein was active, so most of the modified protein retained the native structure.

Second, a commercially available succinimide ester of fluorescein analog was also derivatised with cadaverine (Scheme 1b).[7] This pH-sensitive fluorescent dye was also successfully introduced into the terminal site of GST with the retention of the protein's native activity, using the same method as described previously. As expected, the fluorescent peak position of the labelled protein shifted to longer wavelengths continuously from pH 6–9. It is emphasized that the coupling reaction between cadaverine and succinimide esters of fluorophores is an extremely useful method to obtain novel fluorescent TGase substrates because many other colour variants of succinimide esters of fluorescein analogs are also commercially available. Some of their photophysical features such as pH sensitivity and/or photostabilities are known to be excellent.

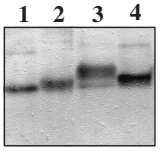


Figure 2. Native-PAGE analysis of terminal-labelled protein. The proteins were applied to 15% native-PAGE, and transferred to PVDF membranes, followed by incubation with an *anti*-GST antibody and alkaliphosphatase-labelled *anti*-goat IgG. The bands were visualized with Western Blue substrate (Promega). Each lane contains the following: lane 1 contains wild-type GST. Lanes 2–4 contain TG1-GST. Lane 3 contains both chelate dye **1** and Tb^{3+}, whereas lane 4 contains only **1**.

In conclusion, two different novel luminescent substrates for TGase were synthesized in a single step, and their enzymatic incorporation into an active protein only at a terminal site was investigated. This is the first example in which a single lanthanide luminophore was covalently bound only to the terminal site of an active protein. The extremely long luminescent lifetime of a lanthanide chelate-active protein conjugate can allow for a unique time-resolved fluoroimmunoassay[9] in which background fluorescent emission of the biological matrix, such as the autofluorescence in a living cell, can be effectively removed. Alternatively, using luminescence resonance energy transfer (LRET) from lanthanide chelate, real-time observation of the interaction between the derivatized active protein and another biological molecule may also be possible at the single molecule level and in the native in vivo context.[6] Using the pH-sensitive fluorescent dye, we may link information directly between a microenvironmental pH change around a target protein and the translocation and/or conformational change of the target protein.[10,11] For these assays, we are currently trying to deliver these luminophore-modified proteins into living cells using a lipid-mediated delivery system.[12]

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References and Notes

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7 Spectroscopic data of **1** and **2** are in the following. **1**: ^{1}H NMR (D_2O) δ 7.52 (1H, d), 7.50 (1H, s), 7.17 (1H, d), 6.23 (1H, s), 2.94–3.80 (18H, m), 2.90 (2H, t), 2.74 (2H, t), 2.27 (3H, s), 0.97–1.44 (6H, m); positive-ESIMS (m/z) Calculated for $(M + H)^+$: 634.320. Found: 634.319. **2**: ^{1}H NMR (D_2O) δ 8.22 (2H, m), 7.89 (1H, d), 7.24 (2H, m), 7.09 (2H, m), 6.99 (1H, s, broad), 6.85 (1H, d), 6.62 (2H, m), 3.36 (2H, t), 2.93 (2H, t), 1.61 (4H, m), 1.39 (2H, m); positive-ESIMS (m/z) Calculated for $(M + H)^+$: 511.186. Found: 511.186.
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