Enzymatic single-step preparation of multifunctional proteins

Supplementary Information

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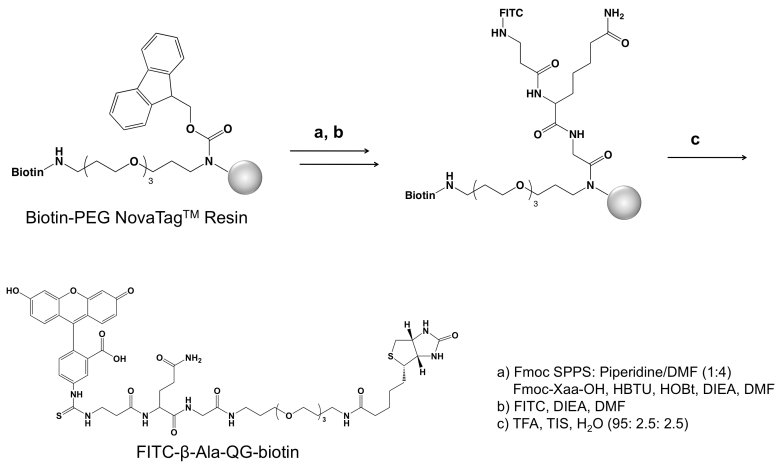
Experimental details

1. Preparation of FITC-β-Ala-QG-biotin

FITC-β-Ala-QG-biotin was manually synthesized on Biotin-PEG Nova Tag™ Resin (0.1 mmol) with an Fmoc strategy using a PD-10 column.[1] Coupling reactions employed 3 equiv. (relative to resin) of Fmoc amino acids activated in situ with 3 equiv. of HBTU and 3 equiv. of HOBt and 6 equiv. of DIEA in DMF for 120 min. Removal of the Fmoc-protecting group was achieved by treatment with PPD/DMF (20%, v/v) for 15 min. The deprotection and cleavage from the resin was accomplished by the addition of TFA/TIS/H_2O (95:2.5:2.5) for 1 h. The decantation was treated with ether to precipitate the peptides. The pelleted material was lyophilized to yield a yellow powder. RP-HPLC purification (absorbance was monitored at 495 nm, 10–80% eluant B in 30 min gradient) was used and the fractions collected were lyophilized. The synthesis of FITC-β-Ala-QG-biotin was confirmed by RP-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Autoflex-III) (Fig. S1). Mass spectrum: $C_{51}H_{65}N_9O_{14}S_2$; MW: 1092.2 g/mol; calculated exact mass: 1091.4; found m/z $[M+H]^+$=1092.2. The material was lyophilized to yield a yellow powder.

Scheme S1 Preparation of FITC-β-Ala-QG-biotin.



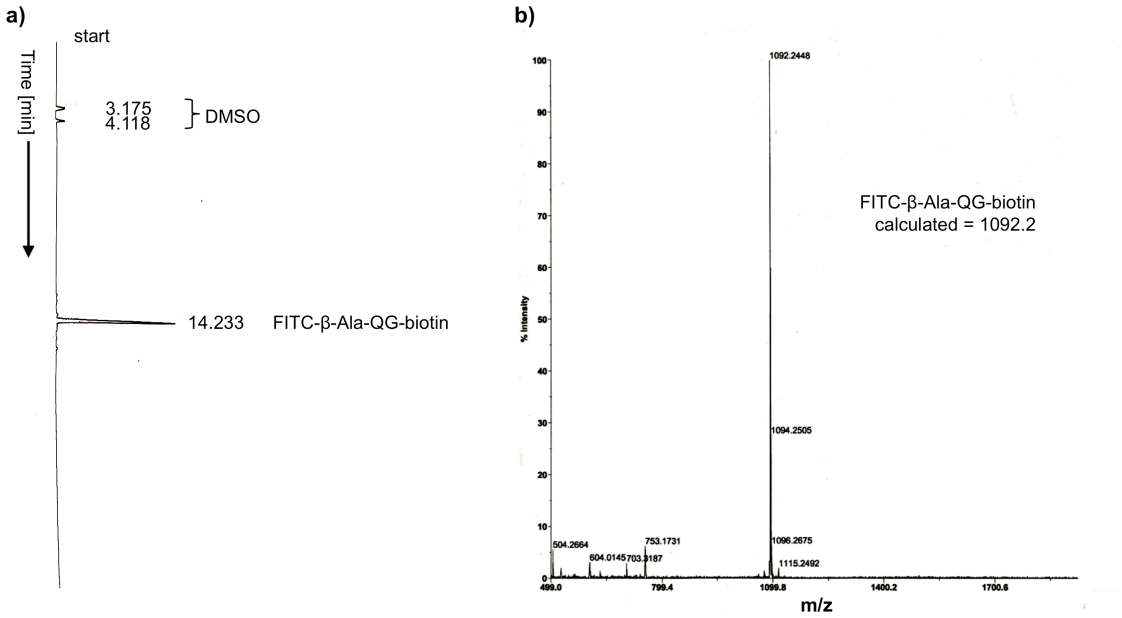


Fig. S1 (a) RP-HPLC chromatogram of the purified FITC-β-Ala-QG-biotin (absorbance was monitored at 230 nm). Analytical conditions were as follows: column; Inertsil ODS-3 (GL sciences, Inc., 10 × 250 mm), mobile phase; CH_3CN/H_2O (containing 0.1% TFA) = 10/90 → 80/20 (30 min) → 95/5 (35 min), flow rate; 5 ml/min. (b) The mass chromatogram of FITC-β-Ala-QG-biotin.

2. Kinetic analysis of MTG-mediated cross-linking using functional glutamine substrates with model acyl acceptor substrate

The reaction mixtures consisting of each glutamine substrate (0.5–2 mM), model substrate (MRHKGS-NH_2; 5 mM), and 0.05 U/ml MTG in 100 mM phosphate buffer (pH 7.0) containing 1 vol% DMSO were incubated for 10 min at 37 °C. After terminating the reaction with 1% aqueous TFA, each mixture was analyzed by RP-HPLC (absorbance was monitored at 440 nm). Analytical conditions were as follows: column; Inertsil ODS-3 (GL sciences, Inc., 4.6 × 250 mm), mobile phase; CH_3CN/H_2O (containing 0.1% TFA) = 10/90 → 80/20 (30 min) → 95/5 (35 min), flow rate; 0.8 ml/min. We assumed that the competing hydrolysis of glutamine substrates is negligible under the experimental conditions used. Support for this assumption was the absence of a signal in the chromatogram derived from the hydrolyzed product.

3. Calculation of the degree of labeling of FITC-β-Ala-QG-biotin-labeled NK6-AP

NK6-AP labeled with FITC-β-Ala-QG-biotin was separated from the unreacted probe and MTG by a Ni-NTA column using the His-tag of the recombinant proteins. First, the reaction mixture was loaded onto a Ni-NTA column (column volume: 1 ml, Amersham Biosciences), which was equilibrated with 20 mM Tris-HCl (pH 7.4) supplemented with 500 mM NaCl and 35 mM imidazole. Next, the column was washed by 10 ml of the equilibrated buffer. The labeled NK6-AP was eluted with 20 mM Tris-HCl (pH 7.4) supplemented with 500 mM NaCl and 500 mM imidazole. The buffer components of the eluted aqueous solution were exchanged using a PD-10 column with 50 mM Tris-HCl (pH 8.0). The UV-vis absorption spectrum of labeled NK6-AP was measured using a NanoDrop ND-1000 (NanoDrop).

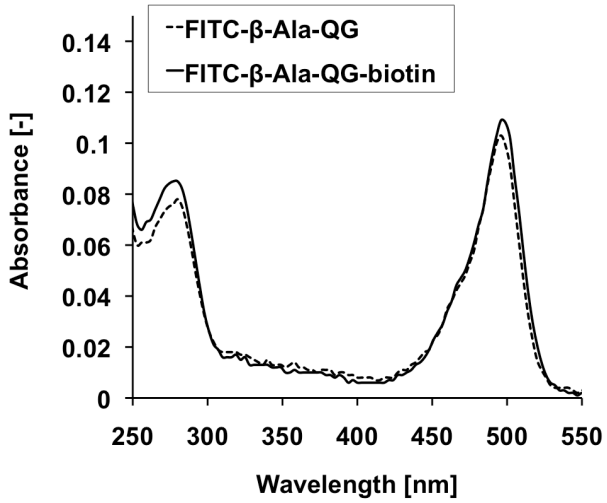


Fig. S2 A representative UV-visible absorption spectrum of purified NK6-AP labeled with FITC-β-Ala-QG or FITC-β-Ala-QG-biotin.

4. Effect of MTG-mediated conjugation of FITC-β-Ala-QG-Biotin on the function of NK6-AP

The catalytic activities of AP were measured with *p*-nitrophenylphosphate (*p*-NPP) as a substrate. In 1 ml of 1 M Tris-HCl buffer (pH 8), the hydrolysis of *p*-NPP (1 mM) was initiated by the addition of each recombinant AP at 25 °C. The initial activity was determined by the increase in the absorbance at 410 nm derived from *p*-nitrophenol on a UV/Vis spectrophotometer V-560 (Jasco). Fig. S3 shows the enzymatic activity of APs following MTG-mediated treatment with FITC-β-Ala-QG-biotin. The results indicate no negative effect of MTG-mediated protein labeling with FITC-β-Ala-QG-biotin.

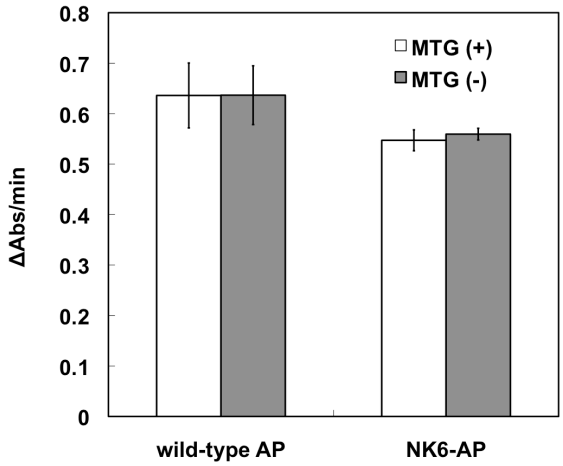


Fig. S3 Enzymatic activity of APs following MTG-mediated treatment with FITC-β-Ala-QG-biotin. Only NK6-AP was quantitatively labeled with the new substrate.

5. Immobilization of FITC-β-Ala-QG-biotin-labeled NK6-AP on a streptavidin-coated resin

All the NK6-APs treated with MTG were purified by a Ni-NTA column using the His-tag of NK6-AP, as mentioned in the preceding section. Prior to applying the NK6-AP samples, the buffer was exchanged using a gel filtration column (PD-10, GE healthcare) with 50 mM Tris-HCl (pH 8).

Immobilization of FITC-β-Ala-QG-biotin-labeled NK6-AP was carried out with a Pierce Streptavidin Plus UltraLink® Resin (Pierce) and a fluorescent microscope (Olympus). Prior to use, 5 µl settled resin (10 µl of the 50% slurry) was pre-washed three times with washing buffer (50 mM Tris-HCl (pH 8)). After removing the supernatant, the resin was resuspended in 50 mM Tris-HCl (pH 8) containing 2 µM labeled NK6-AP (100 µl), incubated at 37 °C for 30 min and washed three times with the washing buffer. The resin was resuspended in the washing buffer (100 µl of the 5% slurry). Subsequently, a fluorescence of the FITC-β-Ala-QG-biotin-labeled NK6-AP immobilized resin was measured by a fluorescent microscope. Finally, the catalytic activity of immobilized AP was measured with *p*-NPP as the substrate. The 0.5 µl settled resin (10 µl of the 5% slurry) was incubated in 1 ml *p*-NPP (1 mM, 1 M Tris-HCl (pH 8)) for 1 min. The mixture was centrifuged immediately, and the supernatant was collected. Enzymatic activity was determined by an increase in the absorbance of the supernatant at 410 nm (derived from *p*-nitrophenol).

6. MTG-mediated conjugation of an anti-PSA monoclonal IgG antibody with FITC-β-Ala-QG-biotin

An anti-prostate specific antigen (PSA) monoclonal IgG1 antibody was purchased from Cosmo Bio Co., Ltd., and employed without further purification.

The reaction mixture consisted of the anti-PSA antibody (0.5 mg/ml) and FITC-β-Ala-QG or FITC-β-Ala-QG-biotin (1 mM) in PBS containing 5 vol% of DMSO. The antibody labeling reaction was initiated by the addition of MTG (1 U/ml) and the reaction mixture was kept at 37 °C for 24 h. After incubation, the reaction products were analyzed by SDS-PAGE. The in-gel fluorescence analysis was performed with Molecular imager® FxPro (Bio-Rad, excitation: 488 nm, band pass filter: 530 nm). The analysis revealed clear fluorescence detection as a single band corresponding to the heavy chain of the antibody (Fig. S4, lanes 1 and 3), whereas fluorescent bands were not detected when no MTG was added (Fig. S4, lane 2).

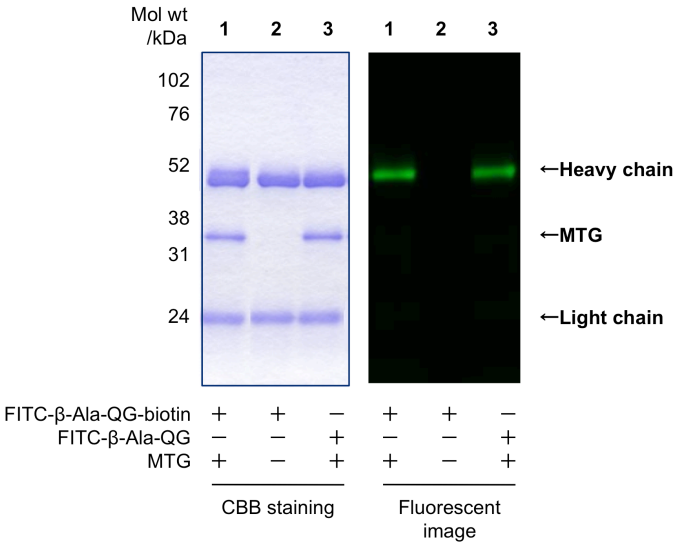


Fig. S4 SDS-PAGE and in-gel fluorescent analyses of the MTG-mediated conjugation of an antibody with FITC-β-Ala-QG-biotin or FITC-β-Ala-QG.

References

1. Koda, D.; Maruyama, T.; Minakuchi, N.;Nakashima, K.; Goto, M. *Chem. Commun.* **2010**, *46*, 979-981.