Supporting information for:

Photo-induced Conjugation of Tetrazoles to Modified and Native Proteins

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Materials & Methods

Phosphate buffer saline (PBS, 1X, pH 7.4) was purchased from Invitrogen. Absolute ethanol and dimethylformamide (DMF) were from Merck. Ethyl acetate and dichloromethane were from Alfa Aesar. Terephthaldehydic acid, benzenesulfonyl hydrazide, aniline, allyl chloride, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and L-tryptophan were bought from TCI. Poly(ethylene glycol)monomethyl ether-3300 (PEG-3300) was from Polymer Source (Toronto, Canada). Sodium nitrite, sodium chloride, hydrochloric acid, pyridine, 4-hydroxy-3-methoxycinnamic acid, and β-lactoglobulin A from bovine milk (BLG-A), horseradish peroxidase Type I (HRP), *Candida Antarctica* lipase B (recombinant from *Aspergillus oryzae*; CalB), and lysozyme from chicken egg white were bought from Sigma-Aldrich. The polypeptide JR2EC was kindly donated by Dr D. Aili (Linköping University, Dept of Physics, Chemistry and Biology; sequence: NH_2-NAADL EKAIE ALEKH LEAKG PCDAA QLEKQ LEQAF EAFER AG-COOH). The tryptophan-functional peptide and the control peptide were from Optimal Biotech (Singapore) with sequences NH_2-GHALHLAHALYW-COOH (MW = 1388.57) and NH_2-GHALHLAHALYC-COOH (MW = 1305.5), respectively (purity > 95%).

Absorbance and fluorescence measurements were performed on a Tecan Infinite M200 pro plate reader using 96-well plates (Corning). Nuclear Magnetic Resonance (NMR) spectra were obtained from a 300-MHz Bruker Avance Ultrashield. UV irradiation was conducted using a UVP UVM-57 Handheld UV lamp at 302 nm (6 Watt, 0.16 Amps). SDS-PAGE was performed using a Bio-Rad Mini-PROTEAN Tetra Cell. MALDI-ToF measurements were performed on a Shimadzu Kratos Axima TOF2 using 4-hydroxy-3-methoxycinnamic acid as a matrix.

Preparation of 4-(2-phenyl-2H-tetrazol-5-yl)benzoic acid (tetrazole)

This compound was synthesized as reported previously.[1]

Preparation of Tetrazole-functionalized Poly(ethylene glycol), PEG-3300-Tetrazole

The synthesis of PEG-tz has been reported previously.[2]

A solution of EDC (10.9 mg, 57.2 μmol) in 0.5 mL of dichloromethane was added drop wise to a mixture of PEG-3300 (100.0 mg, 28.6 μmol), tetrazole (9.7 mg, 34.3 μmol), and DMAP (0.8 mg, 2.86 μmol) in 1.0 mL of dichloromethane at -20 °C. After complete addition, the reaction mixture was allowed to stir for 20 hours at room temperature. Then, the reaction mixture was extracted with 0.1 M HCl (1.5 mL x 2), followed by saturated sodium chloride (1.5 mL x 2). The organic layer was dried with magnesium sulfate before rotary-evaporation and drying under high vacuum overnight. The orange crystalline solid was dissolved in water (milli-Q) and centrifuged at 14,000 rpm for 10 minutes to remove non-dissolved material. The filtrate was freeze-dried overnight to yield 79.9 mg (74.5%) of a white powder.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.31 (d, 2H), 8.18 (m, 4H), 7.56 (m, 2H), 7.48 (m, 1H), 4.48 (t, 2H), 3.83 (t, 2H), 3.51 (m, 296H), 3.36 (s, 3H). MALDI-ToF: m/z 3139.84.

Preparation of allyl-modified β-lactoglobulin (BLG-allyl)

Allyl-functionalized BLG was prepared as detailed in literature by mixing 2.0 mL of 2.5 mg/mL BLG (0.13 mM) in 5.0 mM of PBS, pH 7.5 with 300 µL of allyl chloride (0.65 M) in DMF.[3] The mixture was incubated under agitation using a thermomixer (VWR 5355, Eppendorf) at 37 °C for 2.0 hours, followed by removal of the small molecules using a PD-10 column, eluting with 5.0 mM PBS, pH 7.5. An Ellman's test was performed to determine the concentration of free thiol. The product was freeze-dried overnight to yield a white powder.

	BLG-A concentration (mg/mL) [a,b]	**Conc. of Free -SH (µM)[b]**	**Free thiol / BLG-A (mole/mole)**
BLG-A before reaction	2.5 (± 0.3)	3.3 (± 0.1)	1.3
BLG-A after reaction with allyl chloride	0.41 (± 0.04)	-0.1 (± 0.2)	-0.03

a) BLG-A concentration was determined by the absorbance of protein at 280 nm using UV/Vis spectrometer.

b) Average of three independent measurements

Preparation of Protein-Polymer Conjugates using Photoinduced Cycloaddition Chemistry

The protein-polymer conjugates were prepared by UV irradiation of an aqueous solution of BLG-allyl and PEG-tetrazole (UVP UVM-57 Handheld UV lamp, 302 nm, 6 Watt). Thus, 1.0 mL of 2.0 mg/mL BLG-allyl (0.11 µmol, 1.0 equiv.) in milliQ was mixed with 0.75 mL of 5.0 mg/mL PEG-3300-Tetrazole (1.1 µmol, 10.0 equiv.) in milliQ in a quartz cuvette (10 mm path length). Under stirring, the mixture was irradiated at 302 nm with a handheld UV-lamp at about 5 cm distance for 15 minutes, resulting in yellow/orange solution. For purification, the mixture was filtered three times through Vivaspin 10 kDa centrifuge filter at 10,000 rpm for 10 min (Legend Micro 21 Centrifuge, Thermo Scientific, Sorvall) to remove excess PEG-Tetrazole. The resulting PEG-Pyr-BLG was re-dispersed in 1.0 mL of PBS, and freeze-dried overnight yielding a yellowish powder. The product was characterized by fluorescence measurements, SDS-PAGE and MALDI-ToF.

The same procedure was followed for the conjugation of PEG with lysozyme, HRP, CalB and the three polypeptides.

Figure S1

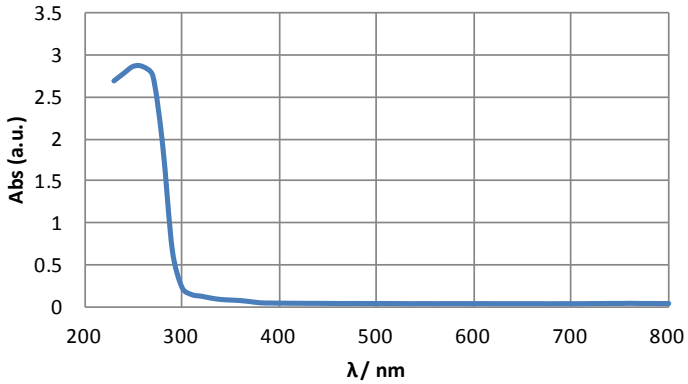

Figure S1: Absorbance spectrum of BLG (1 mg mL^{-1} in PBS) in the range of λ = 230 – 600 nm.

Figure S2

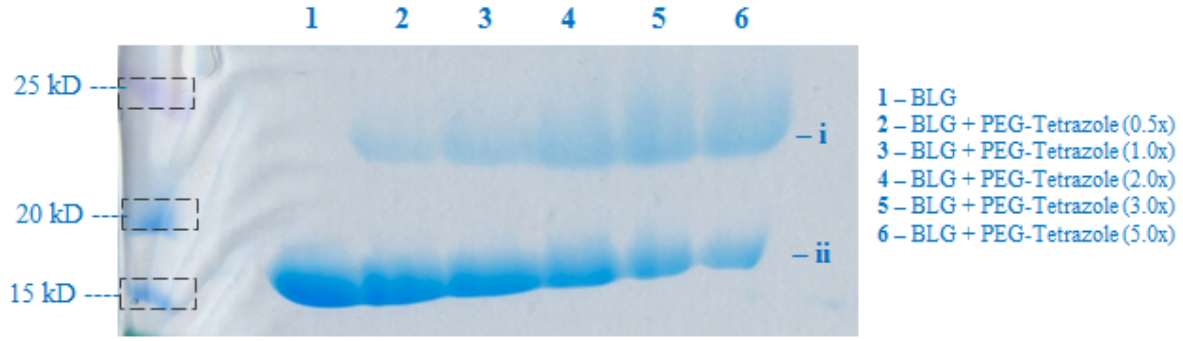

Figure S2: SDS-PAGE of the product of PEG-tz and unmodified BLG, after photo-irradiation at increasing eqs of PEG-tz (0.5, 1.0, 2.0, 3.0 and 5.0 eqs). Bands indicated by **i** represent the conjugated product, while **ii** represents native BLG.

Figure S3

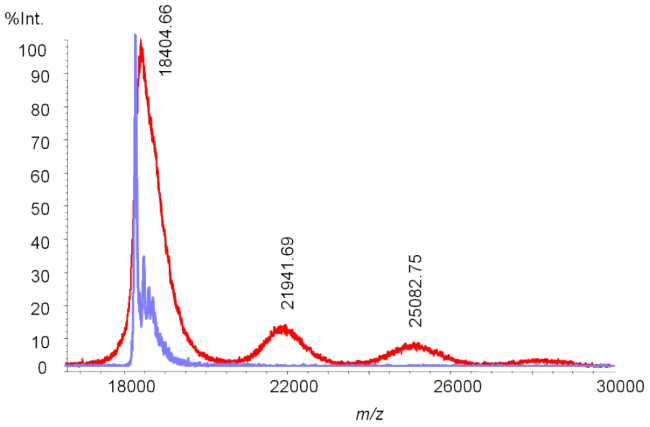

Figure S3: MALDI-ToF spectrum of native BLG (blue line) and the PEG-tz:BLG conjugate (red).The peaks at m/z 21.9 kDa and 25.1 kDa represent singly conjugated and doubly conjugated BLG, respectively.

Figure S4

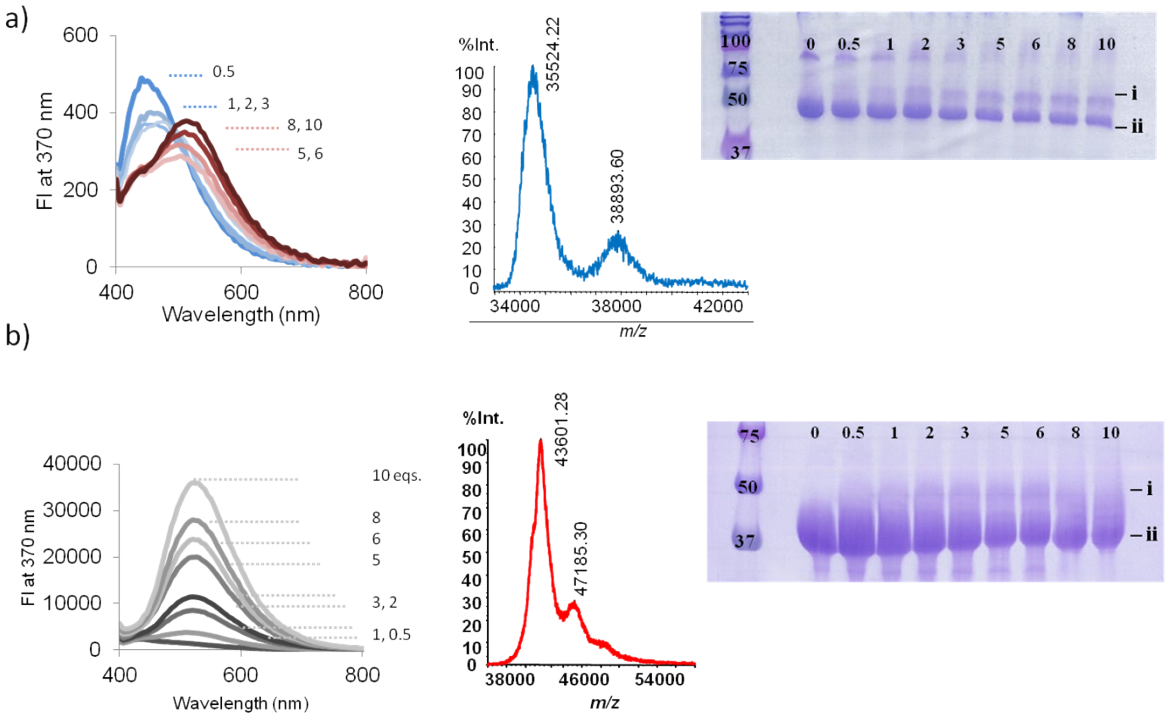

Figure S4: Fluorescence and MALDI-ToF spectra, as well as SDS-PAGE analysis of conjugates formed after irradiation of PEG-tz with native CalB (a) and HRP (b). HRP shows a relatively simple unimodal fluorescence spectrum that steadily increases in intensity with higher PEG-tz / HRP ratios, whereas the evolution of the PEG-tz:CalB spectrum is more complex. For CalB, MALDI-ToF indicates the formation of predominantly mono-conjugated enzymes, while HRP is both doubly and singly conjugated enzyme. For SDS-PAGE, bands indicated by **i** represent the conjugated product, while **ii** represents the free enzyme.

Figure S5

JR2EC: NH2-NAADL EKAIE ALEKH LEAKG PCDAA QLEKQ LEQAF EAFER AG-COOH

Figure S5: Amino acid sequence of JR2EC. A cysteine is present at position 22.

Figure S6

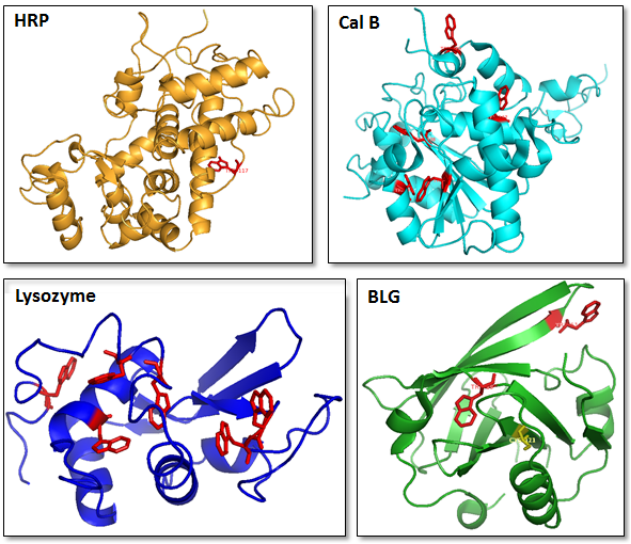

Figure S6: Graphic representations of HRP, CalB, Lysozyme and BLG, with tryptophan residues shown in red. The picture was rendered using PyMOL (PDB ID for HRP, CalB, lysozyme, and BLG: 1ATJ, 3ICV, 6LYZ and 1QG5, respectively). HRP, CalB, BLG and lysozyme contain one (Trp-117), five (Trp-86, Trp-99, Trp-138, Trp-147, and Trp-189), two (Trp-19 and Trp-61), and six tryptophan residues (Trp-28, Trp-62, Trp-63, Trp-108, Trp-111, and Trp-123), respectively.

References:

1 S. Ito, Y. Tanaka, A. Kakehi, K. Kondo, K., *Bull. Chem. Soc. Jpn.*, 1976, **49**, 1920-1923
2 W. Song, Y. Wang, J. Qu, M. M. Madden, Q. Lin, *Angew. Chem., Int. Ed.*, 2008, **47**, 2832–2835
3. J. M. Chalker, Y. A. Lin, O. Boutureira, B. G. Davis, *Chem. Commun.*, 2009, 3714-3716