Supporting Information

An efficient reagent for covalent introduction of alkyne into proteins

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Experimental part

1. Synthesis of the diazonium reagent 2

![Chemical Structure](image)

4-Ethynyl Benzene Diazonium Hexafluorophosphate (2). 4-Ethynylaniline (1.00 g, 8.5 mmol) was dissolved in 30 mL cold concentrated HCl. The water solution of NaNO₂ (1.77 g, 25.6 mmol) was slowly added to the mixture at -10 °C. After 1.5 h reaction, 60% HPF₆ in water (2.5 mL, 17.0 mmol) was added at -10 °C and stirred for 1 h. Then the mixture was stirred at room temperature for 30 min. The product were collected by filtration and washed with ice-cold water, yielding a light yellow solid of 2 (1.34 g, 57%). ¹H NMR (600 MHz, DMSO-d₆), δ 8.70-8.65 (m, 2H), 8.01 (d, J = 8.4 Hz, 2H), 5.04 (s, 1H). ¹³C NMR (151 MHz, DMSO-d₆), δ 133.9, 133.8, 132.9, 115.3, 91.2, 81.1. ³¹P NMR (243 MHz, DMSO-d₆), δ -144.23 (h, Jₚ-F = 711.2 Hz). ¹⁹F NMR (376 MHz, DMSO-d₆), δ -70.30 (d, Jₚ-F = 711.2 Hz). HRMS (ESI): m/z [M]+ calcd. for C₆H₅N₂: 129.0447; found: 129.0447.

2. Synthesis of fluorescence probe (6).

![Chemical Structure](image)

A mixture of fluorescein isothiocyanate isomer I 5 (77.9 mg, 0.2 mmol) and
11-azido-3,6,9-trioxaundecan-1-amine (40 µL, 0.2 mmol) was dissolved in 9 mL dry tetrahydrofuran. DIPEA (38 µL, 0.22 mmol) was added to the reaction mixture and stirred at room temperature for 2 h. The crude product was purified by silica gel column chromatography (CH₂Cl₂/CH₃OH). TLC Rf = 0.74 (CH₂Cl₂/CH₃OH = 9:1). ¹H NMR (400 MHz, MeOD), δ 8.17 (s, 1H), 7.73 (d, J = 7.7 Hz, 1H), 7.10 (d, J = 8.1 Hz, 1H), 6.64 (d, J = 7.3 Hz, 4H), 6.50 (d, J = 8.5 Hz, 2H), 3.70 (bs, 2H), 3.64-3.55 (m, 12H), 3.26 (s, 2H). ¹³C NMR (101 MHz, MeOD), δ 182.6, 171.2, 161.8, 154.3, 148.8, 142.3, 131.4, 130.8, 130.4, 129.2, 125.8, 119.8, 113.9, 111.6, 103.5, 71.5, 71.2, 70.9, 70.1, 51.6, 45.4.

3. Synthesis of azido-PEG2000 (9a) and azido-PEG6000 (9b).

PEG2000-N₃ (9a). 7a (4.0 g, 2.0 mmol) was heated at 80 °C under reduced pressure to remove trace water. The solid was dissolved by 40 mL DMSO after cooling down to room temperature. CDI (1.62 g, 10.0 mmol) was added to the solution and stirred for 19 h. Then the solution was diluted with 500 mL EtOAc and put at -20 °C for 5 h. The resulting white precipitate was collected by filtration and washed by Et₂O to give 8a (397 mg, 0.2 mmol), which was dissolved in 3 mL DMSO. 11-Azido-3,6,9-trioxaundecan-1-amine (60 µL, 0.3 mmol) and DIPEA (103 µL, 0.6 mmol) were added to the DMSO solution. After heated at 80 °C for 24 h, the mixture was washed by brine and extracted with CH₂Cl₂ (3 × 25 mL). The CH₂Cl₂ solution was dried by Na₂SO₄ and concentrated under reduced pressure. And the resulting oil-like mixture was diluted with 125 mL EtOAc and put at -20 °C overnight. After pouring the supernate and diluted with Et₂O, the white precipitate was collected by centrifugation (4000 rpm, 0 °C, 10 min). Then the solid was washed with Et₂O and was centrifuged again. The product 9a was obtained by drying the solid under reduced pressure, yield 84.4 mg (18.8%). ¹H NMR (400 MHz, DMSO-d₆) δ 7.18 (t, J = 5.4
Hz, 1H), 4.04 (t, J = 9.2 Hz, 2H), 3.73-3.33 (m, 171H), 3.16-3.06 (m, 2H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 156.2, 72.3, 69.8, 69.7, 69.5, 69.3, 69.1, 68.8, 63.1, 60.2, 50.0.

**PEG6000-N$_3$ (9b).** PEG6000 7b (12.0 g, 2.0 mmol) was heated at 80 °C under reduced pressure to remove water. The solid was dissolved by 40 mL DMSO (with 5% CH$_2$Cl$_2$ to facilitate the dissolution of PEG6000) after cooling down to room temperature. CDI (1.56 g, 9.6 mmol) was added to the solution and stirred for 2 days. After removing CH$_2$Cl$_2$ under reduced pressure, the solution was diluted with 500 mL EtOAc and put at -20 °C overnight. The resulting white precipitate was collected by filtration and washed by Et$_2$O to give 8b (5.86 g, 48%). 8b (1.20 g, 0.2 mmol) was dissolved in 5 mL DMSO. 11-Azido-3,6,9-trioxaundecan-1-amine (60 µL, 0.3 mmol) and DIPEA (103 µL, 0.6 mmol) were added to the DMSO solution. After heated at 80 °C for 40 h, the mixture was washed by brine and extracted with CH$_2$Cl$_2$ (3 × 25 mL). The CH$_2$Cl$_2$ solution was dried by Na$_2$SO$_4$ and concentrated under reduced pressure. And the resulting oil-like mixture was diluted with 150 mL EtOAc and put at -20 °C overnight. The resulted white precipitate was collected by centrifugation. The product 9b was given by drying the solid under reduced pressure, yield 586.3 mg (47.1%). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 7.18 (t, J = 5.5 Hz, 1H), 4.04 (t, J = 9.2 Hz, 2H), 3.80-3.35 (m, 483H), 3.16-3.07 (m, 2H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 156.2, 72.3, 69.8, 69.7, 69.5, 69.3, 69.1, 68.8, 63.1, 60.2, 50.0.

### 4. Reaction with Tyr-containing small molecule.

**Product 4 for HRMS.** 10 µL 100 mM 2 in CH$_3$CN and 10 µL 100 mM 3 in DMSO were co-incubated in 80 µL 50 mM PBS (pH 8.0, containing 30% CH$_3$CN ) for 1 h at...
room temperature. Then the reaction mixture was submitted into ESI-MS without purification.

**UV-Vis analysis of the reaction.** To 2 mL PBS (50 mM, pH 7.0 or pH 8.0, containing 30% CH$_3$CN) was added 2 µL 2 (100 mM) in CH$_3$CN and 2 µL 3 (100 mM) in DMSO. The progress of the reaction was monitored by UV-Vis spectrophotometry from 250 to 600 nm. In order to explore the kinetics of the reaction more accurately, the reaction progress was monitored by UV-Vis spectrophotometry at 400 nm. The reaction condition is 2 µL 2 (100 mM) in CH$_3$CN and 20 µL 3 (100 mM) in DMSO was added to 1.98 mL PBS (50 mM, pH 7.0 or pH 8.0, containing 30% CH$_3$CN).

### 5. Fluorescence labeling of BSA.

500 µL BSA (100 µM) solution in 50 PBS (mM, pH 8.0) was added 1 µL 2 (100 mM) in DMSO and the mixture was co-incubated for 2 h at room temperature. The fluorescence labelling was based on click reaction by copper catalyst. The **click catalyst** (10 mM) was prepared as a mixture of 50 µL TBTA (20 mM), 30 µL deionized water, 10 µL Cu$^{2+}$ (100 mM) and 10 µL ascorbic acid (vitamin C, 200 mM, fresh preparation). 1 µL fluorescence probe 6 (100 mM in DMSO) and 25 µL catalyst was added to 475 µL alkyne-modified BSA or native BSA (negative control) for 2 h reaction at room temperature. The labeled BSA suspension was directly electrophoresed in 15% SDS-PAGE. The PAGE was firstly imaged under the UV light to detect whether the BSA was labelled. After the UV imaging, the PAGE was stained by Coomassie brilliant blue for 1 h and destained by the glacial acetic acid/alcohol/water mixture (1:1:8).

### 6. Fluorescence labeling of TMV particles.

To label the TMV particles, 10 mg/mL TMV suspension (99 µL) was incubated with 1 µL 2 (100 mM) in PBS buffer (0.1 M, pH 7.0) for 2 h at room temperature. The reaction mixture was then transferred into a centrifugal filtration device (3 KDa, Millipore) and eluted by PBS. 80 µL 2-labelled TMV was incubated with 1 µL fluorescence probe 6 (100 mM in DMSO) and 10 µL **click catalyst** for 2 h at room
temperature. The same reaction except the native TMV suspension was set as the negative control. The labelled TMV suspension was denatured by base and then electrophored in 10% SDS-PAGE. The PAGE was firstly imaged under the UV light and then stained by Coomassie brilliant blue.

7. Protein PEGylation.
To achieve the protein PEGylation, we selected a small protein lysozyme (14.4 KDa). A 10 µL lysozyme (2 mM) solution was incubated with 1 µL 2 (100 mM) in 89 µL PBS buffer (50 mM, pH 8.0) for 2 h at room temperature. The reaction mixture was transferred into a centrifugal filtration device (10 KDa, Millipore) and eluted by PBS buffer (50 mM, pH 8.0). 85 µL lysozyme elution was then incubated overnight with 5 µL 20 mM PEG2000-N₃ (or PEG6000-N₃) dissolved in DMSO and 10 µL click catalyst at 4 ºC. The bioconjugations were analyzed by 10% SDS-PAGE. The gel was stained by R250 (Coomassie brilliant blue) for 1 h and destained by the glacial acetic acid/alcohol/water mixture (1:1:8). The band shift was imaged by the Gel Doc XRi instrument (Bio-Rad) and processed with the Quantity One software.
Supporting figures

Fig. S1. $^1$H-NMR and $^{13}$C-NMR spectra of 2.
Fig. S2. HRMS spectrum of 2.

Fig. S3  Time-dependent UV-Vis absorbance spectra of 2 (100 µM) upon treatment with 3 (100 µM) in 50 mM PBS (pH 7.0, containing 30% CH<sub>3</sub>CN) at room temperature. The different reaction time is indicated inset.
Fig. S4  The absorbance at 400 nm versus reaction time of 2 (100 µM) upon treatment with 3 (1 mM) in 50 mM PBS (pH 7.0 (left) or pH 8.0 (right), containing 30% CH$_3$CN) at room temperature. The red line represents the best fitting with single-exponential function.

Fig. S5  Competitive reaction tests of 2 (0.1 mM) and 3 (1 mM) in the presence of some amino acids (1 mM). The reaction was performed at pH 8.0 for 30 min. Absorbance spectra of each reaction solution were checked (a) and the absorbance at 400 nm was shown in (b). Lane 1, only 2; lane 2, 2 and 3; lanes 3-7, 2 and 3 in the presence of Thr, Lys, Arg, Ser, Phe, respectively. Because the specific reaction of 2 and 3 gives azo with unique absorbance at 400 nm, the results of similar absorbance value at 400 nm for lanes 2-7 imply that the reaction of 2 and 3 was nearly not disrupted in the presence of amino acids Thr, Lys, Arg, Ser and Phe.
Fig. S6 $^1$H NMR and $^{13}$C NMR spectra of 6.
Fig. S7 $^1$H NMR and $^{13}$C NMR spectra of azido-PEG 9a.
Fig. S8 Infrared Spectrum of azido-PEG 9a.
Fig. S9 $^1$H NMR and $^{13}$C NMR spectra of azido-PEG 9b.