Supporting Information for

Site-selective protein modification via disulfide rebridging for fast tetrazine/trans-cyclooctene bioconjugation

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1. General experimental details
2. Synthetic Protocol of IC-Tetrazine
3. Synthesis of tetrazine modified somatostatin (SST-Tetrazine)
4. Synthesis of tetrazine modified Fab fragment (Fab-Tetrazine)
5. Synthesis and characterization of CytC-PEG3-TCO
6. SST-Tetrazine conjugation with TCO-PEG12, TCO-Cy5 and CytC-PEG3-TCO
7. Fab-Tetrazine conjugation with TCO-Cy5, TCO-PEG113
8. Stability study of SST-Tetrazine, Fab-Tetrazine and Fab-Cy5
9. CD experiment of SST-Tetrazine and Fab-Tetrazine
10. Enzyme-linked immunosorbent assay (ELISA) experiment
11. Reference
1. General experimental details

Unless otherwise stated, all the operations were performed without taking precautions to exclude moisture and air. All the organic solvents (CH$_2$Cl$_2$, CHCl$_3$, Ethyl Acetate (EA), acetonitrile (ACN)) were bought from commercial sources (Merck, Sigma Aldrich and so on) and are used directly without further purification. IgG Fab Fragment from Human (polyclonal) was obtained from Dianova GmbH and used as bought. Methylene tetrazine N-hydroxysuccinimide (NHS) ester, trans-cyclooctene (TCO)-PEG3-maleimide were bought from Click Chemistry Tools. Sulfo-Cy5-TCO was bought from Santa Cruz Biotechnology. Tetrazine-5-FAM were purchased from Jena Bioscience. TCO-PEG13 was obtained from NANOCS. TCO-PEG12 was bought from Broadpharm. Iso-yeast Cytochrome C was purchased from Sigma-Aldrich. Glutathione was bought from Sigma-Aldrich. Protein L coated well plate, BCA assay and enhanced chemiluminescence (ECL) solution were purchased from Thermo Fisher Scientific. 4,4′-dithiodipyridine was bought from ACROS organics. Zeba spin desalting column (7K MWCO) was obtained from Thermo Fisher Scientific. H$_2$O used for the reactions was obtained from the Millipore purification system. Vivaspin sample concentrators were purchased from GE Healthcare. Reaction progress was monitored by thin layer chromatography (TLC) using Merck 60 F$_{254}$ pre-coated silica gel plates and visualized under ultraviolet lamp (254 nm) or using appropriate staining solution (KMnO$_4$, ninhydrin, iodine). Flash column chromatography was carried out using Merck silica gel 60 mesh. High performance liquid chromatography (HPLC) was carried out using Shimadzu Analytical HPLC system. NMR spectra were recorded on Bruker Avance 300 NMR spectrometer and the chemical shifts (δ) were reported as parts per million (ppm) referenced with respect to the residual solvent peaks. MALDI-TOF-MS spectra were acquired on a Bruker Time-of-flight MS rapifleX. Fluorescence (or absorbance) spectra or intensities were measured on microplate reader (Tecan Spark 20M). The chemiluminescence was measured by GloMax Discover Microplate Reader.

2. Synthetic Protocol of IC-Tetrazine

Scheme 1 Synthesis of tetrazine-containing disulfide rebridging reagent (IC-Tetrazine). (a) Di-tert-butyl dicarbonate, CH$_2$Cl$_2$, overnight. (b) methacryl chloride, Et$_3$N, CH$_2$Cl$_2$, 90%. (c)
1. I₂, sodium p-toluenesulfinate, CH₂Cl₂, 3 days. 2. Et₃N, CH₂Cl₂, overnight. 3. Et₃N, ethyl acetate, 95 °C, overnight, yield after all three steps: 60%. (d) Trifluoroacetic acid (TFA), CH₂Cl₂, 98%. (e) methyltetrazine NHS ester, Et₃N, CH₂Cl₂, overnight, 40%.

**Synthesis of 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azaheptadecan-16-yl methacrylate (compound 3)**

**Step a:** 2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethanol (1, 0.50 g, 2.59 mmol) was dissolved in 6 mL CH₂Cl₂. Di-tert-butyl dicarbonate (0.68 g, 3.10 mmol) was also dissolved in 6 mL CH₂Cl₂ and then was added to the former solution dropwise with a syringe pump. The reaction mixture was stirred at room temperature overnight and the solvent was removed under vacuum. The compound 2 was got as a colorless oil and used directly for the next step without further purification.

**Step b:** To a 10 mL CH₂Cl₂ solution of crude product from step a (200 mg, 0.68 mmol) was added Et₃N (82.9 mg, 114 µL, 0.82 mmol) followed by methacryloyl chloride (85.7 mg, 0.82 mmol) at 0 °C. The mixture was stirred at room temperature overnight. The solvent was evaporated under high vacuum and the crude product was dissolved in 30 mL CH₂Cl₂. Subsequently, the crude product was washed with 1 M HCl and brine solution. The organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed under vacuum. The crude product was purified by column chromatography (EA: hexane = 1:1) to afford the compound 3 as colorless oil in 90% yield.

**1H NMR** (300 MHz, chloroform-d) δ 6.10 (s, 1H), 5.57–5.53 (s, 1H), 4.32–4.23 (m, 2H), 3.77–3.54 (m, 12H), 2.47 (t, J = 6.6 Hz, 2H), 1.92 (s, 3H), 1.42 (s, 9H).

**13C NMR** (75 MHz, chloroform-d) δ 167.09, 155.92, 135.98, 125.83, 70.58, 70.24, 69.16, 63.86, 28.42, 18.31.

**LC-MS:** m/z = 362 [M+H]⁺, 384 [M+Na]⁺ (calculated exact mass: 362.21 [M+H]⁺, 384.21 [M+Na]⁺, formula:C₁₇H₃₁NO₇)

**Synthesis of 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azaheptadecan-16-yl2-(tosylmethyl)-acrylate (compound 4)**

Compound 3 (211 mg, 0.58 mmol) was dissolved in 5 mL CH₂Cl₂ followed by adding the sodium p-toluenesulfinate (156 mg, 0.87 mmol) and I₂ (222 mg, 0.87 mmol) sequentially. The reaction mixture was stirred at room temperature for 3 days. Next, Et₃N (176 mg, 242 µL, 1.74 mmol) was added to the mixture and stirred overnight. Then the organic layer was washed with 1 M HCl solution, saturated NaHCO₃ solution, Na₂S₂O₃ solution and brine solution. The organic layer was dried over MgSO₄ and the solvent was removed by high vacuum. The residue was dissolved in 10 mL ethyl acetate and Et₃N (176 mg, 242 µL, 1.74 mmol) was added dropwise at 0 °C. The mixture was refluxed overnight. After that, the solvent was evaporated
and the crude product was purified by column chromatography (EA : hexane = 3:1) to afford compound 4 as slight yellow oil in 60% yield.

$^1$H NMR (300 MHz, chloroform-d) $\delta$ 7.72 (d, $J = 8.3$ Hz, 2H), 7.31 (d, $J = 8.0$ Hz, 2H), 6.50 (s, 1H), 5.86 (s, 1H), 4.17 – 4.11 (m, 4H), 3.68 – 3.58 (m, 10H), 3.52 (t, $J = 5.2$ Hz, 2H), 3.28 (t, $J = 4.8$ Hz, 2H), 2.42 (s, 3H), 1.42 (s, 9H).

$^{13}$C NMR (75 MHz, chloroform-d) $\delta$ 164.82, 155.98, 144.87, 135.37, 133.44, 129.61, 128.67, 79.25, 70.49, 70.23, 68.81, 64.50, 57.57, 40.52, 28.42, 21.54.

LC-MS: $m/z$ = 516 [M+H]$^+$, 538 [M+Na]$^+$ (calculated exact mass: 516.22 [M+H]$^+$, 538.22 [M+Na]$^+$, formula:C$_{24}$H$_{37}$NO$_9$S)

**Synthesis of 1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl 2-(tosylmethyl)acrylate (IC-Tetrazine)**

**Step d:** In a 10 mL flask, compound 4 (15.1 mg, 0.03 mmol) was dissolved in 3 mL CH$_2$Cl$_2$. Trifluoroacetic acid (TFA, 171.2 mg, 1.54 mmol) was also added. The resulting mixture was stirred at room temperature overnight. The solvent and TFA was removed in vacuum to obtain 11.9 mg boc-deprotected product in 98%.

**Step e:** Boc-deprotected product (11.9 mg, 0.03 mmol) was dissolved in 2 mL CH$_2$Cl$_2$ followed by adding N,N-disopropylethylamine (DIEA, 7.5 mg, 0.06 mmol) to ensure a basic condition. Methyltetrazine-NHS ester (11.4 mg, 0.03 mmol) was dissolved in 1 mL CH$_2$Cl$_2$ and added to the aforementioned mixture above. The reaction mixture was stirred at room temperature overnight and the solvent was evaporated under high vacuum. The residue was dissolved in 20 mL CH$_2$Cl$_2$ and was washed with brine solution. The organic layer was dried over anhydrous MgSO$_4$ and the solvent was removed under high vacuum. The residue was purified by column chromatography (CH$_2$Cl$_2$: MeOH = 20:1) to afford compound IC-Tetrazine as red oil in 40% yield. The purify of IC-Tetrazine is 98% based on the quantification of the peak at 214 nm in LC-MS.

$^1$H NMR (300 MHz, chloroform-d) $\delta$ 8.54 (d, $J = 7.5$ Hz, 2H), 7.72 (d, $J = 7.6$ Hz, 2H), 7.51 (d, $J = 7.8$ Hz, 2H), 7.33 (d, $J = 7.5$ Hz, 2H), 6.50 (s, 1H), 5.83 (s, 1H), 4.25 – 4.08 (m, 4H), 3.75 – 3.37 (m, 1H), 3.09 (s, 3H), 2.44 (s, 3H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.10, 167.24, 164.70, 163.85, 145.37, 144.96, 140.00, 135.50, 135.21, 133.46, 130.23, 129.73, 128.93, 128.73, 128.28, 70.62, 70.49, 70.25, 69.77, 68.81, 64.48, 57.60, 55.28, 43.48, 39.53, 35.78, 21.72, 21.12.

LC-MS: $m/z$ = 628 [M+H]$^+$, 650 [M+Na]$^+$ (calculated exact mass: 628.24 [M+H]$^+$, 650.24 [M+Na]$^+$, formula:C$_{30}$H$_{37}$N$_5$O$_8$S)
3. Synthesis of tetrazine modified somatostatin (SST-Tetrazine)

Somatostatin (SST, 2 mg, 1.22 µmol) was dissolved in 1 mL ACN/phosphate buffer (PB, 50 mM, pH = 7.8) mixture (v/v = 2:3). Tris(2-carboxyethyl)phosphine (TCEP, 0.7 mg, 2.44 µmol) was dissolved in 100 µL ACN/PB mixture and added to the somatostatin solution. The mixture was incubated at room temperature for 30 min. Afterwards, IC-Tetrazine (1.9 mg, 3.05 µmol) was dissolved in 1 mL ACN/PB mixture as well which was added to the SST and TCEP solution. The reaction mixture was degassed for several minutes to exclude air in the solvent and gently shaken at room temperature overnight. The crude product was purified by Prep HPLC using Atlantis Prep OBD T3 Column (19×100 mm, 5 µm) with the mobile phase starting from 100% solvent A (0.1% TFA in MilliQ water) and 0% solvent B (0.1% TFA in ACN), reaching 45% B at 35 min and finally reaching 100% B at 42 min with a flow rate of 10 mL/min. The absorbance was monitored at 280 nm, 254 nm and 515 nm. The retention time for SST-Tetrazine was 34 min. About 0.8 mg SST-Tetrazine was obtained after lyophilisation (30% yield).

**LC-MS:** \( m/z = 1056 \ [M+2H]^2+ \), 705 \([M+3H]^3+ \), 1054 \([M-2H]^2- \).
MALDI-TOF-MS (matrix: sinapinic acid): $m/z = 2110.95$ [M+H]$^+$ (calculated exact mass: 2110.95 [M+H]$^+$, formula: C$_{99}$H$_{135}$N$_{23}$O$_{25}$S$_2$)

Figure S2 LC-MS data of purified SST-Tetrazine

Figure S3 The chemical structure of the fragmentation part (in the frame) of SST-Tetrazine in MALDI-TOF-MS spectrum

Furthermore, SST-Tetrazine is also showing no obvious degradation after one year storage in the freezer based on the LC-MS data.

Figure S4 LC-MS data of SST-Tetrazine after one year storage in the freezer (−20 °C)
4. Synthesis of tetrazine modified Fab fragment (Fab-Tetrazine)

First, native IgG Fab was incubated with different amounts of TCEP to determine how much TCEP is necessary to totally reduce the interchain disulfide bond of IgG Fab. IgG Fab (3.2 µg, 0.067 nmol, 4.8 mg/mL, 1 eq) was mixed with 1 eq, 5 eq, 10 eq, 25 eq, 50 eq, 100 eq TCEP for incubation for 1 hour. Then, the mixture was loaded to the SDS-PAGE. SDS-PAGE data (Figure S5A) showed that 50 eq TCEP is necessary to fully reduce the interchain disulfide bond of IgG Fab.

Second, the buffer of the Fab solution (100 µg, 4.8 mg/mL, 2.08 nmol) was changed to PB (50 mM, pH = 7.8) by using 10 kDa MWCO ultrafiltration tube and diluted to 1 mg/mL with PB (50 mM, pH = 7.8). TCEP (30 µg, 0.10 µmol) was dissolved in 10 µL PB (50 mM, pH = 7.8) and added to the Fab solution. Next the mixture was incubated at room temperature for 1 hour. IC-Tetrazine (65.5 µg, 0.10 µmol, 20 mg/mL in dimethyl sulfoxide (DMSO)) was first dissolved in 5.5 µL DMSO and then added to the Fab solution. The mixture was incubated at room temperature overnight. Excess reagents were removed by repeated ultrafiltration in water using Vivaspin sample concentrator (10 kDa MWCO). The sample was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Based on the concentration calculated from gel densitometry, about 80% of the Fab fragment was successfully modified (SDS-PAGE is shown in Figure S5B).

SDS gel electrophoresis: 10% separating gel was prepared in the following order: 2.5 mL 40% acrylamide solution, 5 mL water, 2.5 mL 4× Bis-Tris gel buffer, 100 µL of 10% sodium dodecyl sulfate buffer, 50 µL ammonium persulfate (v/w) solution, 5 µL tetramethylethylenediamine. The mixed gel solution was added to the Bio-Rad Mini-Gel apparatus and stand for 15 min for polymerization. The isopropanol was added above the separating gel to exclude air. Next, 6% stacking gel was prepared in the following order: 0.75 mL 40% acrylamide solution, 3 mL water, 1.25 mL 4× Bis-Tris gel buffer, 30 µL 10% sodium dodecyl sulfate buffer, 25 µL ammonium persulfate (v/w) solution, 2.5 µL tetramethylethylenediamine. The stacking gel was also added above the separating gel after totally removing the isopropanol. A 10-well comb was inserted and the stacking gel was polymerized for another 10 min. For the sample loading to the SDS-PAGE, 16 µL protein solution was mixed with 6 µL loading dye and 1 µL TCEP solution. The mixture was incubated for one hour and then loaded to the gel.
On the other hand, a thiol-quantification experiment was performed to quantify how much thiol groups are still left in Fab-Tetrazine sample. 4,4'-Dithiodipyridine is a disulfide-containing reagent which reacts with protein thiols to form stoichiometric amounts of the chromogenic compound 4-thiopyridone (4-TP), absorbing at 324 nm. In this experiment, cysteine was selected as a standard compound. The procedure is described below:

1. 30 µL of 4,4'-dithiodipyridine (2 mM in 100 mM citrate buffer, pH = 4.5) incubated with 30 µL cysteine solution (concentration: 0.01 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, dissolved in 100 mM citrate buffer, pH = 4.5) for 15 minutes at room temperature. 50 µL of cysteine and 4,4'-dithiodipyridine mixture were taken in 384-well plate. Absorbance was monitored at 324 nm and the standard curve is shown in Figure S5C.

2. 50 µL of Fab-Tetrazine sample was mixed with 100 eq TCEP (100 mM PB, pH = 7.8) and incubated for 1 hour to tally reduce the unmodified disulfide bonds.

3. Zeba spin desalting columns (7K MWCO) was buffer exchanged to the 100 mM citrate buffer first following the protocol provided by the supplier. Next, the Fab-Tetrazine solution was loaded to the column and the desalting steps was repeated six to eight times to tally remove the residue TCEP. After desalting, the protein concentration was determined to be 3.21 mg/mL based on the BCA assay following the protocol provided by the supplier.

4. Next, 30 µL of 4,4'-dithiodipyridine (2 mM in 100 mM citrate buffer, pH = 4.5) was incubated with 30 µL the Fab-Tetrazine solution at room temperature for 15 minutes. 50 µL of the protein and 4,4'-dithiodipyridine mixture were taken in 384-well plate. Absorbance was monitored at 324 nm and 0.51 was obtained for the Fab-Tetrazine sample.

Based on the standard curve, 0.51 indicated the concentration of thiol groups in 60 µL protein and 4,4'-dithiodipyridine mixture is 0.018 mM. The concentration of unmodified disulfide bond is 0.009 mM. The concentration of Fab-Tetrazine is 1.6 mg/mL (0.033 mM) in 60 µL protein and 4,4'-dithiodipyridine mixture. From this readout, about 73% of IgG Fab was successfully modified.

Figure S5 (A) SDS-PAGE analysis of Fab fragment which was incubated with different amounts of TCEP (M: Protein Marker, 1: Fab + 1 eq TCEP, 2: Fab + 5 eq TCEP, 3: Fab + 10 eq TCEP, 4: Fab + 20 eq TCEP, 5: Fab + 40 eq TCEP, 6: Fab + 50 eq TCEP ) (B) MALDI-
TOF-MS of native IgG Fab (found: 48.0k [M+H]^+, calculated: 48.0k [M+H]^+) and Fab-Tetrazine (found: 48.5k [M+H]^+, calculated: 48.5k [M+H]^+) (C) standard curve based on checking the absorbance of cysteine and 4,4’-dithiodipyridine solution at 324 nm

5. Modification and characterization of Cytochrome C (CytC) with trans-cyclooctene (TCO) group

Scheme S4 Modification of Cytochrome C with TCO-PEG3-Maleimide

CytC (212 μg, 0.02 μmol) was dissolved in 212 μL PB (50 mM, pH = 7.0) and mixed with TCEP (9.7 μg, 0.03 μmol). The mixture was shaken at room temperature for 1 hour. TCO-PEG3-Maleimide (267 μg, 0.51 μmol) was added to the mixture above and shaken at room temperature for 4 hours. Afterwards, the crude product was purified by repeated ultrafiltration in water using Vivaspin sample concentrator (5 kDa MWCO). The MALDI-TOF-MS of the modified product (CytC-PEG3-TCO) is shown in Figure S6B. Compared to the native CytC (Figure S6A), the molecular weight of CytC-PEG3-TCO increased by 525 Da which corresponds to the molecular weight of TCO-PEG3-Maleimide (523.62 Da).

Figure S6 MALDI-TOF-MS of (A) native CytC (found: 12710 [M+H]^+, calculated: 12588 [M+H]^+) and (B) CytC-PEG3-TCO (found: 13233 [M+H]^+, calculated: 13235 [M+H]^+)
In order to check the modification yield of CytC with the TCO group, CytC-PEG3-TCO was used to react with a dye (Tetrazine-FAM) in H₂O. After reaction, the modification yield was estimated based on the absorbance of CytC at 280 nm and the FAM dye at 490 nm. CytC-PEG3-TCO (70 μg, 5.29 nmol) was mixed with 2.27 μL Tetrazine-FAM (25.7 μg, 0.05 μmol, 10 mg/mL stock solution in DMSO) in H₂O. The mixture was shaken at room temperature for 1 hour. After that, the crude product was purified by repeated ultrafiltration in water using Vivaspin sample concentrator (5 kDa MWCO) to get the purified CytC-PEG3-FAM. The degree of labelling (DOL) was calculated based on the following equation:

\[
DOL = \frac{A_{\text{max}} \times \varepsilon_{280} (\text{protein})}{(A_{280} - A_{\text{max}} \times CF) \times \varepsilon_{\text{max}}}
\]

We check the absorbance of CytC-PEG3-TCO at 280 nm and 490 nm, the values are listed below:

- \(A_{280}\) (the absorbance of the sample at 280 nm) = 0.42,
- \(A_{490}\) (the absorbance of the sample at 490 nm) = 0.89
- \(\varepsilon_{280}\) (the extinction coefficient of CytC at 280 nm) = 13075 M⁻¹cm⁻¹
- \(\varepsilon_{\text{max}}\) (the extinction coefficient of FAM dye at 490 nm) = 83000 M⁻¹cm⁻¹
- CF (correction factor) = 0.3

Based on the calculation, the DOL is 90%.

Absorbance at 280 nm (\(A_{280}\)) is used to determine the protein concentration in a sample. However, fluorescent dyes also absorb at 280 nm. Therefore, a correction factor must be used to adjust for \(A_{280}\) contributed by the dye. The correction factor (CF) equals the \(A_{280}\) of the dye divided by the \(A_{\text{max}}\) of the dye as shown in the equation above.

6. SST-Tetrazine conjugation with TCO-PEG12, TCO-Cy5 and CytC-PEG3-TCO
Scheme S6 Bioconjugation between SST-Tetrazine, TCO-PEG12 and TCO-Cy5

SST-Tetrazine (2 mg, 0.95 μmol) was dissolved in 1.332 mL PB (50 mM, pH = 7.4). TCO-PEG12 (730 μg, 1.00 μmol) was dissolved in 667 μL PB (50 mM, pH = 7.4) and added to the SST-Tetrazine solution. The reaction mixture was shaken for 30 min. After mixing the two components, the pink color originating from the tetrazine group disappeared immediately. The crude product was purified by semi-preparative HPLC using Agilent Eclipse XDB-C18 column (9.4 × 250 mm, 5 μm) with the mobile phase starting from 100% solvent A (0.1% TFA in MilliQ water) and 0% solvent B (0.1% TFA in ACN), reaching 43% B at 35 min and finally reaching 100% B at 38 min with a flow rate of 4 mL/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for SST-PEG12 was 32 min. 2.56 mg SST-PEG12 was obtained (yield: 95%).

LC-MS: \( m/z = 718 \ [M+Na+3H]^4+, \ 959 \ [M+Na+2H]^3+, \ 966 \ [M+2Na+H]^2+, \ 1438 \ [M+Na+H]^2+, \ 1424 \ [M-H]\)

MALDI-TOF-MS (matrix: sinapinic acid): \( m/z = 2852.2102 \ [M+H]^+ \) (calculated exact mass: 2852.3873 [M+H]^+, formula: \( C_{135}H_{202}N_{22}O_{41}S_{2} \)).

Figure S7 HPLC chromatogram (280 nm detection wavelength) of SST-PEG12
SST-Tetrazine (180 mg, 0.85 μmol) was dissolved in 1.26 mL PB (50 mM, pH = 7.4). TCO-Cy5 (1.03 μg, 0.9 μmol) was dissolved in 252 μL DMSO and added to the SST-Tetrazine solution. The reaction mixture was stirred for 30 min. The crude product was purified by Semipreparative HPLC using Agilent Eclipse XDB-C18 column (9.4×250 mm, 5 μm) with the mobile phase starting from 100% solvent A (0.1% TFA in MilliQ water) and 0% solvent B (0.1% TFA in ACN), reaching 43% B at 35 min and finally reaching 100% B at 38 min with a flow rate of 4 mL/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for SST-Cy5 was 28.5 min. 2.3 mg SST-Cy5 was obtained (yield: 90%).

LC-MS: m/z = 1014 [M+3H]^3+, 1520 [M-2H]^2+, 1012 [M-3H]^3-

MALDI-TOF-MS (matrix: sinapinic acid): m/z = 3041.1742 [M+H]^+ (calculated exact mass: 3041.2939 [M+H]^+, formula: C\textsubscript{145}H\textsubscript{197}N\textsubscript{25}O\textsubscript{37}S\textsubscript{5})
Figure S10 LC-MS data of SST-Cy5

Scheme S7 Bioconjugation between SST-Tetrazine and CytC-PEG3-TCO
SST-Tetrazine (32 μg, 0.015 μmol) was mixed with CytC-PEG3-TCO (132 μg, 0.010 μmol) in PB (50 mM, pH = 7.4). The mixture was shaken for 30 min. Afterwards, the reaction mixture was purified by repeating ultrafiltration in water using Vivaspin sample concentrator (5 kDa MWCO) to get the purified SST-PEG3-CytC. The desired SST-PEG3-CytC was characterized by MALDI-TOF-MS and SDS-PAGE.

7. Fab-Tetrazine conjugation with TCO-Cy5 and TCO-PEG113

Scheme S8 Bioconjugation between Fab-Tetrazine, TCO-Cy5 and TCO-PEG113
Fab-Tetrazine (48 μg, 1.00 nmol) was dissolved in 85 μL PB (50 mM, pH = 7.4). TCO-Cy5 (5.7 μg, 5.00 nmol, 20 mg/mL in DMSO stock solution) was added to the Fab-Tetrazine solution. The reaction mixture was incubated at room temperature for 30 min. Afterwards, the crude product was purified by repeated ultrafiltration in water using Vivaspin sample
concentrator (10 kDa MWCO). Based on Figure S11A, Fab-Cy5 showed obvious fluorescence compared to the Fab-Tetrazine indicating the successful conjugation.

The degree of labelling of Fab-Cy5 was calculated using two different methods. One method to determine the degree of labeling of Fab-Cy5 is based on the following equation:

\[
\text{DOL} = \frac{A_{\text{max}} \times \varepsilon_{280}}{(A_{280} - A_{\text{max}} \times CF) \times \varepsilon_{\text{max}}}
\]

After extensive desalting steps using Zeba spin desalting columns (repeated six to eight times to totally remove the free dye), we check the absorbance of Fab-Cy5 at 280 nm and 650 nm, the values are listed below:
- \( A_{280} \) (the absorbance of the sample at 280 nm) = 0.23
- \( A_{\text{max}} \) (the absorbance of the sample at 650 nm) = 0.56
- \( \varepsilon_{280} \) (the extinction coefficient of native Fab at 280 nm) = 70000 M\(^{-1}\)cm\(^{-1}\)
- \( \varepsilon_{\text{max}} \) (the extinction coefficient of Cy5 dye at 650 nm) = 250000 M\(^{-1}\)cm\(^{-1}\)
- CF (correction factor) = 0.03

Based on the calculation, the DOL is 73%.

The other method to determine the degree of labelling is based on the gel densitometry. The absorbance of Fab-Cy5 at 650 nm is 0.56. The Cy5 concentration was 13.3 \( \mu \)g/mL (13.8 \( \mu \)M) calculated using the calibration curve (Figure 11C). Fab-Cy5 solution was diluted 10 times and then loaded to the SDS-PAGE. Based on the gel densitometry (Figure S11B), the concentration of Fab-Cy5 (1:10 dilution) is 0.094 mg/mL (1.96 \( \mu \)M). So the concentration of the original Fab-Cy5 (no dilution) is 0.94 mg/mL (19.6 \( \mu \)M). Therefore, the labelling efficiency was 70% (= 13.8/19.6 \( \times \)100%).

Figure S11 (A) Fluorescence spectra of Fab-Cy5 and Fab-Tetrazine (B) SDS-PAGE of IgG Fab and Fab-Cy5 (M: Protein marker, 1: Fab-Cy5 (based on the gel densitometry, the concentration of Fab-Cy5 is 0.094 mg/mL), 2: IgG Fab (0.1 mg/mL)) (C) calibration curve of TCO-Cy5 based on the absorbance of 650 nm (D) MALDI-Tof-MS of Fab-PEG113 showing a signal at 53.5 kDa (found: 53.5k [M+H]\(^+\), calculated: 53.5k [M+H]\(^+\])

TCO-PEG113 (molecular weight: 5000 Da containing 113 repeating units on average) (25 \( \mu \)g, 5.00 nmol) was dissolved in PB (50 mM, pH = 7.4) and added to the Fab-Tetrazine (48 \( \mu \)g, 1.00
nmol) solution. The reaction mixture was stirred at room temperature for 30 min. After the reaction, the crude product was purified by repeated ultrafiltration in water using Vivaspin sample concentrator (10 kDa MWCO). The sample was analyzed by SDS-PAGE. Based on the gel, the band of Fab-Tetrazine totally disappeared and shifted to higher molecular weight (Fig. 3d in the main text). Because TCO-PEG113 is not monodisperse, the band of Fab-PEG113 also became broaden after conjugation. MALDI-ToF-MS of Fab-PEG113 also showed a signal at 53.55 kDa indicating the successful modification (Fig. S11D).

8. Stability test of SST-Tetrazine and Fab-Tetrazine

0.01 mM SST-Tetrazine solution was incubated with 20 µM Glutathione at 37 °C for 0 hours, 12 hours and 24 hours. For each sample, 10 µL of 10 mg/mL Fmoc-L-phenylalanine was added as internal standard. After incubation, the samples were injected into LC-MS. Identification of SST-Tetrazine product was performed simultaneously by UV-VIS detection at 254 nm and 214 nm and selective ion monitoring (SIM). The amount of SST-Tetrazine in each sample was determined as a ratio of the integration of the chromatogram at 254 nm of SST-Tetrazine to the internal standard.

Scheme S9 Reaction of GSH-mediated cleavage of SST-Tetrazine
Figure S12 (A) LC-MS chromatogram of SST-Tetrazine incubating with GSH at 214 nm (the peak at 2 min is from DMSO). (B) The area ratio of SST-Tetrazine at 214 nm compared to internal standard. (C) selective ion monitoring profile of m/z 1085 (GSH-Tetrazine) at 0 h and 24 h showing no degradation products were detected.

As suggested in the reference\(^1\), the stability of Fab-Tetrazine and Fab-Cy5 were investigated through incubation with fetal bovine serum (FBS) at 37 °C. 0.01 mM Fab-Tetrazine and Fab-Cy5 were incubated with 1% FBS for 0 hour, 12 hours and 24 hours at 37 °C. After incubation, the mixture was analyzed by SDS-PAGE as described in the protocol in the section above. From the SDS-PAGE data, the protein band of Fab-Tetrazine did not have obvious change showing the good stability of Fab-Tetrazine. The stability of Fab-Cy5 was also investigated based on quantifying the fluorescence of the conjugate. The intensity of the fluorescence band of lane 5–7 is 23226.886, 24341.886 and 23008.664 using Image J. The data indicated good stability of Fab-Cy5 after incubation with FBS for 24 hours (Fig. 5d)

9. CD experiment of SST-Tetrazine and Fab-Tetrazine

Native SST, SST-Tetrazine and SST-PEG12 were dissolved in PBS to prepare 0.1 mg/mL solution. CD measurements were performed on JASCO J-1500 spectrometer in a 1 mm High Precision Cell by Hellma Analytics from 260 to 190 nm with a bandwidth of 1 nm. The data pitch was set to 0.2 nm, while the scanning speed was 10 nm/min. Each sample was measured
three times and the signal from the buffer blank was subtracted from the sample scan. The data was processed in the software Spectra Analysis and CD Multivariate SSE by JASCO.

Native IgG Fab, Fab-Tetrazine and Fab-PEG113 conjugate were dissolved in PBS at a final concentration of 0.1 mg/mL. CD experiment was performed as SST and SST-Tetrazine using the same machine with the same procedure. Each sample was measured three times and the signal from the buffer blank was subtracted from the sample scan. The data was processed in the softwares Spectra Analysis and CD Multivariate SSE by JASCO.

10. Enzyme-Linked immunosorbent assay (ELISA) experiment

Protein L coated 96-well plate was washed with 100 μL wash buffer (PBS with added 0.05% Tween-20 detergent). Then dilution buffer (2% BSA in washing buffer) was used to dilute the native IgG Fab and Fab-Tetrazine to the following concentration: 1 nM, 10 nM, 50 nM, 100 nM, 200 nM and 100 μL of dilution solution were added to the corresponding well incubating for 2 hours. After 2 hours, the dilution solution was removed and each well was washed three times with wash buffer. Next, 100 μL of anti-human IgG, Fab-specific-HRP solution (prepared by taking 4 μL of a 1:5000 diluted solution and further diluting with 20 mL of PBS) was added to each well and incubate for 1 hour. Next, the solution was removed and washed with wash buffer 3 times (200 μL). For each well, 100 μL enhanced chemiluminescence solution (ECL solution, from Thermo Fisher Scientific) was added to each well and chemiluminescence was measured after 5 minutes.

11. Reference

$^1$H NMR of compound 3

$^{13}$C NMR of compound 3
$^1$H NMR of compound 4

$^{13}$C NMR of compound 4
$^1$H NMR of IC-Tetrazine

$^{13}$C NMR of IC-Tetrazine