Synthesis of Bispecific Antibodies with Genetically Encoded Unnatural Amino Acids

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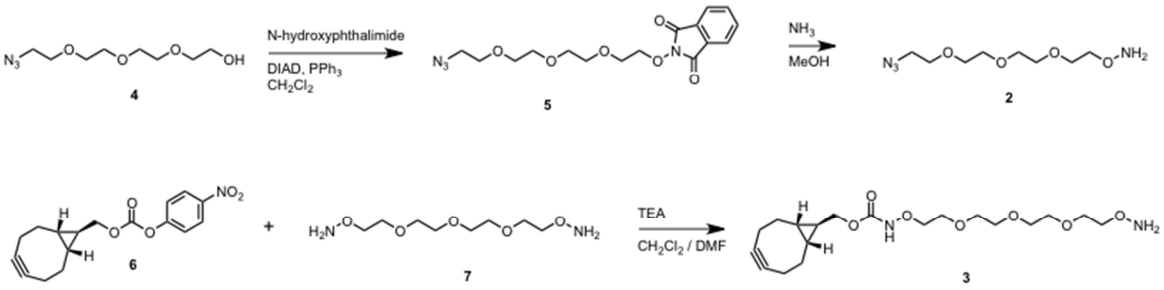
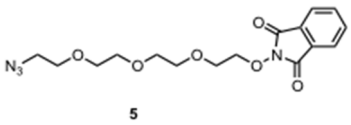
Supporting Information

Synthesis

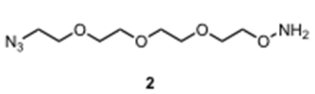
All chemicals were obtained from commercial sources and used without further purification. 1H- and 13C-NMR spectra were obtained on a Varian INOVA-399 (400 MHz) or MER-300 (300 MHz) spectrophotometer. Chemical shift values were recorded as parts per million relative to tetramethylsilane as an internal standard. Protein mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, CA).




5

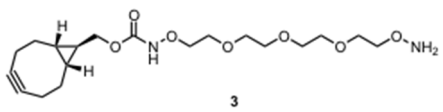
Triphenylphosphine (430 mg, 1.64 mmol), *N*-hydroxyphthalimide (270 mg, 1.66 mmol) and DIAD (0.32 mL, 1.6 mmol) were added to a solution of 1-azido-3,6,9-trioxaundecane-1-ol (300 mg, 1.37 mmol) in CH_2Cl_2 (20 mL) at 0 ^oC. After stirring overnight at room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (Hex-EtOAc, 1:1) to afford **5** (426 mg, 85 %) . R_f 0.2 (Hex-EtOAc, 1:1). ^1H NMR (400 MHz, $CDCl_3$): δ 7.82 (dd, J = 5.4, 3.1 Hz, 2H), 7.74 (dd, J = 5.5, 3.1 Hz, 2H), 4.38-4.34 (m, 2H), 3.88-3.82 (m, 2H), 3.69-3.62 (m, 4H), 3.62-3.54 (m, 6H), 3.36 (t, J = 5.6 Hz, 2H). ^{13}C NMR

(100 MHz, $CDCl_3$): 163.96, 134.98, 129.53, 124.02, 77.76, 71.34, 71.17, 71.17, 71.12, 70.55, 69.83, 51.23. MS (ESI) calcd. for C16H20N4O6 (M^++Na) 387.1, found 387.1.



2

2-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)isoindoline-1,3-dione (**5**, 426 mg, 1.17 mmol) was dissolved in methanolic ammonia solution (7 N in MeOH, 50 mL) and stirred overnight at 40 ^oC. After the solvent was removed under reduced pressure, the crude mixture was dissolved in CH_2Cl_2 (50 mL), and the white precipitate was filtered-off. After concentration, the residue was purified by flash column chromatography on silica gel (CH_2Cl_2-MeOH, 50:1) to afford **2** (254 mg, 93 %). R_f 0.3 (CH_2Cl_2-MeOH, 20:1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.90-3.82 (m, 2H), 3.70-3.67 (m, 4H), 3.66 (s, 6H), 3.67-3.65 (m, 2H), 3.39 (t, J = 5.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 74.70, 70.69, 70.62, 70.60, 70.52, 70.01, 69.66, 50.72. HRMS (ESI) calcd. for $C_8H_{18}N_4O_4$ (M^++1) 235.1401, found 235.1403.



3

TEA (0.18 mL, 1.3 mmol) was added to a solution of (1R,8S,9R)-bicyclo[6.1.0]non-4-yn-9-ylmethyl(4-nitrophenyl)carbonate (**6**, 270 mg, 0.86 mmol, synthetic details are in ref. 20) in DMF (10 mL). *O*,*O*'-[oxybis(2,1-ethanediyloxy-2,1-ethanediyl)]bis(hydroxylamine) (**7**, 770 mg, 3.4 mmol) in CH_2Cl_2 (40 mL) was added to the solution and stirred overnight at room temperature. The solvent was removed by blowing nitrogen gas, and the residue was purified by flash column chromatography on silica gel (CH_2Cl_2-MeOH, 20:1) to afford **3** (280 mg, 82 %). R_f 0.2 (CH_2Cl_2-MeOH, 10:1). ^1H NMR (300 MHz, $CDCl_3$) δ 8.16 (s, 1H), 5.58 (br. s, 2H), 4.10-4.00 (m, 4H), 3.89-3.81 (m, 2H), 3.78-3.70 (m, 4H), 3.68 (s, 8H), 2.40 (d, J = 13.6 Hz, 2H), 2.27 (d, J = 13.7 Hz, 2H), 2.16 (d, J = 16.1 Hz, 2H), 1.50-1.27 (m, 2H), 0.89-0.62 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 157.95, 98.98, 75.64, 74.99, 70.81, 70.73, 70.25, 69.84, 69.49, 33.46, 23.80, 23.24, 21.59. HRMS (ESI) calcd. for $C_{19}H_{32}N_2O_7$ (M^++1) 401.2282, found 401.2287.

Expression of Antibody Fragments in *E. Coli*

Herceptin-Fab (D. J. Slamon et al., *Science*, **1989**, 244, 707) and UCHT1-Fab (ref.25) sequences were obtained from the literature and inserted into a pBAD vector behind the stII signal sequence. The sites for unnatural amino acid incorporation (LC-Ser202 for Her2 Fab, HC-Lys138 for UCHT1) were quickchanged (Stratagene) to TAG amber nonsense codon. The pBAD vector was co-transformed with pEVOL-pAcF (a vector containing orthogonal *M. jannaschii* tRNA and aminoacyl-tRNA synthetase specific for pAcF (Young, T.S., *et al.* J Mol Biol, 2010. **395**(2), 361-374) in DH10B cells. The cells were grown in LB media (2 L) supplemented with 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 1 mM pAcF at 37 °C and 250 rpm. At OD_{600} 0.8, cells were induced with 0.2 % arabinose and moved to 30 ^{o}C for 16 hours at 270 rpm. Cells were harvested and proteins were extracted by incubating with 150 mL of periplasmic lysis buffer (20 % sucrose, 30 mM Tris, pH 8, 1 mM EDTA, and 0.2 mg/mL lysosyme) for 30 min at 37 ^{o}C. Extracts were clarified by centrifugation (18000 rpm, 30 min), filtered through 0.22 micron filter, and loaded onto Protein G column (GE healthcare). The column was washed with 20 bed volumes of 50 mM NaOAc, pH 5.2, and proteins were eluted with 10 bed volumes of 100 mM glycine, pH 2.8. The elutants were immediately neutralized by adding 10 % of 1 M Tris, pH 8, and dialysed against PBS for long-term storage.

Linker conjugation and Protein coupling reaction

The antibodies were buffer exchanged into 100 mM NaOAc, pH 4.5, and the concentrations were adjusted to ~5 mg/mL. The oxime ligation was conducted with 50 times molar excess of linkers, and the reaction was complete within 24 hours, as monitored by LC-MS (**Supplementary Figure 1**). Excess linkers were removed by size filtration (Amicon 10K) or size exclusion column (Superdex 75) chromatography, the antibodies were buffer exchanged into PBS, pH 7.4, and the protein concentrations were adjusted to 10 mg/mL. Fab-Az conjugate and Fab-cyclooctyne conjugate were mixed at 1:1 molar ratio (~10 mg in total), and incubated for 2 days at 37 °C. Dimeric Fab conjugate was purified from unreacted Fabs by size exclusion column (Superdex 200) (**Supplementary Figure 2**).

Flow cytometry analysis

SK-BR-3 cells were trypsinized (0.05 % trypsin/EDTA, Hyclone) and washed with PBS. Jurkat cells in suspension were directly used without trypsinization. Cells (0.2×10^6) were blocked with 3 % BSA in PBS (200 μL) for 1 hour at 4 °C then incubated with 100 nM of primary antibodies for 1 hour at 4 °C. Cells were washed (cold PBS 1 mL) twice, resuspended in 200 μL of cold PBS, and incubated with secondary goat anti-human kappa PE conjugated antibody (100X, Southern Biotech) for 30 min at 4 ^{o}C. Cells were washed twice with 1 mL of cold PBS before analysis on a BD LSR II flow cytometer (Beckton Dickinson Immunocytometry Systems) with 10,000 cell events per sample. Data was analyzed using FlowJo software (Tree Star Inc.).

Fluorescent Staining

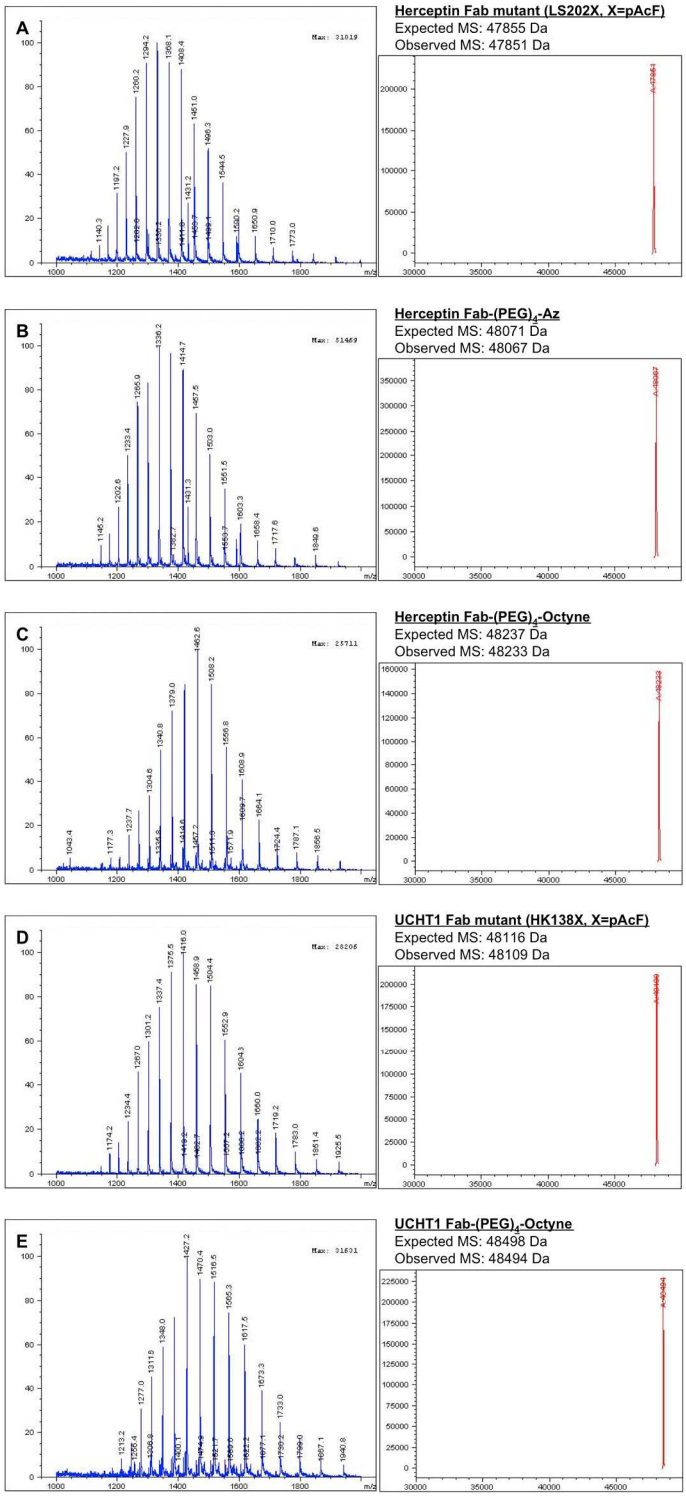
SK-BR-3 and Jurkat cells were stained with Mito Trakcer Red (Invitrogen) and CFSE (carboxyfluorescein succinimidyl ester, Invitrogen), respectively, following the manufacturer's protocol. Jurkat cells (2×10^4) were incubated with anti-HER2/anti-CD3 heterodimer (100 nM) in 200 μL of PBS for 30 min at 4 ^oC. In a separate tube, Jurkat cells were incubated with a 1:1 mixture of anti-HER2 Fab and anti-CD3 Fab (100 nM each) as negative controls. After washing with 1mL of cold PBS, the Jurkat cells were resuspended with 200 μL of RPMI media with 10 % FBS, then mixed with SK-BR-3 cells (2×10^3) in the same media (200 μL). Two hundred microliters of the cell mixture was added into a clear bottom 96 well plate, and incubated at 37 ^oC and 5 % CO_2. After 6 hours, wells were gently washed with PBS (200 μL) 4 times and imaged on a fluorescent microscope (Eclipse Ti, Nikon) under FITC (for CFSE) and rhodamine (for Mitro Traker Red) filters. The images from each filter were combined to produce an overlay image as seen in Figure **3**.

ADCC assays

Peripheral blood mononuclear cells (PBMCs) were purified from fresh healthy human donor blood by conventional Ficoll-Hypaque gradient centrifugation. Purified PBMCs were washed and incubated in flasks in RPMI media with 10 % FBS for 2 hours to remove adherent cells, and then transferred to anti-CD3 (eBioScience) and anti-CD28 (eBioScience) antibodies coated ELISA plates at 37 °C. After 3 days, the PBMCs were transferred into a flask and incubated with 20 units/mL IL2 (R&D Systems) for T cell proliferation. HER2-transfected MDA-MB-435 or non-transfected MDA-MB-435 cells (target cells) were dissociated with 0.05 % tryspin/EDTA solution (HyClone) and washed with RPMI with 10 % FBS. 1×10^4 target cells were mixed with PBMCs at 1:10 ratio in 100 μL, and incubated with different concentrations of conjugated and unconjugated anti-HER2/anti-CD3 Fabs (10 μL in PBS) for 17 hours at 37 °C. Cytotoxicity of each well was measured for LDH (lactate dehydrogenase) levels in supernatant using Cytotox-96 non-radioactive cytotoxicity assay kit (Promega). Lysis solution (10 μL, provided in the same kit) was added to wells with only target cells to get the maximum killing, and spontaneous killing was measured from wells with effector and target cells treated with vehicle (10 μL PBS). The absorbance at 490 nm was recorded using SpectraMax 250 plate reader (Molecular Devices Corp.). Percent cytotoxicity was calculated by:

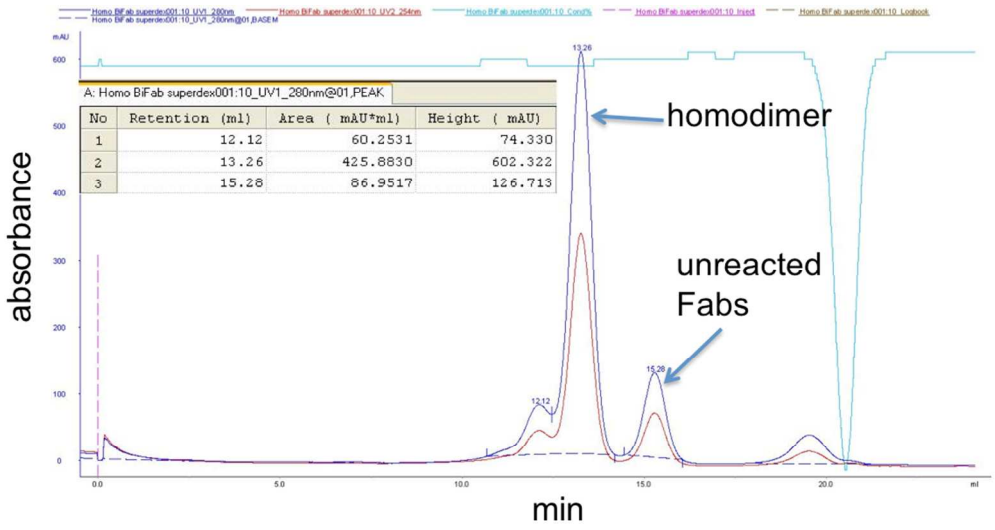
$$\% \text{ Cytotoxicity} = \text{Absorbance}_{\text{expt}} - \text{Absorbance}_{\text{spontaneous average}})/(\text{Absorbance}_{\text{max killing average}} - \text{Absorbance}_{\text{spontaneous average}}).$$

Supplementary Figure 1. ESI-MS analysis of Fab fragments before and after linker conjugation

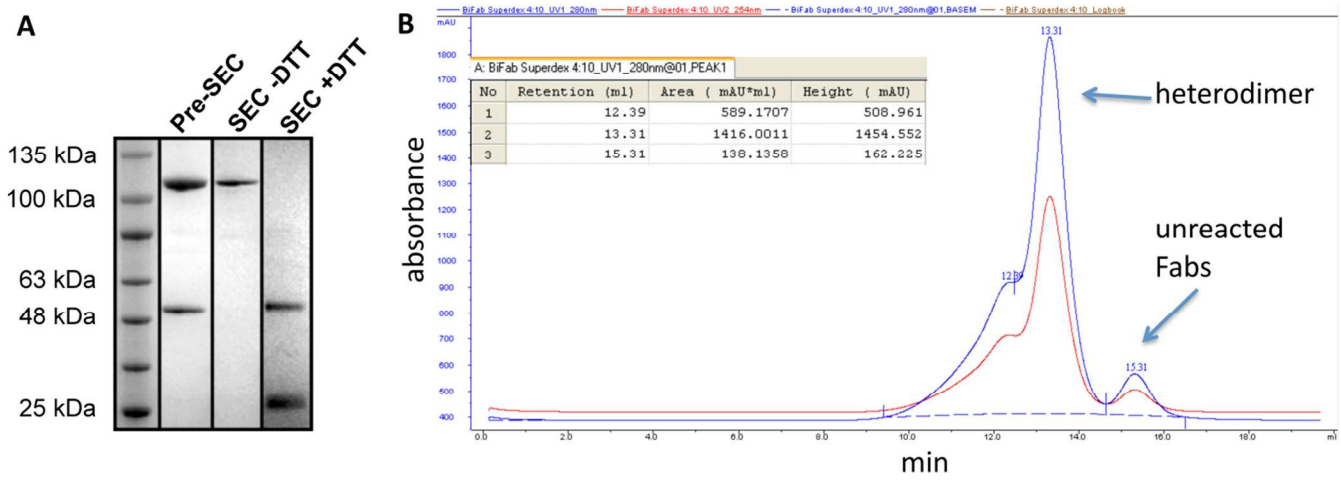


Supplementary Figure 2. Size exclusion chromatography FPLC trace from the purification of anti-HER2

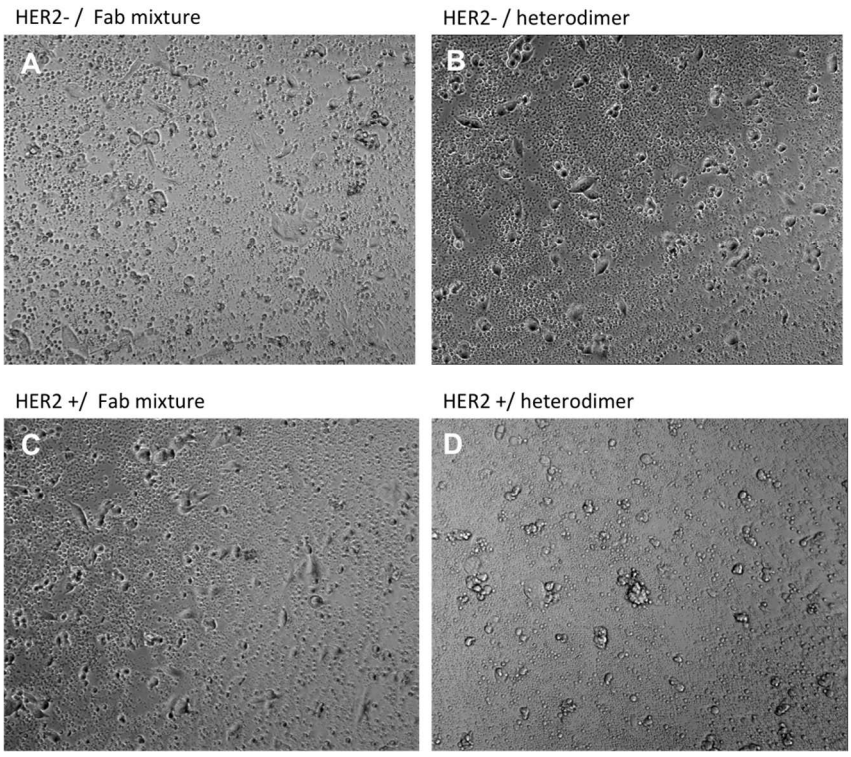
Fab-homodimer



Supplementary Figure 3. SDS-PAGE gel (A) and size exclusion chromatography FPLC trace (B) of the anti-HER2/anti-CD3 heterodimer.



Supplementary Figure 4. Microscopic images from cytotoxicity assay (at 100 pM of compounds)


HER2- / Fab mixture
A
HER2- / heterodimer
B
HER2 +/ Fab mixture
C
HER2 +/ heterodimer
D