Supporting Information:

Site-Specific Dual Antibody Conjugation via Engineered Cysteine and Selenocysteine Residues

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Dedicated to the memory of Carlos F. Barbas III

TABLE OF CONTENTS

EXPERIMENTAL PROCEDURES

Antibody cloning, expression, and purification

The anti-HER2 monoclonal antibody trastuzumab in scFv-Fc format containing one *C*-terminal Sec residue was generated as described previously.[1] The Fc region of this construct contains the hinge-C_H2-C_H3 sequence of human IgG1, followed by a TGA codon, six His codons, a TAA codon, and a SECIS element from the 3'-UTR of the cDNA of human TXNRD1. The antibody without Sec residue was generated by removing TGA codon and SECIS element while keeping the six His codons. The serine-to-cysteine substitution at position 396 in C_H3 was introduced with the Site-Directed Mutagenesis Kit (Agilent Technologies). Following confirmation by DNA sequencing, all plasmids were purified with the QIAGEN Plasmid Maxi Kit for transfections.

Human embryonic kidney (HEK) 293C18 cells (ATCC) were maintained in DMEM (Dulbecco's Modified Eagle's Medium with GlutaMAX; Life Technologies) containing 10% (v/v) Fetal Bovine Serum (FBS; Life Technologies) and 1% (v/v) Penicillin Streptomycin (Pen Strep; Life Technologies) in a humidified 5% CO_2 atmosphere at 37 °C. The mammalian cell expression vectors described above were transiently transfected into HEK 293C18 cells using polyethylenimine (PEI; Polysciences). After 6-12 h of transfections, media were replaced with fresh DMEM without FBS. For antibodies with engineered Sec residues, 1 μM sodium selenite (Na_2SeO_3; Sigma-Aldrich) was added to the medium. Culture supernatants were collected on days 3, 6, and 9 after transfections and filtered using 0.45-μm Stericup filter units (Millipore). The supernatants were loaded on a 1-mL recombinant Protein A column (HiTrap; GE Healthcare) connected to an ÄKTApurifier system (GE Healthcare). PBS was used for column equilibration and washing, 0.5 M acetic acid (pH 3.0) for elution, and 1 M Tris-HCl (pH 8.0) for immediate

neutralization. The neutralized eluate was buffer exchanged into PBS and concentrated simultaneously using 30-kDa cutoff centrifugal filter devices (Millipore). In order to separate scFv-Fc-Sec-His protein from scFv-Fc-stop protein, the purified scFv-Fc protein mixture was 10-fold diluted in loading/washing buffer (500 mM NaCl and 25 mM imidazole in PBS) and loaded on a 1-mL immobilized metal ion affinity chromatography (IMAC) column (HisTrap, GE Healthcare) connected to the ÄKTApurifier system. Subsequently, the column was washed with 10 mL loading/washing buffer, and the bound scFv-Fc-Sec-His protein was eluted with elution buffer (500 mM NaCl and 500 mM imidazole in PBS). Pooled fractions were buffer exchanged into PBS and concentrated as described above. All antibodies were determined to be >95% pure by SDS-PAGE. Yields for trastuzumab scFv-FcS396C-Sec protein reached 4 mg/L compared to 20 mg/L for trastuzumab scFv-FcS396C and scFv-Fc.

Dual antibody conjugation

First step. For selective conjugation at the Sec interface, trastuzumab scFv-Fc (control), scFv-Fc-Sec, scFv- FcS396C (control), and scFv-FcS396C-Sec proteins were buffer-exchanged to 100 mM sodium acetate (pH 5.2) and concentrated to 4 µM (~ 0.5 mg/mL) using a 30-kDa cutoff centrifugal filter device. The proteins were reduced by incubation with 0.1 mM dithiothreitol (DTT) for 20 min at room temperature (RT), followed by incubation with iodoacetamide or methylsulfone derivatives of biotin at a final concentration of 10 µM (1% (v/v) DMSO in reaction buffer) for 0.5-1 h at RT. Unreacted compound and DTT were washed away by using a 30-kDa cutoff centrifugal filter device and the antibodies were buffer-exchanged to PBS at ~ 4 µM.

Second step. For selective conjugation at the Cys interface, a 5-fold molar excess of methylsulfone-ODA-fluorescein (2% DMSO in reaction buffer) was added to above samples. The reactions were allowed to proceed for 1 h at RT. Unconjugated compounds were removed by using a 30-kDa cutoff centrifugal filter device. The conjugates in PBS were stored at 4 ºC for short term use and at -80 ºC in aliquots for long term use. Antibody concentrations were determined with the Bio-Rad Protein Assay, using a known concentration of an unconjugated antibody as standard.

SDS-PAGE

A 5-µL aliquot of samples from each of the final conjugation reaction solutions was incubated at 70 ºC for 5 min in NuPAGE LDS Sample Buffer (Life Technologies) supplemented with 50 mM DTT and then loaded on a NuPAGE Novex Bis-Tris 4-12% gradient gel (Life Technologies). Following SDS-PAGE and prior to staining with SimplyBlue SafeStain (Life Technologies), a picture of the gel was taken under blue light illumination (Life Technologies) to record the fluorescence.

Western blotting

Samples from each of the final conjugation reaction solutions were diluted 10-fold. Next, 1 µL diluted samples were incubated at 70 ºC for 5 min in NuPAGE LDS Sample Buffer supplemented with 50 mM DTT and electrophoresed on a NuPAGE Novex Bis-Tris 4-12% gradient gel, blotted on a PVDF membrane (Millipore), blocked with 3% (w/v) BSA in PBS, and incubated with HRP-conjugated ExtrAvidin (Sigma) diluted 1:1,000 in 3% BSA/PBS. HRP-conjugated donkey anti-human IgG Fcγ pAbs (Jackson ImmunoResearch Laboratories) were

used as positive control. Immunoreactive bands were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using Blue Basic autoradiography film (GeneMate).

Flow cytometry

Human breast cancer cell lines SK-BR-3 and MDA-MB-468 were purchased from ATCC. Both cell lines were maintained at 37 ºC in a humidified 5% CO_2 atmosphere in DMEM completed with 10% FBS and 1% Pen Strep. Cells were harvested using TrypLE (Life Technologies), and 2 x 10^5 cells were distributed in each well of a V-shaped 96-well microtiter plate. Unlabeled, single labeled, or dual labeled antibodies were added at a concentration of 2 µg/mL. The cells were incubated on ice for 30 min and washed three times with 200 µL FACS buffer (PBS, 1% (w/v) BSA, 0.01% (w/v) NaN_3, pH 7.4). APC-conjugated streptavidin (Jackson ImmunoResearch) was diluted 1:500 in 50 µL FACS buffer and added to each sample for 20 min on ice. Samples were washed three times with 200 µL FACS buffer and transferred to filter-top FACS tubes. Fluorescence was measured by flow cytometry (BD LSRII; BD Biosciences). For each sample, 10,000 live events were collected, and data were analyzed using FlowJo software (Tree Star, Inc.).

A flow cytometry-based internalization assay was performed as previously described [2]. Briefly, SK-BR-3 cells were incubated with 20 ng/mL of trastuzumab scFv-Fc or scFv-FcS396C-Sec/biotin/ODA-fluorescein in 1% (v/v) FBS/PBS buffer for 30 min on ice and washed three times with PBS. Cells were transferred into fresh DMEM medium with 10% (v/v) FBS and either warmed to 37 ºC to allow internalization or maintained on ice. After internalization was stopped by transferring the cells on ice, cells were stained with APC-conjugated goat anti-

human Fcγ $F(ab')_2$ fragment (Jackson ImmunoResearch) and analyzed by flow cytometry as above. The percentage of mean fluorescence intensity (MFI) reduction was calculated for each antibody relative to the unspecific human IgG control ($MFI_{background}$) and antibody maintained on ice (MFI_{max}) by using the formula $[(MFI_{max} - MFI_{background})-(MFI_{experimental} - MFI_{background})]/(MFI_{max} - MFI_{background}) \times 100$.

Surface plasmon resonance

Surface plasmon resonance for the measurement of trastuzumab scFv-Fc and scFv-FcS396C-Sec/biotin/ODA-fluorescein affinities to HER2 was performed on a Biacore X100 instrument using Biacore reagents and software (GE Healthcare). Recombined human HER2-Fc (R&D Systems) in 10 mM sodium acetate (pH 4.5) was immobilized on a CM5 sensor chip at a density of 1,418 resonance units (RU). Antibodies were injected at five concentrations ranging from 0.625 to 10 nM. The sensor chip was regenerated with 4.5 M $MgCl_2$ without loss of any binding capacity. The sensorgram data were fit to a bivalent analyte model using Biacore X100 evaluation software.

Confocal immunofluorescence microscopy

A confocal immunofluorescence microscopy-based approach was used to visualize antibody internalization. SK-BR-3 and MDA-MB-468 cells on coverslips were treated with 2 μg/mL of trastuzumab scFv-FcS396C-Sec/biotin/ODA-fluorescein at 37 ºC for 4 h. Next, non-internalized antibody was removed by washing with acidic buffer (Tris-glycine buffer, pH 2.5). Cells were fixed with 4% (v/v) formaldehyde and permeabilized with 0.1% (v/v) Triton X-100. Internalized antibody was revealed with Cy3-conjugated goat anti-human Fcγ $F(ab')_2$ fragment

(Jackson ImmunoResearch). Cells were counterstained with Hoechst 33342 (Sigma-Aldrich). Visualization of internalized antibody was performed using an Olympus IX81 confocal microscope and SlideBook digital microscopy software (Intelligent Imaging Innovations).

Human plasma stability

Trastuzumab scFv-FcS396C-Sec/biotin/ODA-fluorescein at a concentration of 1.3 mg/mL was diluted 1:1 (v/v) into human plasma and incubated at 37 °C for the duration of the time course study. Time points (0, 12, 24, 48, and 72 h) were removed and stored at -80 ºC, and then SDS-PAGE and Western blotting were performed as above without DTT in sample loading buffer. A mouse anti-His mAb (Qiagen) was used as control for detection of the total antibody (conjugated and unconjugated).

Mass spectrometry

Deglycosylation was performed by incubating 100 µg antibody samples with 5 µL N-Glycosidase F (Promega) at 37 ºC overnight. MALDI-TOF mass spectra were recorded on an AB SCIEX 4800 Plus MALDI-TOF/TOF Analyzer in linear high-mass positive mode. All deglycosylated antibody samples were prepared in 1:1 (v/v) H_2O/acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). Any salt from the samples was removed using C4 ZipTip Pipette Tips (Millipore) and 1:1 (v/v) H_2O/acetonitrile containing 0.1% (v/v) TFA for elution. As a MALDI-TOF matrix, 5 mg/mL sinapinic acid (Sigma-Aldrich) in 1:1 (v/v) H_2O/acetonitrile containing 0.1% (v/v) TFA was used. A 5 pmol/µL solution of BSA (AB SCIEX; molecular weight: 66,430 Da) was used as internal standard and for external calibration.

Compounds

The synthesis of *methylsulfone-ODA-fluorescein* (Figure 1B) was described previously.[2] *Biotin-ethylenediamine-iodoacetamide* (Figure 1B) was purchased from Biotium. *Biotin-PEG-iodoacetamide* (Figure S3A) was synthesized on Rink amide resin following a modified solid-phase peptide synthesis protocol. Rink amide resin (200 mg, 0.59 mmol/g loading; Novabiochem) was swollen in dimethylformamide (DMF) for 1 h before deprotecting Fmoc with 20% (v/v) piperidine in DMF. The resin was treated with 4 equivalents of oxyma (Novabiochem), diisopropyl carbodiimide (DIC), and Fmoc-Phe-OH for 1 h at RT. Following Fmoc deprotection as mentioned above, the process was repeated to couple Fmoc-PEG2-OH (Peptides International), Fmoc-Phe-OH, Fmoc-Ahx-OH (EMD Millipore), and Fmoc-PEG3-OH (Peptides International). The Fmoc group was deprotected with 20% (v/v) piperidine in DMF, and the resin was treated with 4 equivalents of oxyma, DIC, and iodoacetic acid (Sigma-Aldrich) for 1 h at RT in the dark. The resin was thoroughly washed with dichloromethane (DCM), dried under vacuum, and treated with 50% (v/v) TFA, 2 % (v/v) triisopropyl silane (TIPS) in DCM for 1 h at RT in the dark to cleave the compounds from resins. The cleavage cocktail was removed from the compounds by using an argon flow in a high capacity Savant SpeedVac system (Explorer 2000; Thermoelectron). The crude sample was purified on a Waters 1525 Binary HPLC system (equipped with Waters 1525 binary HPLC pumps and a 2487 dual λ absorbance detector) with acetonitrile:water gradient (5 to 50 % (v/v) acetonitrile over 30 min) using a Vydac C18 Reverse phase preparative column (Figure S3B). The fractions containing the iodoacetamide compound were confirmed by mass spectrometry using a AB SCIEX 4800 Plus MALDI-TOF/TOF Analyzer (Figure S3C), pooled together, and lyophilized using a Virtis

Benchtop K (Model 4KBTZL) Lyophilizer. All compounds were dissolved in DMSO at 10 mM stock concentration and stored at -80 ºC in aliquots.

SUPPLEMENTARY FIGURES AND TABLE

TABLE

Antibody	k_{a1} ($M^{-1}s^{-1}$)	k_{d1} (s^{-1})	K_{D1} (nM)	k_{a2} ($RU^{-1}s^{-1}$)	k_{d2} (s^{-1})	Chi^2 (RU^2)
scFv-Fc	1.1×10^{5}	4.8×10^{-5}	0.43	2.8×10^{-3}	5.9×10^{-3}	0.261
scFv-FcS396C-Sec/biotin/ODA-fluorescein	8.8×10^{4}	1.1×10^{-4}	1.25	3.8×10^{-3}	8.3×10^{-3}	0.103

Table S1. Kinetic parameters for the interaction between antibodies and HER2-Fc as determined by surface plasmon resonance. The sensorgram data were fit to a bivalent analyte model.

FIGURES

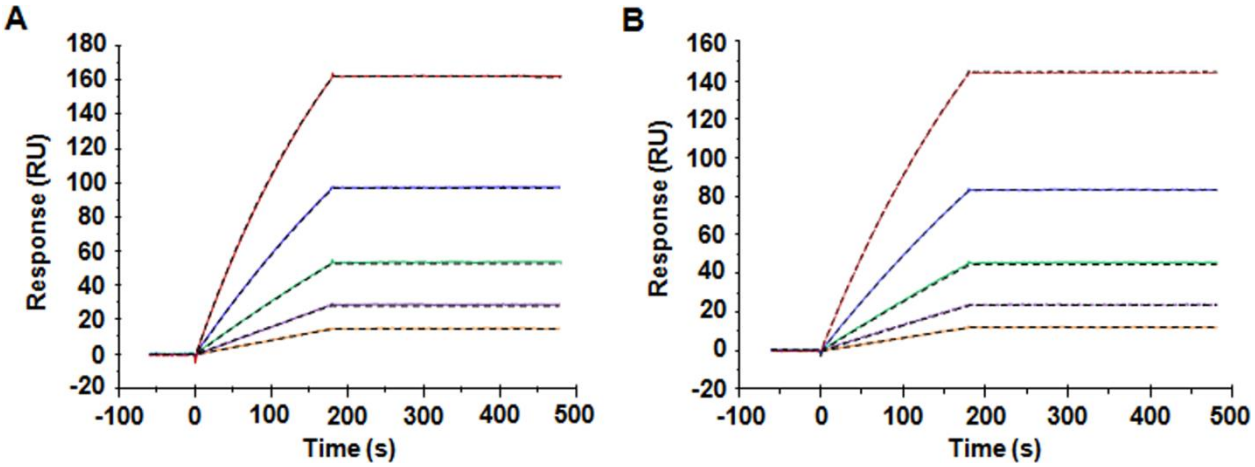

Figure S1. Avidity measurements by surface plasmon resonance. Biacore X100 sensorgrams for the binding of trastuzumab scFv-Fc (**A**) and scFv-FcS396C-Sec/biotin/ODA-fluorescein (**B**) to immobilized human HER2-Fc after instantaneous background depletion. The antibodies were injected at five different concentrations ranging from 0.615 to 10 nM (bottom to top). The dashed black line marks the fitted curve.

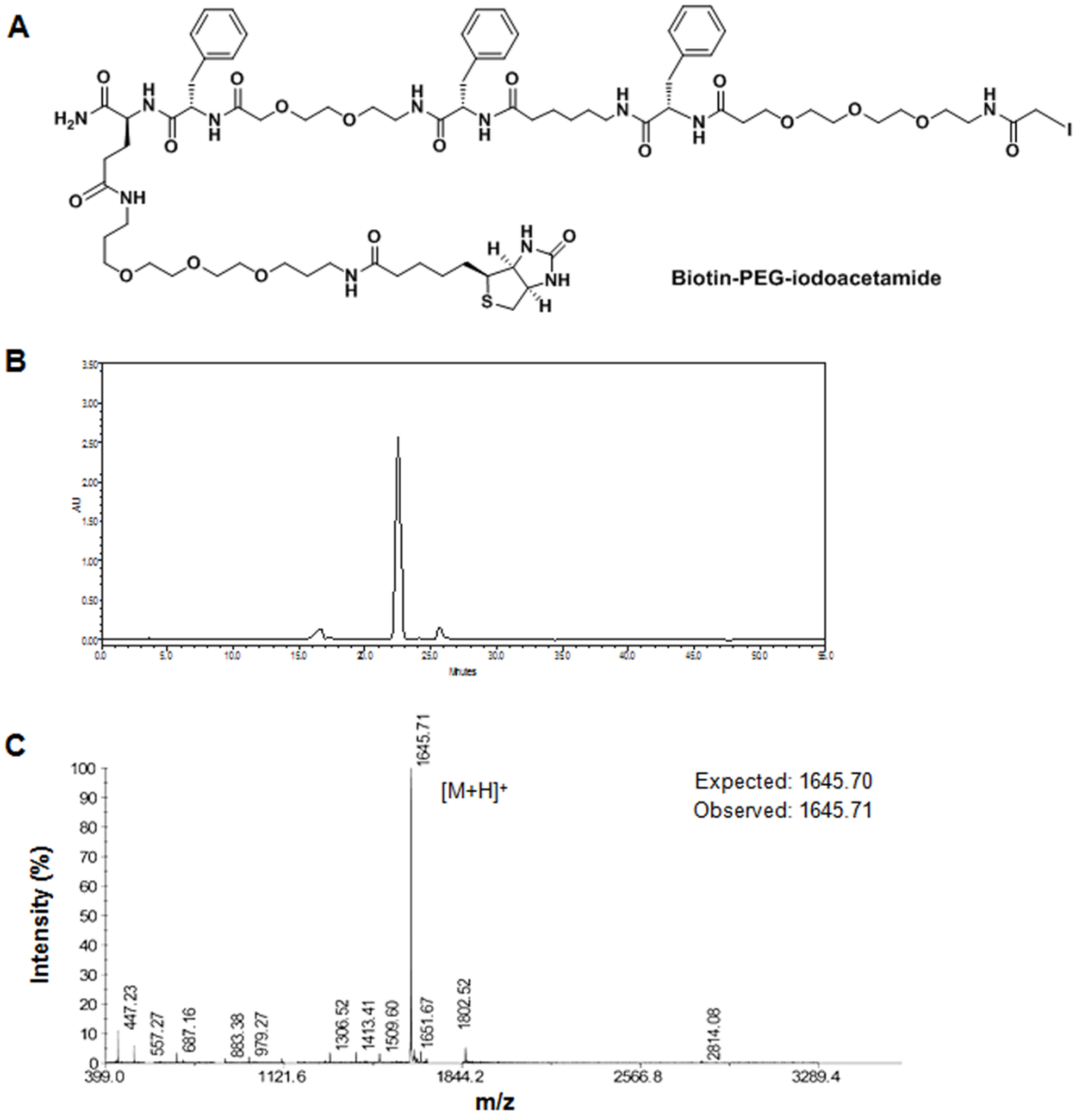

Figure S2. (**A**) Structure of biotin-PEG-iodoacetamide. (**B**) HPLC purification of biotin-PEG-iodoacetamide. (**C**) MALDI-TOF mass spectrum of purified biotin-PEG-iodoacetamide.

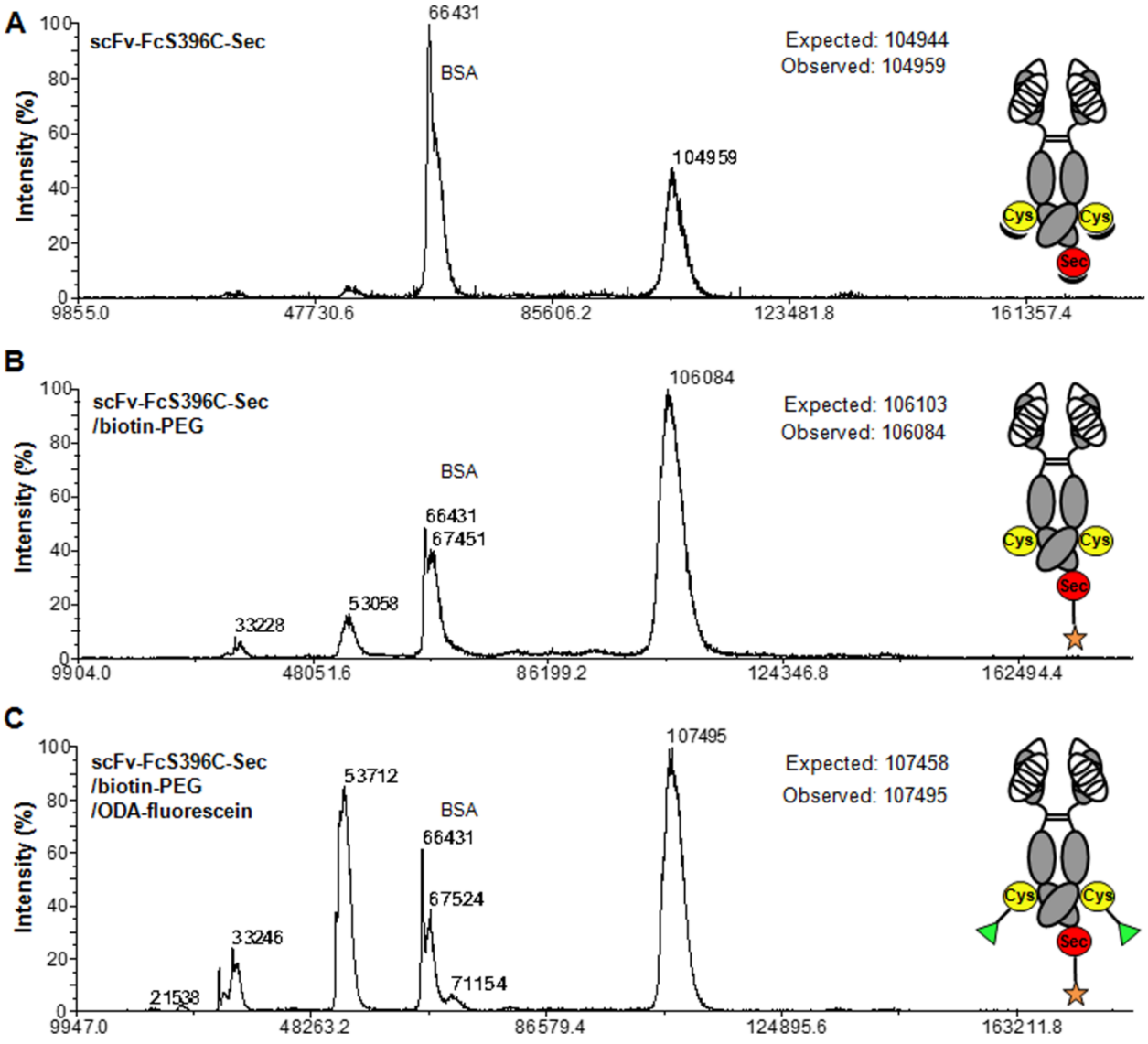

Figure S3. MALDI-TOF mass spectra of unlabeled (**A**), biotin-PEG labeled (**B**), and biotin-PEG/ODA-fluorescein dual labeled (**C**) trastuzumab scFv-FcS396C-Sec. All MALDI-TOF analyses were made with external calibration against bovine serum albumin (BSA) with an average mass of 66,431 Da.

REFERENCES

(1) Li, X., Yang, J., and Rader, C. (2014) Antibody conjugation via one and two C-terminal selenocysteines. *Methods 65*, 133-138.
(2) Toda, N., Asano, S., and Barbas, C. F., 3rd. (2013) Rapid, stable, chemoselective labeling of thiols with Julia-Kocienski-like reagents: a serum-stable alternative to maleimide-based protein conjugation. *Angew. Chem. Int. Ed. Engl. 52*, 12592-12596.