Supporting Information

Template directed synthesis of antibody Fc conjugates with concomitant ligand release

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Abbreviations

ACN: acetonitrile

Boc: tert-butyloxycarbonyl

DCM: dichloromethane

DFO: desferrioxamine B

DIC: N,N'-diisopropylcarbodiimide

DIEA: N,N-diisopropylethylamine

DMF: dimethyl formamide

DMSO: dimethyl sulfoxide

DOTA: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid

DOTA-GA: 2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanedioic acid

DTPA: diethylenetriaminepentaacetic acid

FAM: 5(6)-carboxyfluorescein

FITC: fluorescein isothiocyanate

HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate

HPLC: high-performance liquid chromatography

HRMS: high resolution mass spectrometry

HCD: higher energy collision-induced dissociation

LC-FTMS: liquid chromatography Orbitrap Fourier transform mass spectrometry

LC-FT-MS/MS: liquid chromatography Orbitrap Fourier transform tandem mass spectrometry

NHS: N-hydroxysuccinimide

NMR: nuclear magnetic resonance

NCE: normalized collision energy

PBS: phosphate-buffered saline

PEG: polyethylene glycol

pH: potential for hydrogen

rt: room temperature

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SPPS: solid-phase peptide synthesis

TCEP: tris (2-carboxyethyl) phosphine

TFA: trifluoroacetic acid

TIS: triisopropylsilane

UPLC: ultra-performance liquid chromatography

UV: ultraviolet

Reagents and Solvents

Fmoc-protected amino acids, N,N-diisopropylcarbodiimide (DIC), OxymaPure®, piperazine and Fmoc-Rink Amide AM resin were purchased from Merck KGaA (Darmstadt, Germany). Solvents for synthesis, deprotection reagents, and cleavage reagents used were of synthesis grade and purchased from Merck or Fisher Scientific AG (Reinach, Suisse). Amino acids were purchased from Bachem AG (Bubendorf, Switzerland), Merck KGaA and Aapptec (Louisville, U.S.A.). Solvents and other chemicals used for high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography mass spectrometry (UPLC-MS) were of HPLC reagent grade and purchased from Macherey-Nagel (Düren, Switzerland).

Fc-III peptide and fluorescently labeled Fc-III-FAM peptide were purchased from Genscript (Piscataway, NJ, USA). The DOTA, and fluorescein-carbonate derivatives were synthesized by Genochem (Grasse, France) (Figure 3). The GingisKHAN, FabALACTICA and FabRICATOR proteases were purchased from Genovis (Lund, Sweden), IdeS protease – from Promega (Dübendorf, Switzerland). EndoS protease was purchased from BioConcept (Allschwil, Switzerland). PEG linkers were purchased from BroadPharm (San Diego, U.S.A.). Herceptin® (trastuzumab) and Tecentriq® (atezolizumab) were purchased from Roche (Switzerland). The biosimilar monoclonal IgG1 antibodies were produced by cultivation of recombinant CHO cell lines in Dr. G. Hagens laboratory at the University of Applied Sciences Western Switzerland (HES-SO Valais/Wallis).

General Experimental Details

UPLC-MS

Reactions were monitored by liquid chromatography-mass spectrometry (LC-MS). LC-MS was carried out by Reversed Phase-UPLC (Waters Acquity Ultra Performances LC) connected to MS (Micromass Quattro micro–API Mass Spectrometer) on a Kinetex 1.7 µm XB-C18 100 Å, LC Column 50 x 2.1 mm column (Phenomenex Helvetia) using a mixture of solvent B (acetonitrile with 0.1% TFA) and solvent A (water with 0.1% TFA), 2%–98% gradient, at a flow rate of 0.617 mL/min for 4 min. The peptide signal was monitored at 214 nm. The peptide purity stated in the following sections was determined by integration of the area under the curves of each peak observed in the chromatogram unless otherwise stated.

Concentration determination

Peptide samples were prepared by dissolving purified peptide or peptide conjugate in DMF. The concentrations were determined in 1x PBS pH 7.0 using the absorbance of tryptophan (ε = 5500 M^{-1} cm^{-1}), p-SCN-Bn-CHX-A"-DTPA (ε = 13000 M^{-1} cm^{-1}), p-NCS-Bn-DFO (ε = 21000 M^{-1} cm^{-1}) at 280 nm or the absorbance of FITC (ε = 73000 M^{-1} cm^{-1}) at 496 nm.

FP measurements

The fluorescence polarization measurements were performed on SpectraMax Paradigm Multi-Mode Detection Platform (Molecular Devices) in flat-bottom 384-well Corning microplates (Merck KGaA), using excitation and emission wavelengths of 485 nm and 535 nm, respectively. The acquisition time was 700 ms, and the read height was 1 mm. All reagents used in the assay were diluted in PBS containing 0.05% of Tween 20.

Saturation binding FP assays

Fluorescently labeled peptide Fc-III-FAM was mixed with serial dilutions of IgG1 in PBS containing 0.05% of Tween 20. The final peptide concentration was of 5 nM and the final volume of the sample was of 80 µl. Samples were incubated at 27°C for 15 min and the fluorescence anisotropy was measured in triplicate.

Competitive binding FP assays

Increasing concentrations of peptide were mixed with the Fc-III-FAM tracer and added to the IgG1 in a total volume of 80µL. The final concentration of Fc-III-FAM was kept constant at 5 nM, the final concentration of IgG1 was of 10 - 30 nM and the final volume of sample was of 80 µl. The mixture was incubated at 27°C for 15 min and the fluorescence signal was measured on a Spectramax Paradigm. All sample preparations were done in PBS pH 7.4 or 7.0 containing 0.05% of Tween 20. Each experiment was performed in triplicate.

SDS PAGE

Reducing or non-reducing SDS-PAGE electrophoresis was performed on Bolt 4-12% Bis-Tris Plus Gels (ThermoFisher). The loading buffer was added to the antibody conjugates

(non-reducing Bolt Sample Buffer, ThermoFisher) and the samples were heated at 70 °C for 10 minutes. For reducing SDS-PAGE, reducing buffer was added to the samples prior to the loading buffer. The gel was run at constant voltage (200 V) for 25 - 30 min using Bolt MES Running Buffer. The fluorescence was visualized on FluoroM bio-imaging system (Syngene Cambridge, United Kingdom) prior to staining with Coomassie Blue.

Peptide Synthesis

a) Synthesis. Peptides were synthesized by Fmoc/tBu solid-phase peptide chemistry on a Rink Amide AM resin (loading 0.57 mmol/g) using a Liberty Blue™ automated microwave peptide synthesizer (CEM Corp., Kamp-Lintfort, Germany) following a standard protocol. Each coupling was performed for 4 min at room temperature, using 0.2 M of Fmoc amino acid preactivated with 0.5 M DIC and 1 M OxymaPure® in DMF. Fmoc removal was performed with 10% v/v piperazine in DMF.

b) Cleavage. After completion of the synthesis, peptides were deprotected and cleaved from the resin manually with TFA under gentle agitation over a period of 1.5 h at RT in the presence of scavengers (standard cleavage solution: TFA/TIS/Water 90:5:5). After filtration and evaporation of most of the cleavage solution under a stream of nitrogen, the crude peptides were precipitated by addition of cold diethyl ether, centrifuged, and washed with cold diethyl ether. The resulting amorphous powder was dried, dissolved in ultrapure water/ACN, frozen, and lyophilized.

c) Disulfide bond formation. Crude lyophilized peptides were resuspended in a mixture of DMSO/Acetonitrile/water (2/3/3), then water was added until the peptide became soluble (35 - 50 ml) and the resulting solution was brought to pH 8.5 with 1 M of NH_4HCO_3 or $NaHCO_3$ (C = 0.1 – 0.5 mM). The progress of the oxidation was monitored via analytical UPLC-MS. After completion of the reaction, salts were removed with a Sep-Pak C_{18} Plus Long Cartridge (820 mg Sorbent per Cartridge, 55-105 µm Particle Size, Waters, Baden-Dättwil, Switzerland) and the peptides were lyophilized.

d) Purification. The peptide purifications were performed by Preparative Reversed Phase-HPLC on a Kinetex 5u XB-C18 100A, 100 x 21.2 mm column (Phenomenex Helvetia, Basel, Switzerland) by applying a 15%–55% gradient of solvent B (described in UPLC-MS section above), at a flow rate of 35 mL/min for 25 min. The peptide absorbance was monitored at 214 nm. Fractions were analyzed by UPLC-MS prior to the lyophilization step.

General procedure for the synthesis of PEG_n-Fc-III derivatives

To a solution of Fmoc-PEG_n-COOH (1.2 eq., 14.7 mg, 19.6 µmol) in 100 µl DMF was added HATU (1.1 eq., 18.0 µmol, 33.3 µl), stirred for 1min, followed by the addition of DIEA (2 eq., 32.7 µmol, 16.3 µl). After 3 minutes of pre-activation, Fc-III peptide in 150 µl DMF (1 eq., 25 mg, 16.3 µmol) was added to the reaction mixture and stirred for 1-4 h at room temperature. The reaction completion was monitored by UPLC-MS. The reaction solution was precipitated with cold diethyl ether.

Fmoc deprotection. 300 µl of 20% piperidine in DMF was added to the pellet of Fmoc-PEG_n-Fc-III. The reaction mixture was stirring for 30 min at RT, followed by precipitation with 3ml of cold diethyl ether and purification by HPLC.

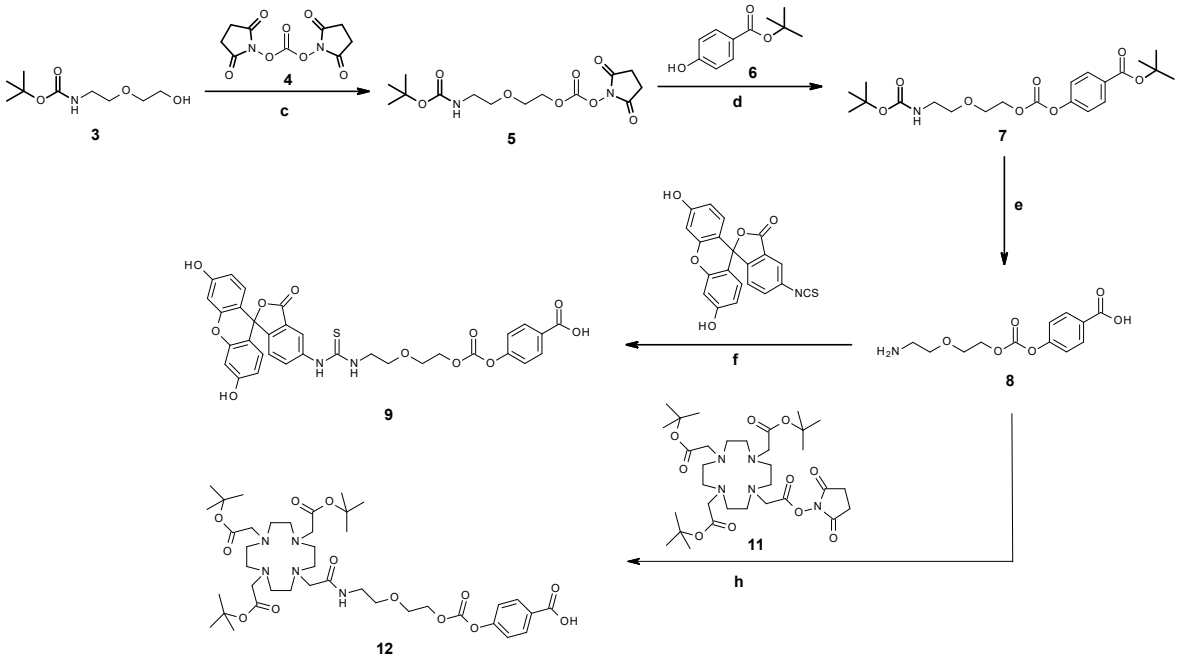
Table S1. Characteristics of Fc-III peptide and its derivatives.

Peptide	**Calculated Exact Mass**	**Calculated $[M + H]^+$**	**Observed $[M + H]^+$**	**Retention time, min[1]**	**Purity**
Fc-III	1529.73	765.87	765.81	1.67	> 95%
PEG_2-Fc-III	1689.13	845.56	845.74	1.69	> 95%
PEG_4-Fc-III	1777.27	889.63	889.87	1.70	> 95%
PEG_6-Fc-III	1865.38	933.69	933.86	1.71	> 95%
PEG_8-Fc-III	1953.53	977.76	977.84	1.74	90%
PEG_{10}-Fc-III	2041.63	1021.81	1021.95	1.75	95%
PEG_{12}-Fc-III	2129.73	1065.86	1065.95	1.77	91%
PEG_{20}-Fc-III	2481.91	828.30	828.50	1.80	> 95%
PEG_{36}-Fc-III	3187.03	1063.34	1063.45	1.90	> 95%
Fc-III-V10W-W11A	1501.68	751.84	751.84	1.70	> 95%
PEG_{20}-Fc-III-V10W-W11A	2453.86	818.95	819.42	1.85	> 95%
Fc-III-C2A-C12A	1467.63	734.82	734.71	1.87	> 95%
PEG_{20}-Fc-III-C2A-C12A	2419.83	807.61	807.53	1.87	> 95%

[1]The retention time was measured as described in UPLC-MS section.

Chemical Synthesis

Synthesis of fluorescein-carbonate 9 and $(tBu)_3$DOTA-carbonate 12 compounds



Scheme S1. Synthesis of compounds **9** and **12**. Reagents and conditions: (c) **4**, Et_3N in ACN at 40°C; (d) **6**, DMAP in DCM at 25°C; (e) TFA/DCM (1/3); (f) **FITC**, DIPEA in ACN/DMF 1/1 at 25°C; (h) **11**, DIPEA in ACN at 25°C. The synthesis of these derivatives was performed by Genochem (Grasse, France).

2-[2-(tert-butoxycarbonylamino)ethoxy]ethyl (2,5-dioxopyrrolidin- 1-yl) carbonate (5). 5.2 g of N,N'-disuccinimidyl carbonate **4** (19 mmol, 2.0 eq) was added to a solution of 2.0 g of 2-(2-Boc-aminoethoxy)ethanol **3** (9.6 mmol) in 70 mL of acetonitrile, followed by 2.7 mL of triethylamine (19 mmol, 2.0 eq) and the suspension was stirred at 40 °C for 1h30. The solvent was removed in vacuo, the residue was dissolved in DCM and filtered through a pad of silica eluting with (dichloromethane/ethyl acetate 80/20) to afford 2-[2-(tert-butoxycarbonylamino)ethoxy]ethyl (2,5-dioxopyrrolidin-1-yl) carbonate **5** (UV purity >80%, yield: 99%). LC-MS : m/z = 247 $[M\text{-}Boc+H]^+$, 369 $[M+Na]^+$. ^1H NMR ($CDCl_3$): δ 4.52 – 4.40 (m, 2H), 3.77 – 3.68 (m, 2H), 3.55 (t, 2H), 3.32 (dd, 2H), 2.84 (s, 4H), 1.44 (s, 9H).

tert-butyl 4-[2-[2-(tert-butoxycarbonylamino)ethoxy]ethoxy carbonyloxy]benzoate (7). A solution of 1.5 g of 2-[2-(tert-butoxycarbonylamino)ethoxy]ethyl (2,5-dioxopyrrolidin-1-yl) carbonate **5** (3.4 mmol, 2.0 eq) in 12 mL of DCM was mixed with 0.35 g of tert-Butyl 4-

hydroxybenzoate **6** (1.7 mmol) and 0.43 g of 4-(dimethylamino)pyridine (3.4 mmol, 2 eq). The reaction mixture was stirred at room temperature for 30 min. 50 mL of water was added to the reaction mixture, the compound was extracted with 3 x 10 mL of dichloromethane, and the combined organic layers were concentrated in vacuo. The residue was purified by Flash Chromatography (cyclohexane/ethyl acetate, 90/10 to 60/40) to afford 0.64 g of tert-butyl-4-[2-[2-(tertbutoxycarbonylamino)ethoxy] ethoxycarbonyloxy] benzoate **7** as a colorless oil (UV purity >98%, yield: 88 %). LC-MS : m/z = 326 $[M\text{-}Boc+H]^+$, 448 $[M+Na]^+$. ^1H NMR (400 MHz, DMSO) δ 7.93 (d, J = 8.8 Hz, 2H), 7.31 (d, J = 8.8 Hz, 2H), 6.75 (t, J = 5.3 Hz, 1H), 4.28 – 4.26 (m, 2H), 3.62 – 3.60 (m, 2H), 3.36 (t, J = 6.0 Hz, 2H), 3.04 – 3,00 (m, 2H), 1.31 (s, 9H); ^{13}C NMR (100 MHz; DMSO) δ 166.49, 155.62, 153.97, 152.62, 130.92, 128.62, 121.44, 77.62, 69.15, 67.97, 67.63, 40 (hidden by d6-DMSO peak), 28.92

4-[2-(2-aminoethoxy)ethoxycarbonyloxy]benzoic acid; 2,2,2-trifluoroacetic acid (8). To a solution of 0.67 g of **7** (1.5 mmol) in 6.3 mL of DCM was added 2.1 ml of TFA (27 mmol, 17 eq) at 0°C and the reaction mixture was stirred at room temperature for 3h. The mixture was concentrated in vacuo to afford 0.73 g of 4-[2-(2-aminoethoxy)ethoxycarbonyloxy]benzoic

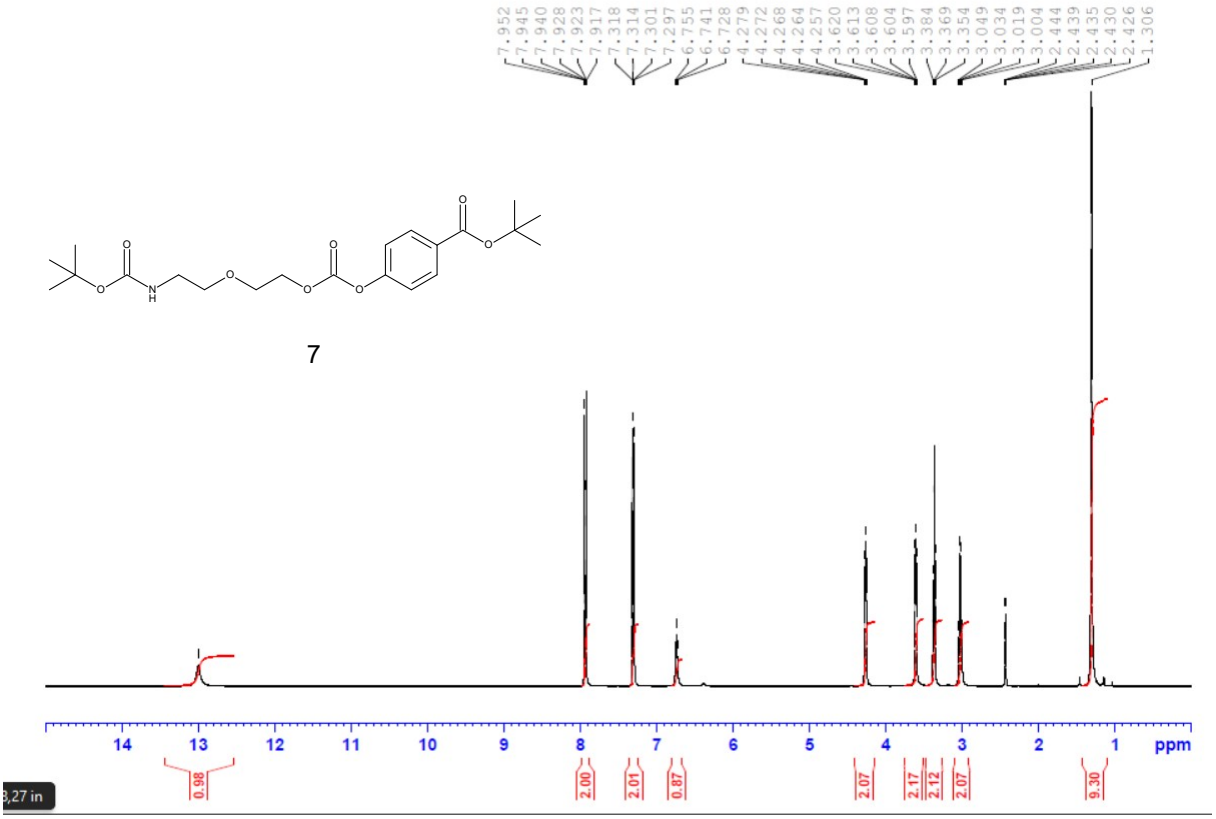
acid; 2,2,2-trifluoroacetic acid **8** as a white solid (UV purity >80%, yield: 97%). LC-MS : m/z = 270 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 8.04-7.99 (m, 2H), 7.98 – 7.77 (broad singlet, 3H), 7.40-7.35 (m, 2H), 4.41-4.37 (m, 2H), 3.77-3-74 (m, 2H), 3.66 (t, J = 5.5 Hz, 2H), 3.06 – 2.97 (m, 2H) ^{13}C NMR (100 MHz, DMSO) δ 166.50, 153.94, 152.55, 131.04, 128.73, 121.43, 67.92, 67.78, 66.66, 38.52.

(4-[2-[2-[(3',6'-dihydroxy-3-oxo-spiro [isobenzofuran-1,9'-xanthene]-5-yl)carbamothioylamino]ethoxy] ethoxycarbonyloxy] benzoic acid (9). To a solution of 0.71 g of 4-[2-(2-aminoethoxy)ethoxycarbonyloxy]benzoic acid, 2,2,2-trifluoroacetic acid **8** (1.2 mmol, 1.2 eq.) in 4.0 mL of ACN were added 0.40 g of fluorescein isothiocyanate **(FITC)** (1.0 mmol) and 4.0 mL of dimethylformamide followed by 1.1 mL of N,N-diisopropylethylamine (6.0 mmol, 6.0 eq.). This mixture was stirred for 10 min at room temperature. The solvent was evaporated under vacuum, and the residue was purified by C_{18} Flash Chromatography (water/acetonitrile 95/5 to 0/1) to yield 0.38 g of compound **9** (4-[2-[2-[(3',6'-dihydroxy-3-oxo-spiro [isobenzofuran-1,9'-xanthene]-5-yl)carbamothioylamino]ethoxy] ethoxycarbonyloxy] benzoic acid as an orange solid) (UV purity: 98%, yield: 56%). LC-MS : m/z = 657 $[M-H]^-$, 659 $[M+H]^+$. ^{1}H NMR (400MHz, DMSO): δ 13.07 (s, 1H), 10.24 – 9.95 (m, 3H), 8.27 (s, 1H), 8.17 (s, 1H), 7.99 (d, 2H), 7.74 (d, 1H), 7.35 (d, 2H), 7.19 (d, 1H), 6.67 (d, 2H), 6.61 – 6.53 (m, 4H), 4.42 – 4.39 (m, 2H), 3.80 – 3.66 (m, 6H); ^{13}C NMR (100 MHz, DMSO) δ 181.04, 168.98, 166.99, 159.92, 154.37, 153.02, 152.33, 147.64, 141.77, 131.44, 129.90, 129.49, 129.19, 127.03, 124.54, 121.87, 116.82, 113.03, 110.17, 102.70, 83.45, 68.88, 68.44, 68.33, 44.08.

4-[2-[2-[[2-[4,7,10-tris(2-tert-butoxy-2-oxo-ethyl)-1,4,7,10-tetrazacyclododec-1-yl]acetyl]amino]ethoxy]ethoxycarbonyloxy]benzoic acid (12). 0.88 mL of DIEA (5.0 mmol, 6.0 eq) and 0.44 g of 4-[2-(2-aminoethoxy)ethoxycarbonyloxy]benzoic acid **8** (0.92 mmol, 1.1 eq) were added to a solution of 0.70 g of DOTA-tris(tBu)ester NHS ester **11** (0.83 mmol) in 3.5 mL of ACN and the reaction mixture was stirred at room temperature for 10 min. The solution was diluted in 3.5 mL of water and purified by C_{18} cartridge Flash Chromatography (water/ACN, 90/10 to 0/100). The fractions were collected, concentrated in vacuo and lyophilized to afford 0.66 g of compound **12** (4-[2-[2-[[2-[4,7,10-tris(2-tert-butoxy-2-oxo-ethyl)-1,4,7,10-tetrazacyclododec-1-yl]acetyl]amino]ethoxy]ethoxycarbonyloxy]benzoic acid) as a white solid (UV purity >95 %, yield: 93%). LC-MS : m/z = 824 $[M+H]^+$, 413 $[M/2+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 8.56 (t, J = 5.8 Hz, 1H), 7.99-7.96 (d, J = 9.6 Hz, 2H), 7.30 (d, J = 9.6 Hz, 2H), 4.36-4.33 (m, 2H), 3.71-3.68 (m, 2H), 3.51 – 3.48 (m, 2H), 3.35 – 3.28 (m,

8H), 3.05 (br s, 2H), 2.81 (br s, 8H), 2.68 (br s, 4H), 2.51 (br s, 4H), 1.41 (s, 27H); ^{13}C NMR (100 MHz, DMSO) δ 170.36, 170.28, 166.79, 153.30, 152.62, 130.78, 120.95, 115.12, 80.10, , 69.12, 67.90, 67.73, 55.64, 53.83 (weak), 51.57, 38.41, 27.83.

NMR spectra of key intermediates (d_6-DMSO 400 MHz)



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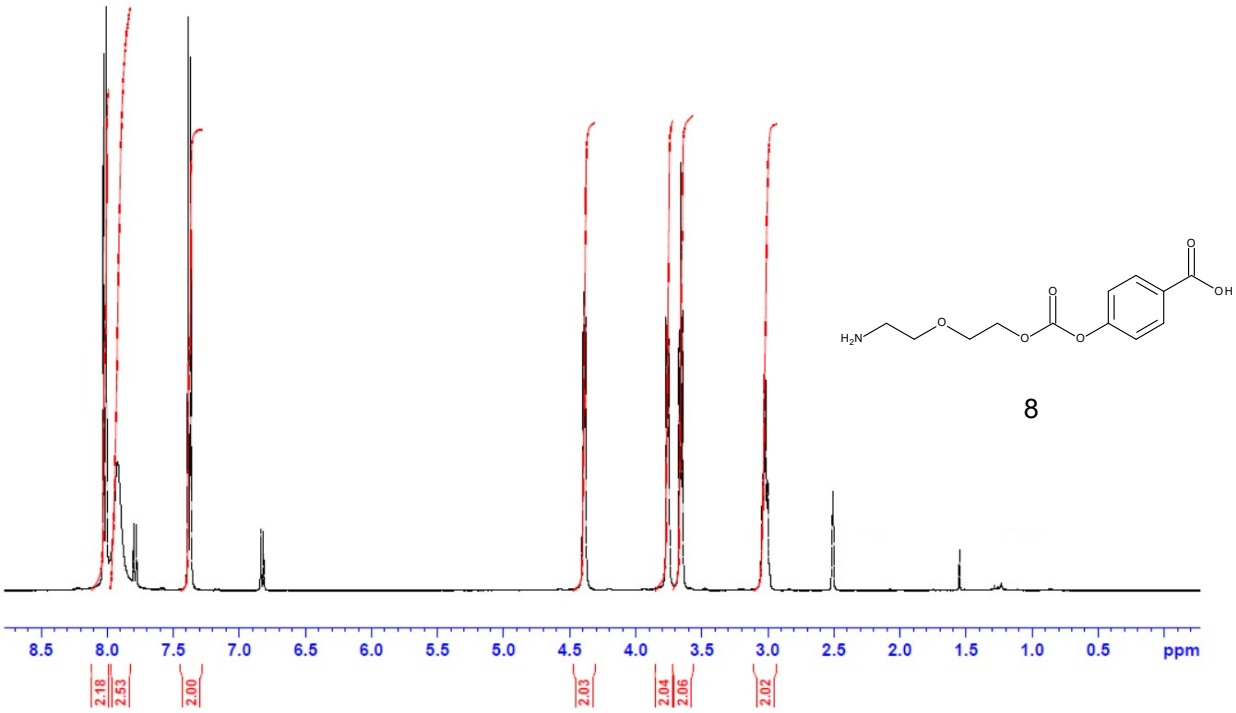
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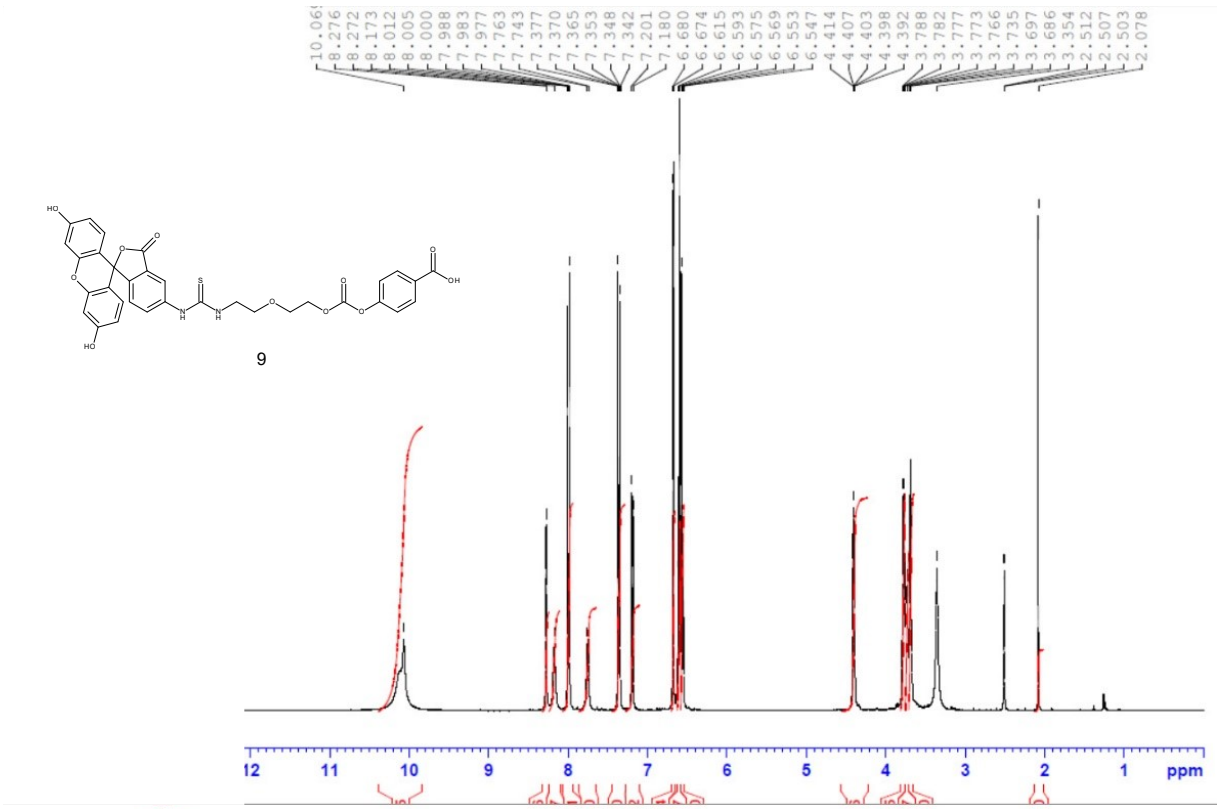
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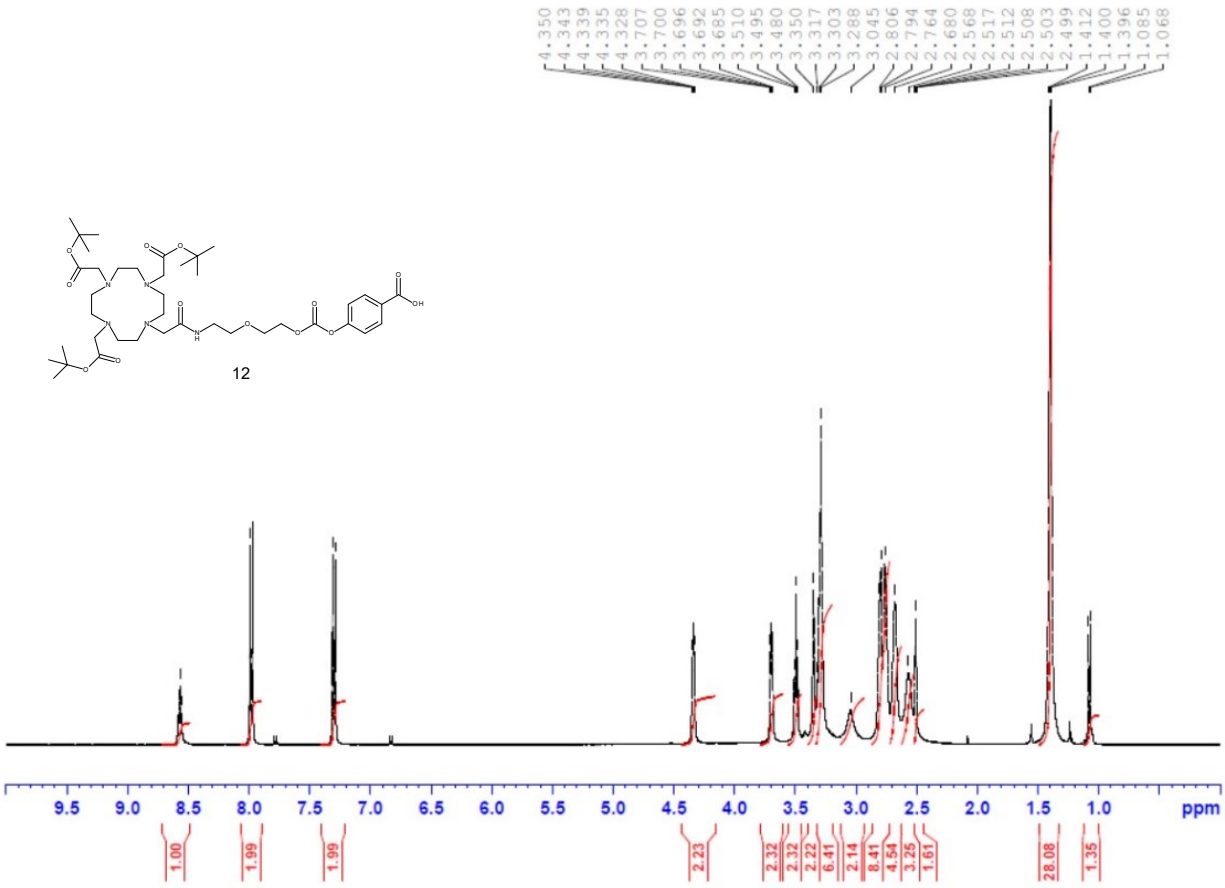


General procedure for the synthesis of payload-PEG$_n$-Fc-III reactive conjugates

Fluorescein-carbonate **9** or $(tBu)_3$DOTA-carbonate **12** (1.2 eq) was dissolved in DMF at a concentration of 1.5mM and and stirred with 1.1 eq. HATU (0.54 M in DMF) for 1min, followed by the addition of 2 eq. of DIEA (2 M in DMF). After 3 minutes, the pre-activated Fluorescein- or $(tBu)_3$DOTA-carbonate was added to a solution of peptide (1 eq., 0.03 M) in DMF and the mixture was stirred for 2-4 h at RT. The reaction was monitored by UPLC-MS. If the reaction was not complete, an additional amount of pre-activated payload-carbonate (1-3 eq.) was added and stirred further for 1-2 h. The reaction solution was precipitated with cold diethyl ether and purified by HPLC.

tBu deprotection. A cleavage cocktail consisting of TFA/TIS/Water 95/2.5/2.5 was added to the pellet of $(tBu)_3$DOTA-carbonate-Fc-III. The reaction mixture was stirring for 2.5h at RT, followed by precipitation with cold diethyl ether.

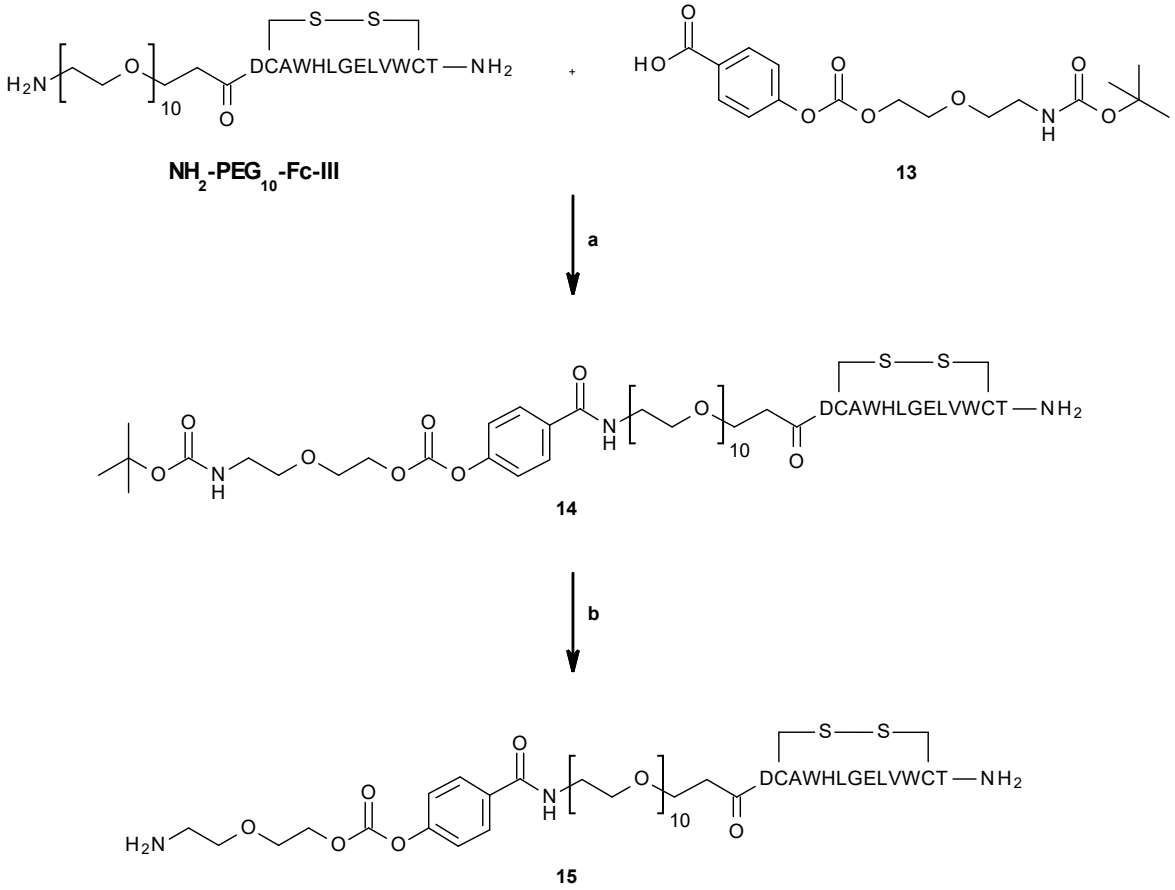
Purification. The purification of peptide conjugates was carried out by Preparative Reversed Phase-HPLC (Waters) on a Kinetex 5 µm C18 100 Å, LC Column 150 x 10.0 mm column (Phenomenex Helvetia) by applying a 15%–55% gradient of solvent B, at a flow rate

of 8 mL/min for 25 min. The peptide signal was monitored at 214 nm. Fractions were analyzed by UPLC-MS prior to lyophilization.

Table S2. Characteristics of payload-PEG_n-Fc-III peptides and its derivatives.

Compound	Calculated Exact Mass	Calculated $[M + H]^+$	Observed $[M + H]^+$	Retention time, min	Purity
Fl-PEG_{20}-Fc-III	3122.91	1041.97	1041.91	2.17	93%
DOTA-PEG_0-Fc-III	2166.73	1084.36	1084.94	1.69	95%
DOTA-PEG_2-Fc-III	2326.13	1164.06	1164.27	1.70	92%
DOTA-PEG_4-Fc-III	2414.27	1208.13	1208.09	1.72	94%
DOTA-PEG_6-Fc-III	2502.38	1252.19	1252.49	1.73	94%
DOTA-PEG_8-Fc-III	2590.53	864.51	865.12	1.74	91%
DOTA-PEG_{10}-Fc-III	2678.63	893.88	893.64	1.75	86%
DOTA-PEG_{12}-Fc-III	2766.73	923.24	923.07	1.77	91%
DOTA-PEG_{20}-Fc-III	3119.91	1040.97	1040.69	1.81	94%
DOTA-PEG_{36}-Fc-III	3824.03	1275.68	1275.55	1.88	> 95%
Fl-PEG_{20}-Fc-III-V10W-W11A	3094.86	1032.62	1033.23	2.58	> 95%
Fl-PEG_{20}-Fc-III-C2A-C12A	3060.83	1021.28	1021.22	2.21	> 95%

Synthesis of NH_2-carbonate-PEG_{10}-Fc-III (S11)



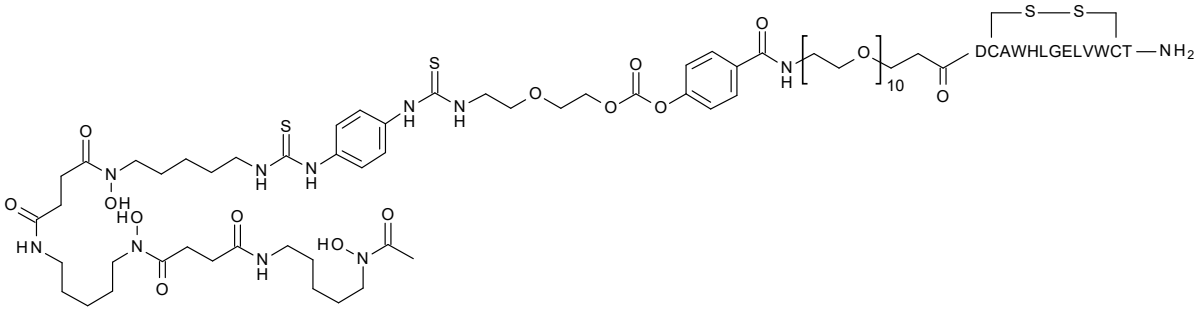
Scheme S2. Synthesis of NH_2-carbonate-PEG_{10}-Fc-III trifluoroacetate (**15**).

a) DIEA was added to a solution of 4-[2-[2-(tert-butoxycarbonylamino)ethoxy]ethoxycarbonyloxy]benzoic acid **13** (2.35 mg, 6.4 µmol, 1.3 eq.) in DMF (0.65 mL) at rt. After stirring at rt for 1 min, HATU (2.81 mg, 5.4 µmol, 1.1 eq.) was added to the reaction mixture. After stirring at rt for 3 min, a solution of NH_2-PEG_{10}-Fc-III (10.0 mg, 4.9 µmol, 1.0 eq.) in DMF (0.65 mL) was added to the reaction mixture. After stirring at rt for 18 h, 2 drops of a 0.1% TFA (aq) was added. Purification by C_{18} reverse phased chromatography (12 g, gradient of 30 to 70% ACN (aq) with 0.1% TFA over 12 CV) afforded Boc-HN-carbonate-PEG_{10}-Fc-III **14** (2.4 mg, 1.0 µmol, purity 95%, yield: 20%) as a white powder after freeze-drying. UPLC-MS: m/z = 1147 $[M\text{-Boc}+2H]^{2+}$, 1195 $[M\text{-}2H]^{2-}$.

b) 0.5 mL of a TFA/DCM solution (1:1) was added to Boc-HN-carbonate-PEG_{10}-Fc-III **14** (23.9 mg, 8.3 µmol, 1.0 eq.) The reaction mixture was stirred at rt for 1.5 h and concentrated *in*

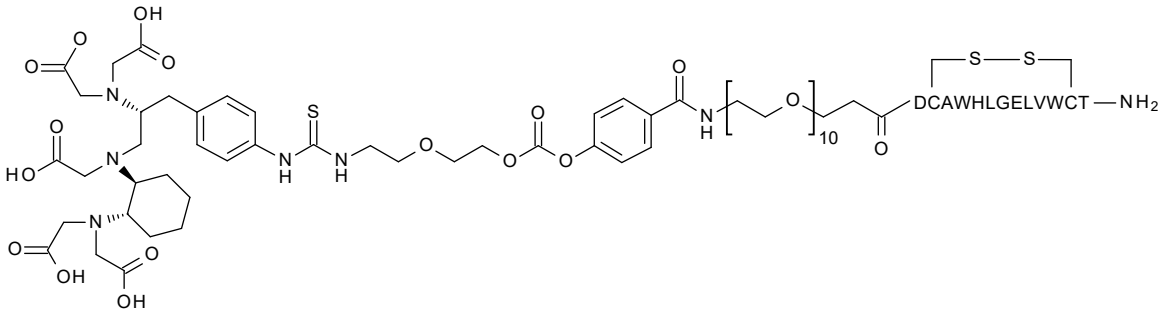
vacuo. A mixture of ACN/Water (1:1, 5 mL) was added and the mixture was freeze dried to yield the trifluoroacetate salt of H_2N-carbonate-PEG_{10}-Fc-III **15** (23.7 mg, 8.3 µmol, purity 99%, quantitative yield) as a white powder. UPLC-MS: m/z = 1147 $[M+2H]^{2+}$, 1145 $[M-2H]^{2-}$.

Synthesis of DFO-carbonate-PEG_{10}-Fc-III



DIEA (10 µL, 80.0 µmol, 16.0 eq.) was added to a solution of NH_2-carbonate-PEG_{10}-Fc-III **15** (12.42 mg, 4.9 µmol, 1.0 eq.) and DFO-NHS (8.2 mg, 5.9 µmol, 1.2 eq.) in DMF (0.4 mL) at rt. After stirring at rt for 3.5 h, ACN/water/TFA (1:1:0.5%, 0.2 mL) was added and the reaction mixture was stirred at rt for 5 min. Purification by preparative HPLC (gradient of 25 to 60% ACN (aq) with 0.1%TFA) afforded DFO-carbonate-PEG_{10}-Fc-III (1.6 mg, 0.45 µmol, purity 86%, yield: 9%) as a white powder after freeze-drying. UPLC-MS: m/z = 1524 $[M+2H]^{2+}$.

Synthesis of DTPA-carbonate-PEG_{10}-Fc-III



p-SCN-Bn-CHX-A"-DTPA.3HCl (4.47 mg, 6.0 µmol, 1.0 eq.) was added to a solution of NH_2-carbonate-PEG_{10}-Fc-III **15** (14.35 mg, 6.0 µmol, 1.0 eq.) in DMF (0.3 mL) at rt. The reaction mixture was stirred at rt for 5 min then triethylamine (4.0 µL, 30.0 µmol, 5.0 eq.) was added. After stirring at rt for 36 h, *p*-SCN-Bn-CHX-A"-DTPA.3HCl (0.90 mg, 1.2 µmol, 0.2 eq.) and triethylamine (0.5 µL, 3.6 µmol, 0.6 eq.) were added and the reaction mixture was stirred at rt

for 18 h. Purification by preparative HPLC (gradient of 30 to 60% ACN (aq) with 0.1% TFA) afforded DTPA-carbonate-PEG_{10}-Fc-III (1.4 mg, 0.52 µmol, purity 95%, yield: 8.7%) as white powder after freeze-drying. UPLC-MS: m/z = 1444.1 $[M+2H]^{2+}$.

Antibody conjugation

Effective local concentration of the chemical reactive site

The effective local concentration was estimated as it follows: if R is the length of the spacer, the reactive chemical site is located within a volume $V = \frac{4}{3} \times \pi \times r^3$. Both the chemical reactive site and the nucleophilic lysine are located within this volume. Consequently, the effective local concentration C_{eff} can be estimated to be $C_{eff} = \frac{\frac{1}{N_A\,(mol^{-1})}}{\frac{4}{3} \times \pi \times r^3(dm^3)}$, C is the molar concentration and N_A the Avogadro number. As a result, C_{eff} = 23mM and 81 mM for a distance of 26 Å and 17 Å to Lys 317, respectively. Given the WLC model, which estimates a distance of 23.8 Å for PEG_{20}, C_{eff} is of 29 mM for this spacer.

General procedure for the preparation of antibody conjugates in solution

Before the conjugation step, the antibody buffer was exchanged with phosphate-buffered saline (PBS, pH 7.4) using a Vivaspin® 500 centrifuge filter (30 kDa MWCO, 0.5 mL volume), and the antibody concentration was measured using an UV plate reader Infinite® 200 PRO. 2 eq. of Fc-III reactive conjugate (1.62 nmol, 0.86 µl) in DMF was added to a solution of antibody (1 eq., 0.81 nmol) in 0.2 M $NaHCO_3$ pH 9.0 and the reaction mixture (24 µl) was stirred for 2 h at RT.

Peptide release from antibody conjugates

Following the payload conjugation to the antibody, the reaction mixture was diluted to 500 µl with 0.1M glycine pH 2.5 and then exchanged 3 times with the same buffer using a 30 kDa MWCO Vivaspin® 500 centrifugal concentrator. The antibody conjugate was then purified by gel filtration chromatography using a pre-equilibrated Bio-spin P-30 Column (bed height: 3.7

cm; overall length: 5 cm, Bio-Rad, Hercules, U.S.A.) and then eluted with 100 µl of 0.1M glycine pH 2.5. The purified antibody conjugate fractions were neutralized with 10 µl of 1M PBS pH 8.5. The buffer was exchanged for PBS (pH 7.4) using a Vivaspin® 500 centrifugal concentrator and then analyzed by SDS PAGE and HRMS. Typically the conjugation yield was more than 80%, in case of the DTPA-trastuzumab it was calculated to be of 89%,

Enzymatic cleavage of antibody conjugates

Cleavage of antibody conjugates into Fab and Fc fragments was achieved by digestion with FabALACTICA protease (50 units per 50 µg of antibody conjugate in 150 mM phosphate buffer, pH 7.0, for 16-18 hours at 37 °C) or GingisKHAN protease (50 units per 50 µg of antibody conjugate in 0.1 M Tris pH 8.0 in the presence of 2.8 µl of GingisKHAN reducing agent in a total volume of 28 µl, for 1 hour at 37 °C). The cleavage of antibody conjugates into $F(ab)_2$ and Fc/2 fragments was achieved by digestion with FabRICATOR from Genovis (67 units per 50 µg of antibody conjugates in PBS, pH 7.4 for 30 min at 37 °C).

Samples preparation for LC-FTMS analysis

Prior to LC-FTMS experiments, antibody conjugates were desalted against 50 mM ammonium acetate solution buffered at pH 7.0 using four cycles of concentration/dilution on micro-concentrators (Vivaspin, 30 kD cutoff, Sartorius, Gottingen, Germany). The deglycosylation of antibody conjugates was achieved by incubating units 200 units of Endo S with 50 µg of antibody conjugate (10 µl) with 1.2 µl of the formulation buffer in a total volume of 12.2 µl for 1h at 37°C.

Table S3. Results of intact mass LC-FTMS analysis for trastuzumab-DOTA conjugate cleaved with different enzymes.

Peptide conjugate	Enzyme	DoC mAb	Labeled mAb	Selectivity Fc/F(ab)$_2$	DoC Fc	DoC F(ab)$_2$
DOTA-PEG$_{20}$-Fc-III	EndoS™	1.09	71%			
	GingisKHAN™			3.2	0.74	0.23
	FabALACTICA™			3.8	0.77	0.20

Table S4. Impact of different PEG length on the intact mass LC-FTMS results of trastuzumab-DOTA conjugate.

Peptide conjugate	Spacer length	DoC mAb	Labeled mAb	Selectivity Fc/F(ab)$_2$	DoC Fc	DoC F(ab)$_2$
DOTA-Fc-III	-	0.53	43	0.1	0.03	0.37
DOTA-PEG$_2$-Fc-III	~8.9 Å	0.39	33	0.5	0.07	0.16
DOTA-PEG$_4$-Fc-III	~11.7 Å	0.47	39	0.7	0.12	0.17
DOTA-PEG$_6$-Fc-III	~13.7 Å	0.68	51	1.7	0.29	0.17
DOTA-PEG$_8$-Fc-III	~16.0 Å	0.98	66	2.9	0.55	0.19
DOTA-PEG$_{10}$-Fc-III	~17.4 Å	1.20	72	4.1	0.90	0.22
DOTA-PEG$_{12}$-Fc-III	~18.9 Å	1.31	77	3.5	0.73	0.21
DOTA-PEG$_{20}$-Fc-III	~23.8 Å	1.09	71	3.2	0.74	0.23
DOTA-PEG$_{36}$-Fc-III	~32.3 Å	1.31	77	2.8	0.90	0.32

Samples preparation for middle-down analysis

Trastuzumab-DOTA conjugates were digested into F(ab')$_2$ and Fc/2 subunits with FabRICATOR™ from Genovis (67 units per 50 µg of antibody conjugates in PBS, pH 7.4 for 30 min at 37 °C). TCEP was then added to 30 mM final concentration and reaction mixture was incubated for 30 min at 50 °C to achieve rapid complete reduction of all disulfide bonds for Lc, Fd, and Fc/2 subunits.

High-resolution mass spectrometry analysis

Direct injection HRMS for peptide analysis was performed on a QExactive HF-HT-Orbitrap-FT-MS instrument, (Thermo Fisher Scientific, Bremen, Germany) coupled to an automated

chip-based nanoelectrospray device (Triversa Nanomate, Advion, Ithaca, U.S.A.). Electrospray ionization was conducted at a capillary voltage of 1.4 kV and nitrogen nanoflow of 0.15 psi. MS experiments were performed with a nominal resolution of 45000 and in the positive ion mode. Data deconvolution was performed with Protein Deconvolution (Thermo Fischer Scientific, Sunnyvale, CA, U.S.A.) using the Xtract algorithm with a 90% fit factor.

LC-FTMS and middle-down analysis

For both intact mass measurement (LC-MS) and middle-down analysis (LC-FT-MS/MS, conjugation site localization), samples were separated onto an Acquity UPLC Protein column BEH C4 (300 Å, 1.7 µm, 1 x 150 mm, Waters, Milford, MA, U.S.A.) using a Dionex Ultimate 3000 analytical RSLC system (Dionex, Germering, Germany) coupled to a HESI source (Thermo Fisher Scientific, Bremen, Germany). The separation was performed with a flow rate of 90 µl/min by applying a gradient of solvent B from 15 to 25 % in 2 min, then from 25 to 40 % within 12 or 20 min, followed by column washing and re-equilibration steps. Solvent A was composed of water with 0.1 % formic acid, while solvent B consisted of acetonitrile with 0.1 % TFA.

Eluting proteoforms were analyzed on a QExactive HF-HT-Orbitrap-FT-MS benchtop instrument (Thermo Fisher Scientific, Bremen, Germany). For intact mass measurements MS1, the scan was performed in protein mode with 15 000 resolution and averaging 10 µscans. Middle-down analysis (MS/MS) for localization of conjugation site was performed in PRM mode isolating species at 950 m/z for Fc/2-DOTA, with 300 Th isolation window, using 240 000 resolution and averaging 10 µscans. HCD (higher energy collision-induced dissociation) was used as a fragmentation method with a normalized collision energy (NCE) of 10, 12, 15 and 17-8 %. Each NCE analysis was performed in triplicates.

Intact mass measurement data were analyzed with Protein Deconvolution (Thermo Fischer Scientific, Sunnyvale, CA, U.S.A.) using a Respect algorithm with 99% noise rejection confidence and 20 ppm accuracy of average mass identification. Middle-down data were grouped by used NCE values for Fc/2-DOTA proteoform and spectral averaging was performed across the elution peak of this proteoform as well as across NCE replicates using Peak-by-Peak software (Spectroswiss, Lausanne, Switzerland). The resulting averaged fragmentation mass spectra were deconvolved using Deconvolution option of Peak-by-Peak software (Spectroswiss, Lausanne, Switzerland). Data obtained with 4 different NCE values were combined together to create a fragmentation map with assigned b- and y-fragment ions using

ProSight Lite software (Kelleher research group, Northwestern University) with 10-5 ppm mass accuracy tolerance.

Average degree of conjugation (DoC) calculation from LC-FTMS analysis

The average DoC values were calculated using the intact mass LC-FTMS data with Eq. 1. These results were derived from the relative peak intensities in deconvoluted mass spectra.

$$DoC = \frac{\sum_{k=0}^{k=n} k \times I(DoC_k)}{\sum_{k=0}^{k=n} I(DoC_k)}$$

where I(DoCk) is relative peak intensity of conjugates with k add-on molecules per antibody.

Conjugation site identification of trastuzumab-DOTA by LC-MS/MS-based peptide mapping

This experiment was performed by M-Scan (Geneva, Switzerland). A 500 µg sample aliquot was subjected to EndoS treatment at room temperature for 15 min using deGlycIT™ Microspin column (Genovis), following supplier's protocol. An aliquot of de-N-glycosylated sample solution equivalent to 100 µg protein was buffer-exchanged against freshly prepared 6 M guanidine hydrochloride, 25 mM ammonium bicarbonate solution using Zeba spin desalting columns (0.5 ml, 7K MWCO). The buffer-exchanged sample solution was reduced with 5 mM TCEP for 1 hour at 60°C. The reduced sample solution was alkylated with 15 mM iodoacetamide (IAA) for 30 min at room temperature and protected from light. The excess of IAA was then quenched through the addition of 10 mM DTT. The alkylated sample solution was buffer exchanged against 25 mM ammonium bicarbonate using Zeba spin desalting columns (0.5 ml, 7K MWCO). The buffer-exchanged sample was digested using a trypsin/Lys-C mixture for 4 hours at 37°C under 300 rpm shaking (weight-to-weight trypsin/Lys-C mixture/substrate ratio of 1/50). The enzymatic digestion was quenched using 2% formic acid. The UPLC-UV-MS^E analysis were performed using a Waters Acquity UPLC H-Class integrated system coupled to a Waters Synapt G2-Si HDMS Q-TOF mass spectrometer. Mass accuracy was better than 5 ppm for the major m/z signals observed prior to sample analysis. In addition, Leu-Enkephaline solution was regularly sprayed into the source of the instrument to allow real time mass correction during the acquisition (Lockspray). Aliquots of sample

solutions were injected on a C18 reversed phase column connected to the source of the mass spectrometer and analyzed using the conditions described below.

The samples were separated on an Acquity UPLC column BEH C18 (1.7 µm, 100 mm x 2.1 mm, Waters, Milford, MA, U.S.A.) using a flow rate of 0.4 ml/min by applying a gradient of solvent A 100% for 3 min, solvent B from 0 to 50 % in 50 min, followed by column washing and re-equilibration steps. Solvent A was composed of water with 0.05 % formic acid, while solvent B consisted of 90% acetonitrile/10% water (v:v) with 0.05 % formic acid.

UPLC-UV-MS^E data were acquired and processed using MassLynx™ software version 4.1 (Waters). The protein sequence supplied by the Study Sponsor was used for raw data interpretation which was supported by both BiopharmaLynx™ software version 1.3.4 (Waters) and BioLynx™ software supplied with the current version of MassLynx™. Briefly, the raw data were first processed using the BiopharmaLynx™ software by considering DOTA conjugation on Lysine residues as a possible modification (working hypothesis). Signals assigned to DOTA-modified peptides were retained for manual verification to confirm or reject software proposed assignments. To this end, a reporting threshold of 1% of the major assigned signal intensity was considered for manual verification, which was carried through examination of both MS and MS^E fragmentation data.

Following manual verification of software-proposed assignments, HC302-320 (heavy chain residue 302 to 320) peptide, belonging to the Fc region, was found conjugated to a DOTA payload. No conjugated peptides belonging to the Fab region could be reliably identified. Among Lysine residues contained in peptide HC302-320, discriminating fragment ions observed in MS^E data suggest that DOTA conjugation is carried by K317 (Figure S15).

Radiolabeling

Radiolabeling of CHX-A”-DTPA -trastuzumab with Indium-111

200 µg of CHX-A”-DTPA -trastuzumab (7.6 mg/mL) and 120 MBq of ^{111}In (Indium chloride (In-111) solution, 370 MBq/ml, in 0.02 M HCl, Mallinckrodt Medical, Le Petten, Netherlands) were mixed in a NH_4OAc aqueous buffer (0.4 M, pH 5.5, 150 µL) at 37 °C for 1 h. [^{111}In]In-DTPA-trastuzumab was then used without further purification; radiochemical purity was tested by radio-SEC and iTLC as described below.

Radio-SEC

Radio-SEC analysis was performed using an Ultimate 3000 SD System (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a GabiStar detector (Raytest, Straubenhard, Germany). 5 µg of [^{111}In]In-DTPA-trastuzumab was loaded and separated with a size exclusion column, XBridge protein BEH 200 Å SEC 3.5 µm, dimension 7.8 × 300 mm (Waters, Baden-Dättwil, Switzerland). Elution was performed using a phosphate buffer saline (PBS) at pH 6.8 (1 mL/min) as mobile phase and was monitored via absorbance at 220/280 nm and γ detection.

iTLC

iTLC analysis was performed using a dried iTLC-SG Glass microfiber chromatography paper impregnated with silica gel (Agilent Technologies, Folsom, CA, USA) dipped in citrate buffer (0.1 M, pH 5.0) as a mobile phase. Under these conditions, [^{111}In]In-DTPA-trastuzumab didn't migrate while unbound ^{111}In migrated with the solvent front. The radioactivity was measured with a miniGITA scanning device (Raytest, Straubenhard, Germany).

In vitro and *in vivo* characterization

Stability in human serum

Immediately after radiolabeling, [^{111}In]In-DTPA-trastuzumab was added to 1 mL of human serum and incubated at 37°C under gentle shaking (350 rpm). Sample aliquots were taken after 24, 48, 72 and 144 hours of incubation, and analyzed by iTLC and radio-SEC as described above.

Cell lines and tumor models

The human ovarian adenocarcinoma SK-OV-3 (HER2 high expression) and human breast adenocarcinoma MDA-MB-231 (HER2 low expression) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

Both cell lines were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA,

USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Xenografts of SK-OV-3 or MDA-MB-231 tumors were established by subcutaneous injection of 3.5×10^6 cells in the right shoulder of 6 – 8 weeks old female BALB/c Nude mice (CAnN.Cg-*Foxn1*^{nu}/Crl, Charles River Laboratories, Wilmington, MA, USA). All animal experiments were performed in accordance with the Swiss legislation for the care and use of laboratory animals under the license VD-2993 (09/2018) delivered after approbation by the Veterinarian Office of the canton of Vaud and the ethics committee.

Radio-immunoreactive fraction

The immunoreactive fraction of radiolabeled DTPA-trastuzumab was tested by incubating a constant amount of [^{111}In]In-DTPA-trastuzumab (15 ng/mL) with increasing numbers (0.25 – 8×10^6) of SK-OV-3 or MDA-MB-231 cells in PBS containing 0.5% Bovine Serum Albumin (PBS/BSA) for 3 h at 37 °C on a shaking platform. Non-specific binding was assessed by adding an excess of non-radiolabeled DTPA-trastuzumab (100-fold excess).

The cells were centrifuged at 1000 rpm to remove unbound conjugate, and then washed twice with PBS/BSA. The radioactivity of the cell bound conjugate was measured with a gamma counter (AMG Automatic Gamma Counter, Hidex, Turku, Finland). The binding curve was extrapolated to an infinite number of cells by nonlinear regression to calculate the theoretical maximum of cell-bound conjugate (Bmax) using Graphpad Prism 7 (GraphPad Software, San Diego, CA, USA).

[^{111}In]In-DTPA-trastuzumab biodistribution study

[^{111}In]In-DTPA-trastuzumab (20 kBq corresponding to 0.05 µg [^{111}In]In-DTPA-trastuzumab completed with 49.95 µg of cold trastuzumab in 100 µl of 0.9% NaCl injectable saline solution) was injected into the lateral tail vein of mice bearing SK-OV-3 or MDA-MB-231 xenografts without anesthesia and sterile filtration. Additionally, the HER2 specificity of [^{111}In]In-DTPA-trastuzumab in SK-OV-3 bearing mice was assessed in a competition experiment whereby cold DTPA-trastuzumab was coinjected in excess (2 mg).

At different time points (2, 72 and 144 h for SK-OV-3 bearing mice, 72 h for MDA-MB-231 bearing mice and 72 h for inhibition by competition in SK-OV-3 bearing mice) post injection, mice were euthanized by CO_2 inhalation and exsanguinated. Blood was collected, organs and tumors were removed, weighed, and the radioactivity was measured with a gamma counter

(AMG Automatic Gamma Counter, Hidex, Turku, Finland). The results shown in Table 2 are expressed as the percentage of injected activity per gram of organs (%IA/g) ± standard deviation (n = 3).

[^{111}In]In-DTPA-trastuzumab SPECT imaging study

SPECT/CT images were acquired using an Albira Si PET/SPECT/CT (Bruker Biospin Corporation, Woodbridge, CT, USA). 18 MBq of [^{111}In]In-DTPA-trastuzumab (corresponding to 26 µg [^{111}In]In-DTPA-trastuzumab and 24 µg of cold trastuzumab) was injected to mice bearing SK-OV-3 xenografts, and SPECT/CT images were acquired at 3, 24, 48, 72 and 144 h post injection. Mice were anesthetized for the duration of the imaging experiments by inhalation of 1.5% isoflurane/O_2 and placed on a heated bed (30 – 35 ºC).

For SPECT imaging, static acquisitions of 1 to 3 h (accumulation of 1 – 2 x 10^6 events) were acquired with the following parameters: photopeak at 171 keV ± 30%, axial FOV 82.5 mm using a single pinhole collimator. The reconstruction algorithm used was an ordered subset expectation-maximization algorithm (OSEM) with two iterations and corrected for scatter. For the CT the following parameters were used: 400 µA intensity and 35 kV voltage, 600 projections, and images were reconstructed using a filtered back-projection (FBP) algorithm with de-ringing correction. Fused representative SPECT/CT images were acquired using the PMOD software (PMOD technologies, version 3.709, Zurich, Switzerland).

Figures

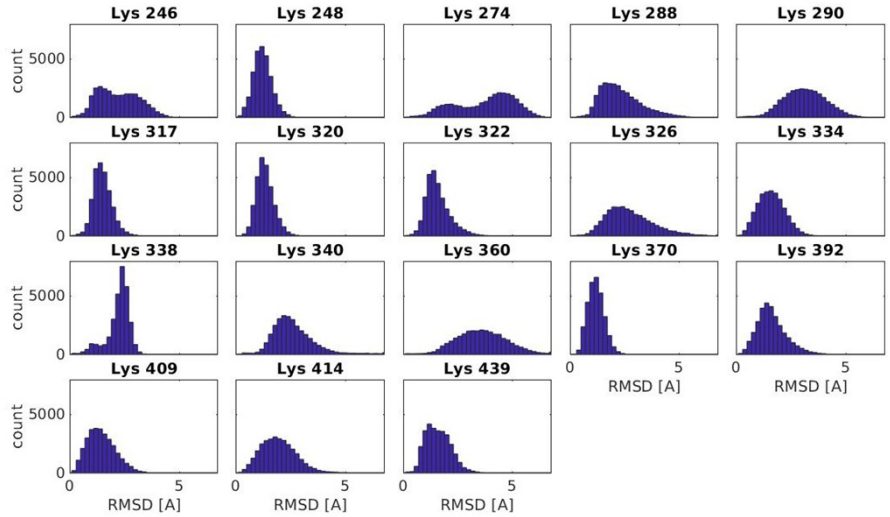


Figure S1. RMSD lysines peaked distributions around the X-ray structure of the Fc_III/ IgG bound complex (PDB accession number, 1DN2). Histograms of per-residue RMSD values were calculated after pooling all 6 protomers together.

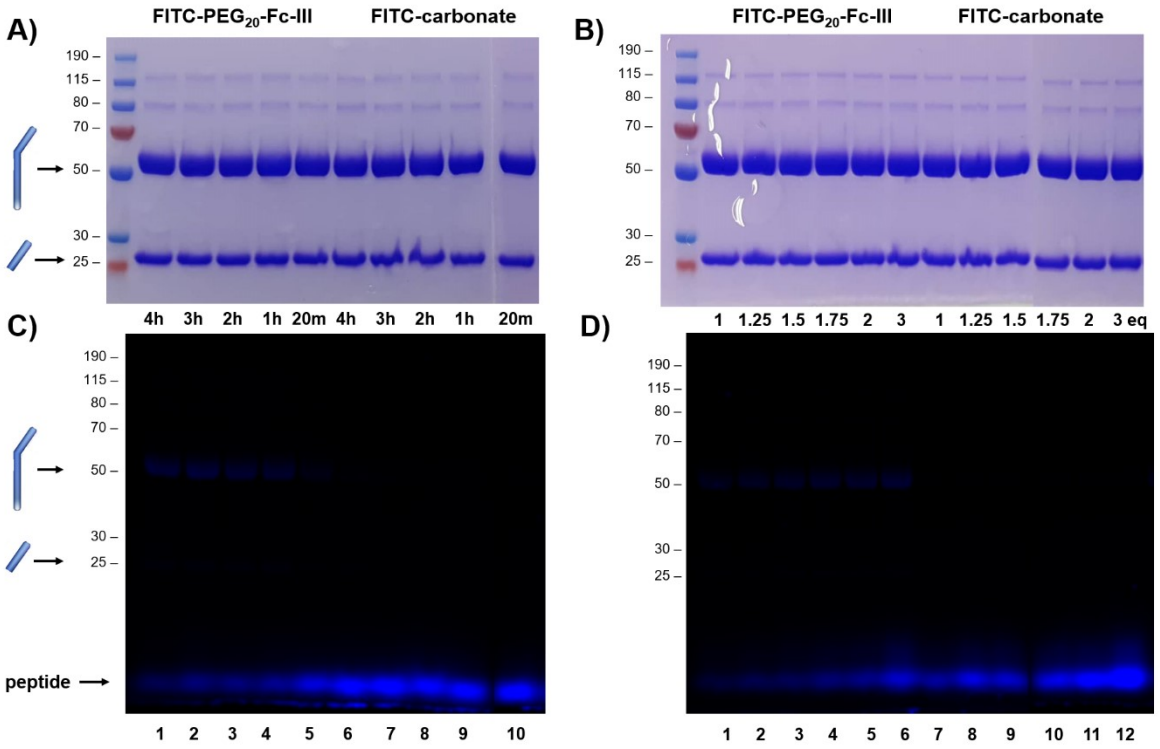


Figure S2. Optimization of the antibody labeling conditions. The incubation time (A, C) and peptide equivalents (B, D) were varied during the conjugation reaction between trastuzumab and FITC-PEG_{20}-Fc-III. Sample aliquots were reduced and analyzed by SDS gel electrophoresis (top panel – Coomassie blue staining, bottom panel – fluorescence imaging).

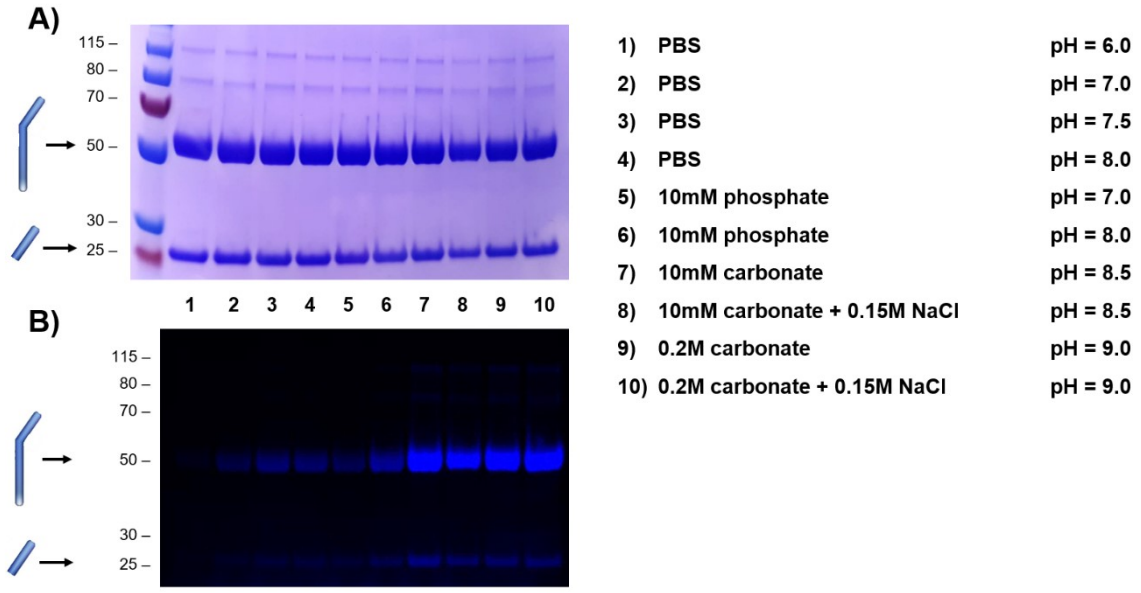


Figure S3. Conjugation of FITC-PEG_{20}-Fc-III to trastuzumab in different buffers (pH 6.0 - 9.0) by SDS gel electrophoresis. Final conjugates were reduced and analyzed using Coomassie blue staining (A) and fluorescence (B).

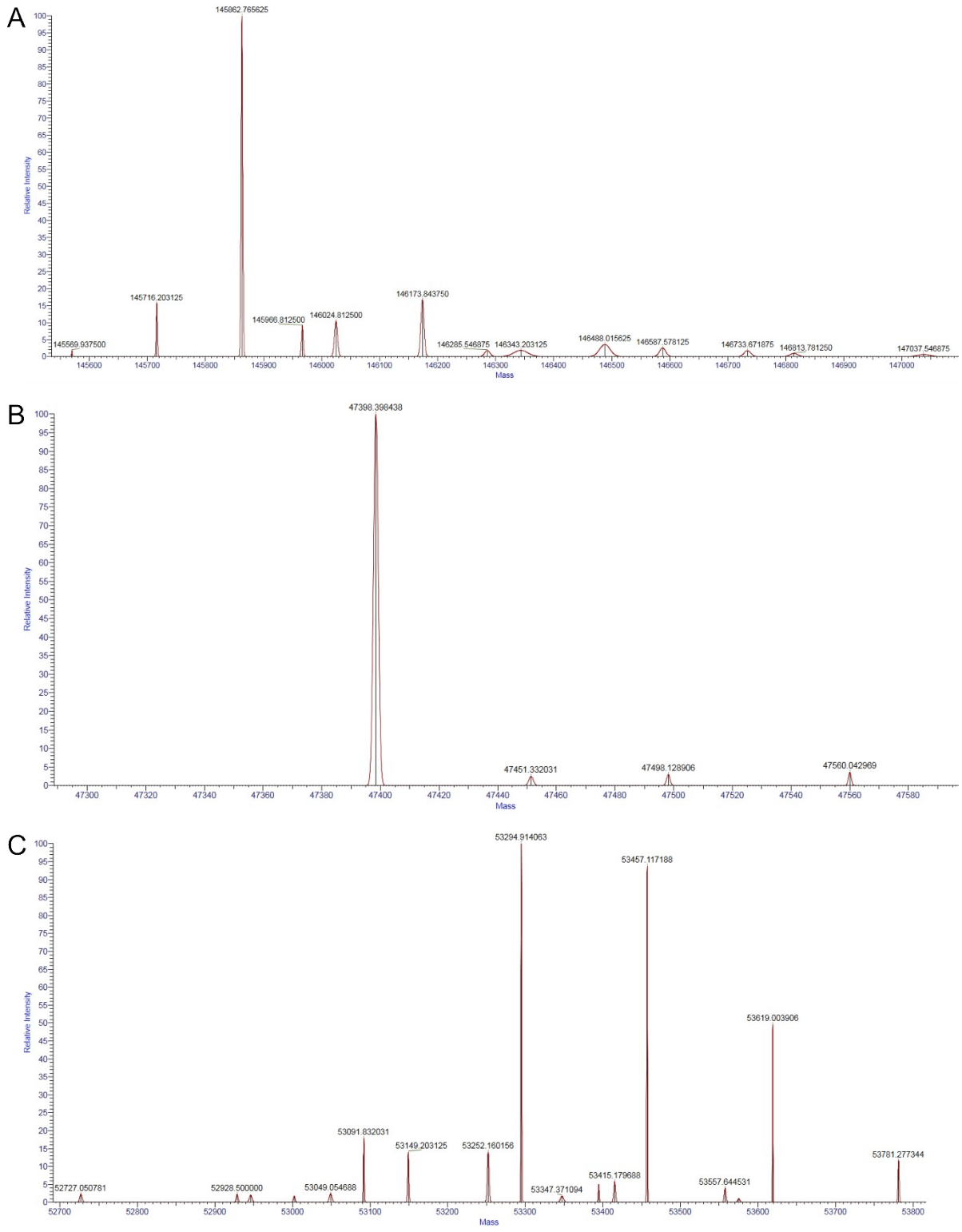


Figure S4. Deconvolved intact mass spectra of trastuzumab: deglycosylated (A); glycosylated trastuzumab cleaved into Fab (B) and Fc (C).

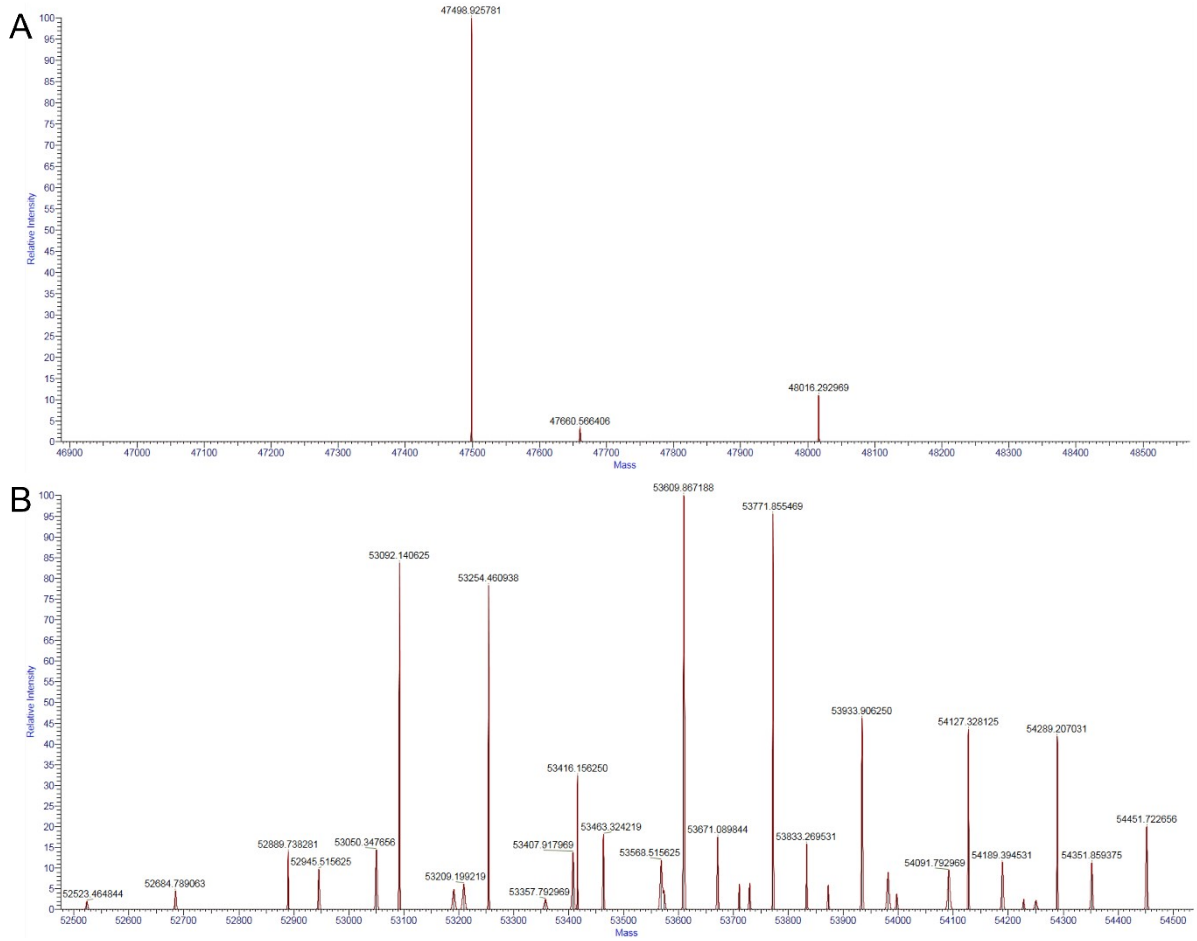


Figure S5. Deconvolved intact mass spectra of trastuzumab-DOTA cleaved into Fab (A) and Fc (B) with FabALACTICA^{TM} enzyme (after the reaction of DOTA-PEG$_{20}$-Fc-III with trastuzumab).

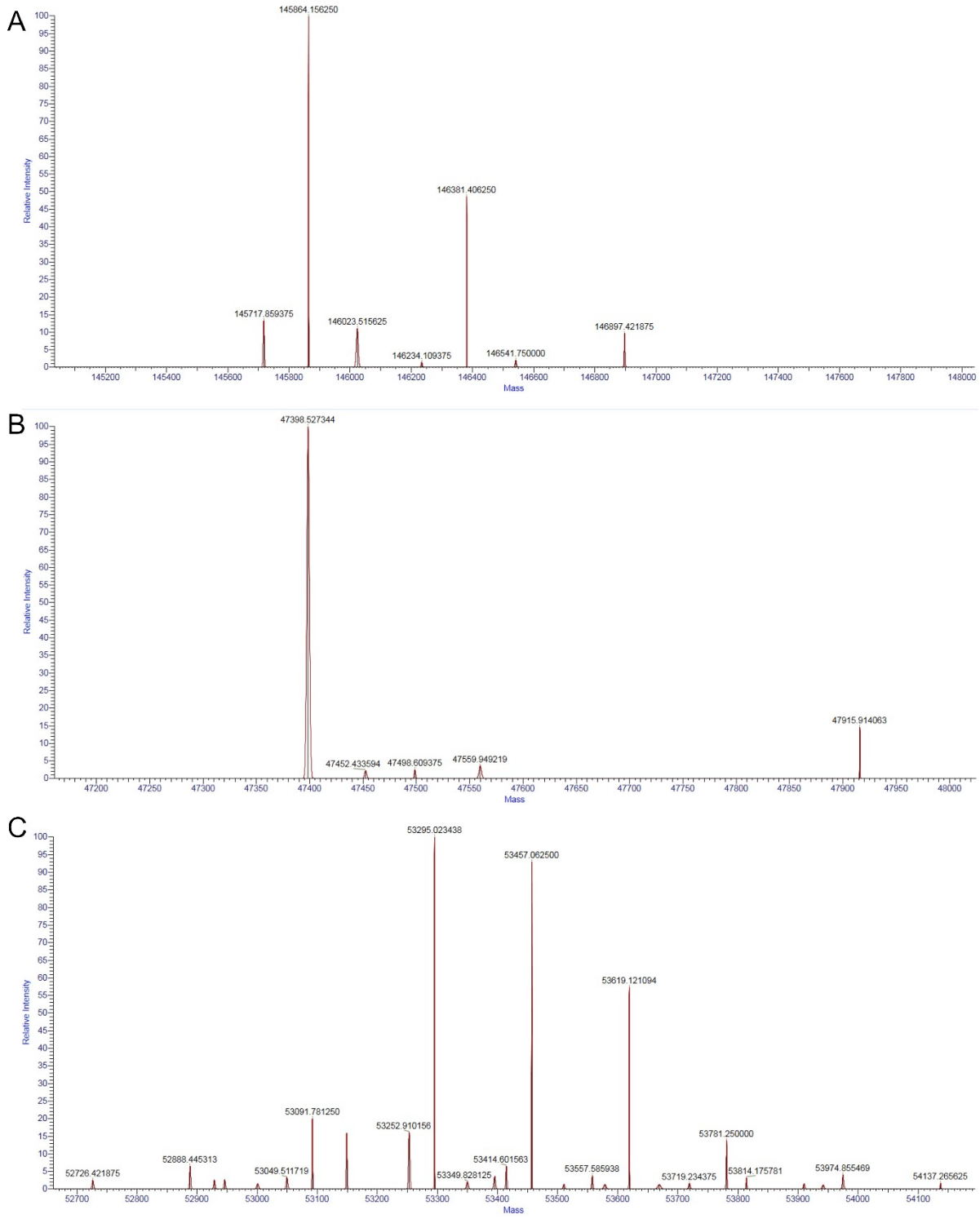


Figure S6. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

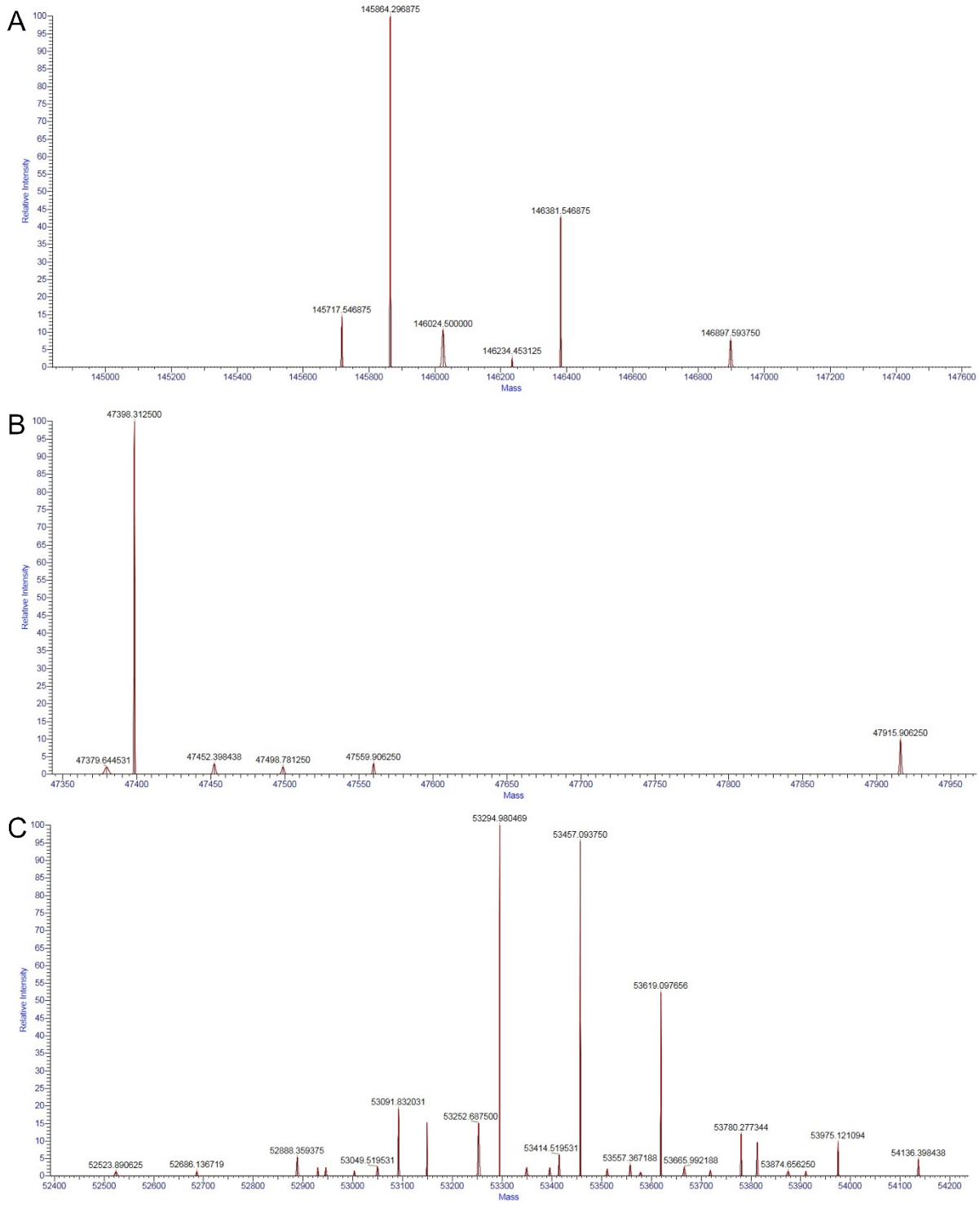


Figure S7. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG_2-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

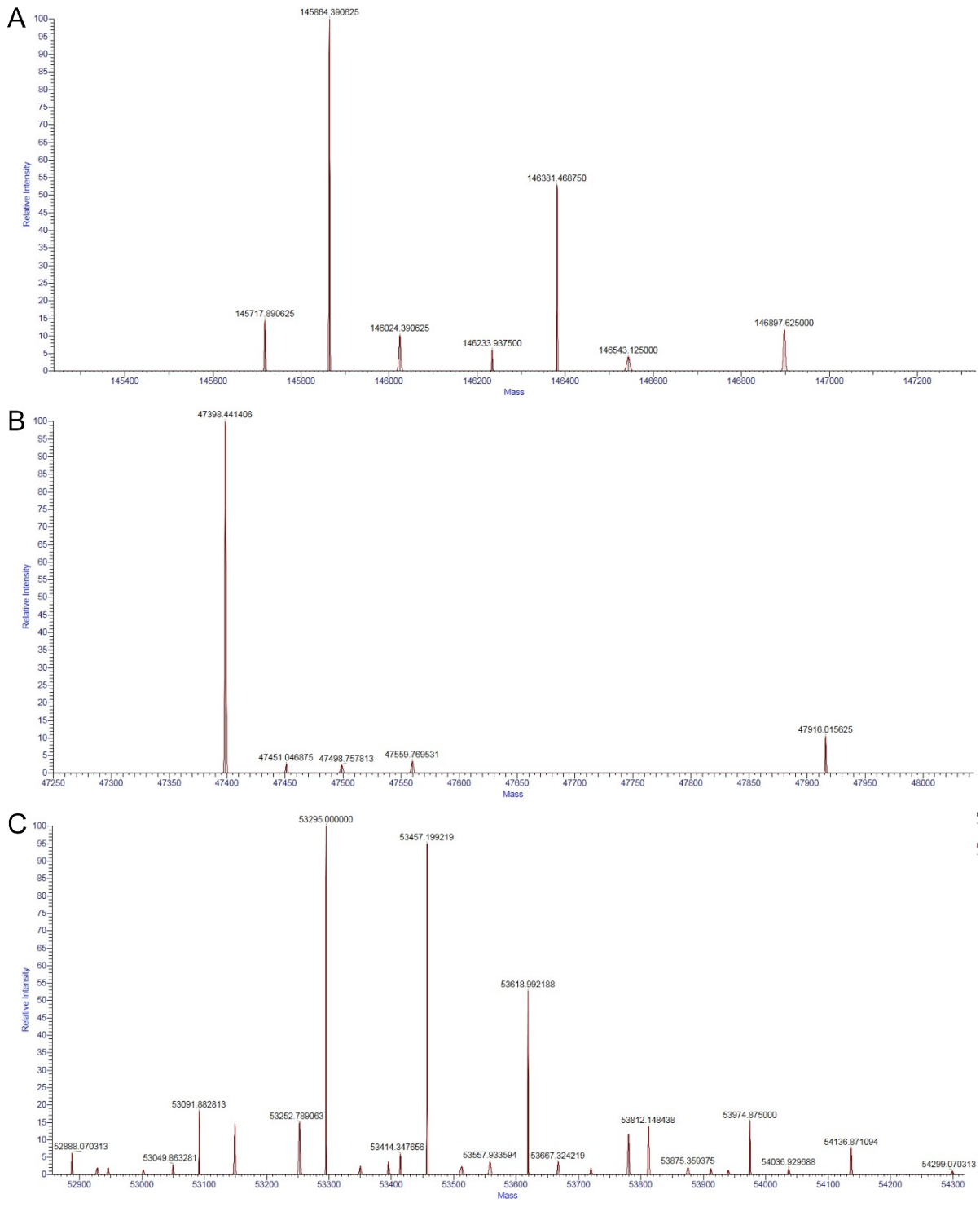


Figure S8. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG$_4$-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

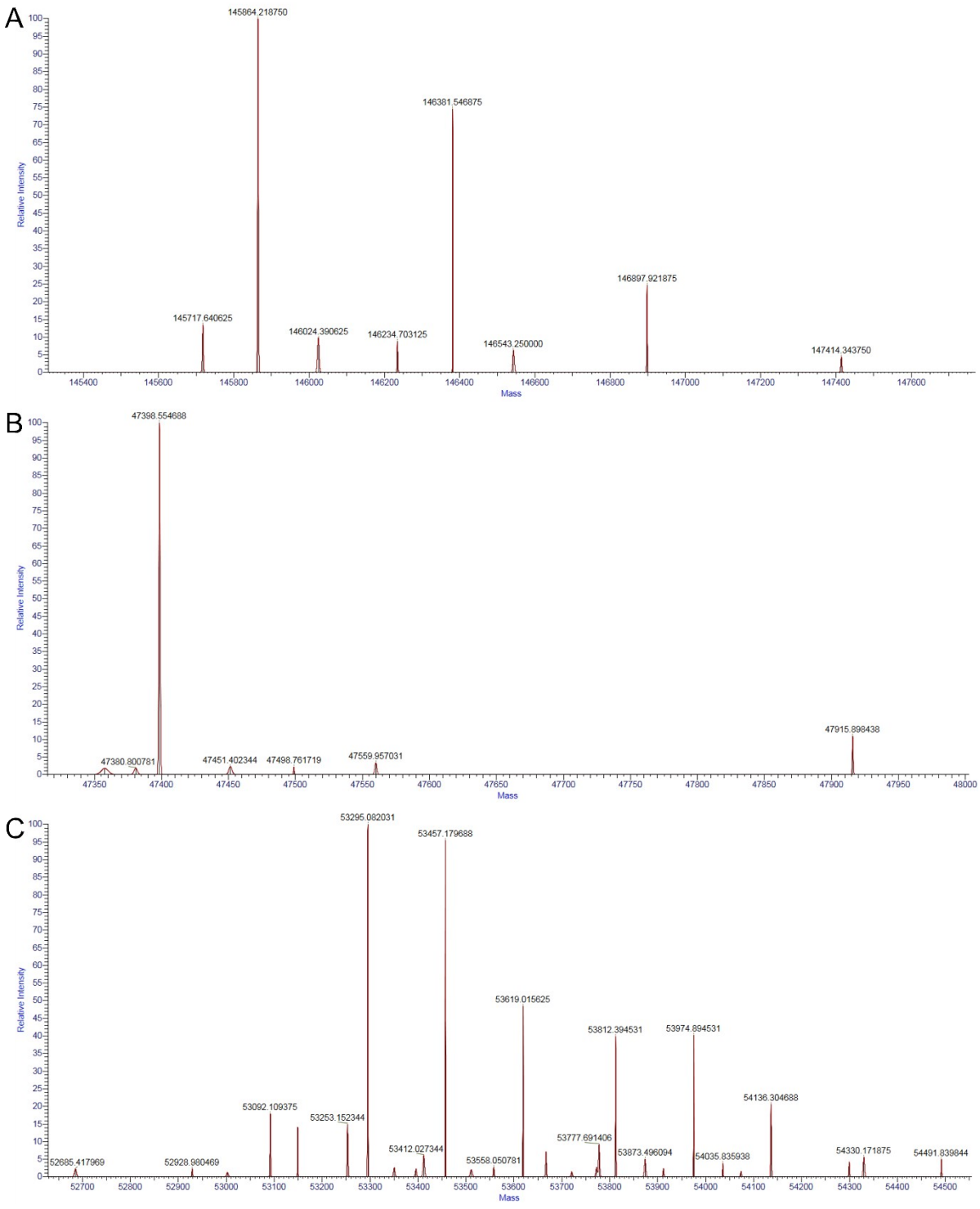


Figure S9. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG$_6$-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

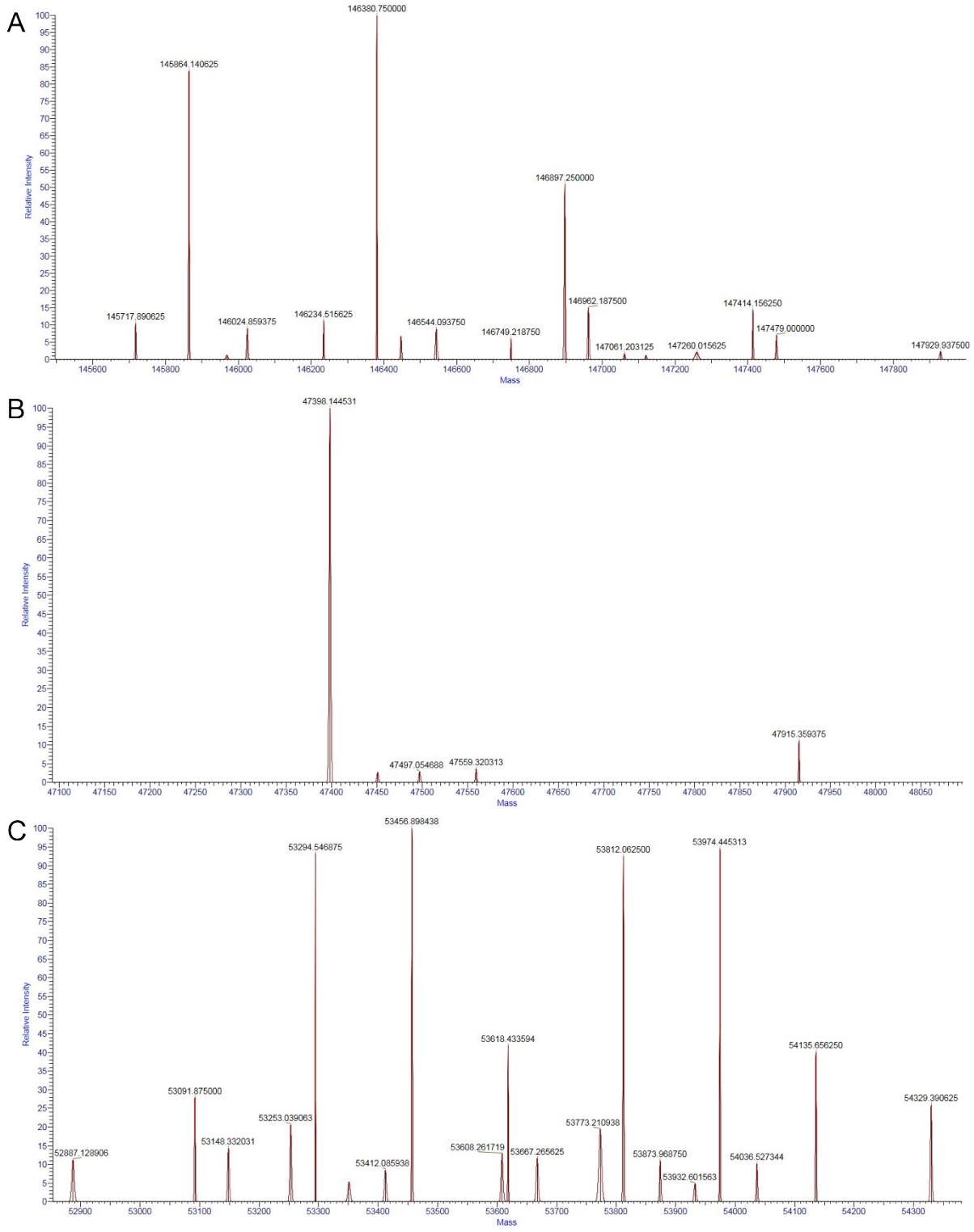


Figure S10. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG$_8$-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

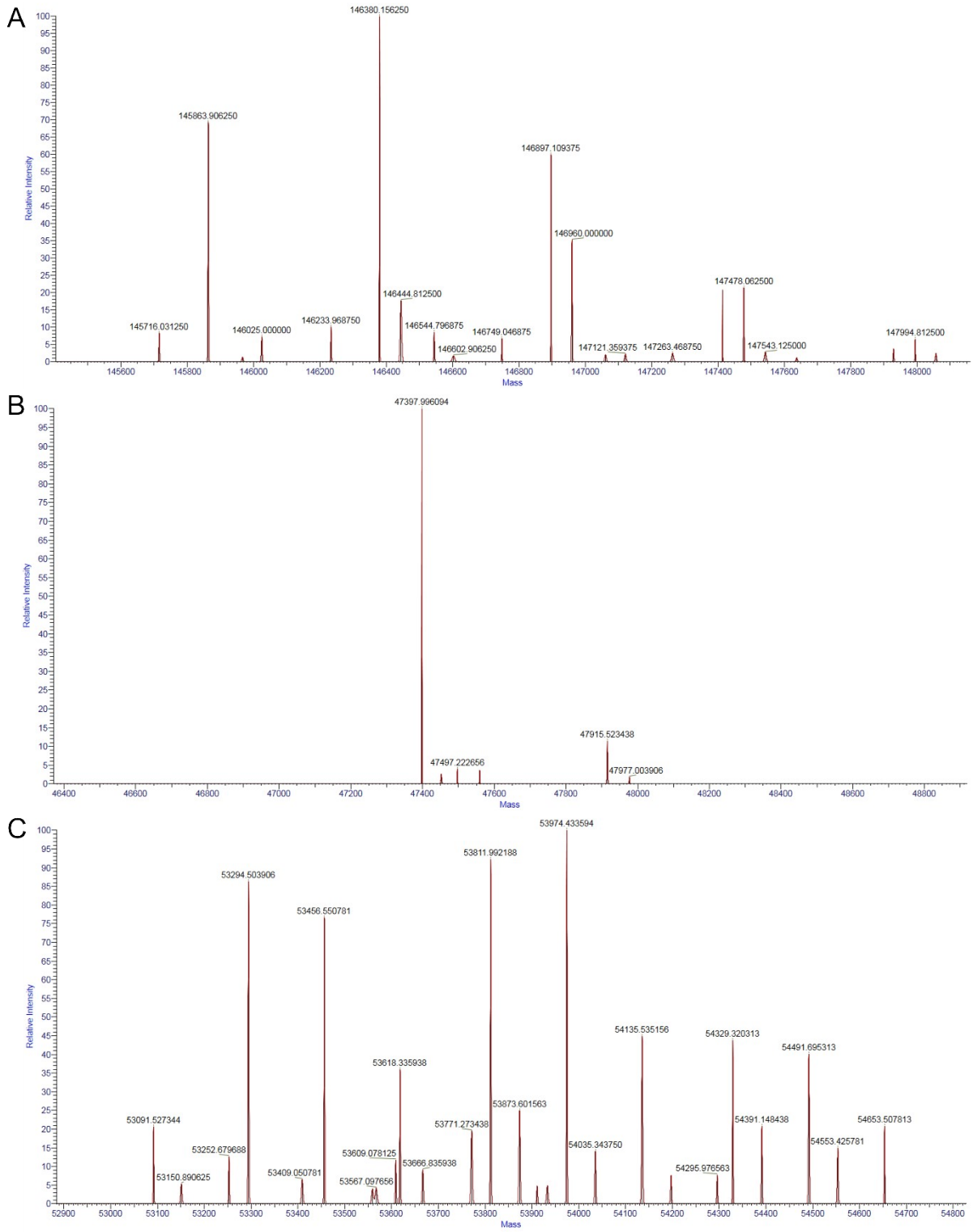


Figure S11. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG_{10}-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

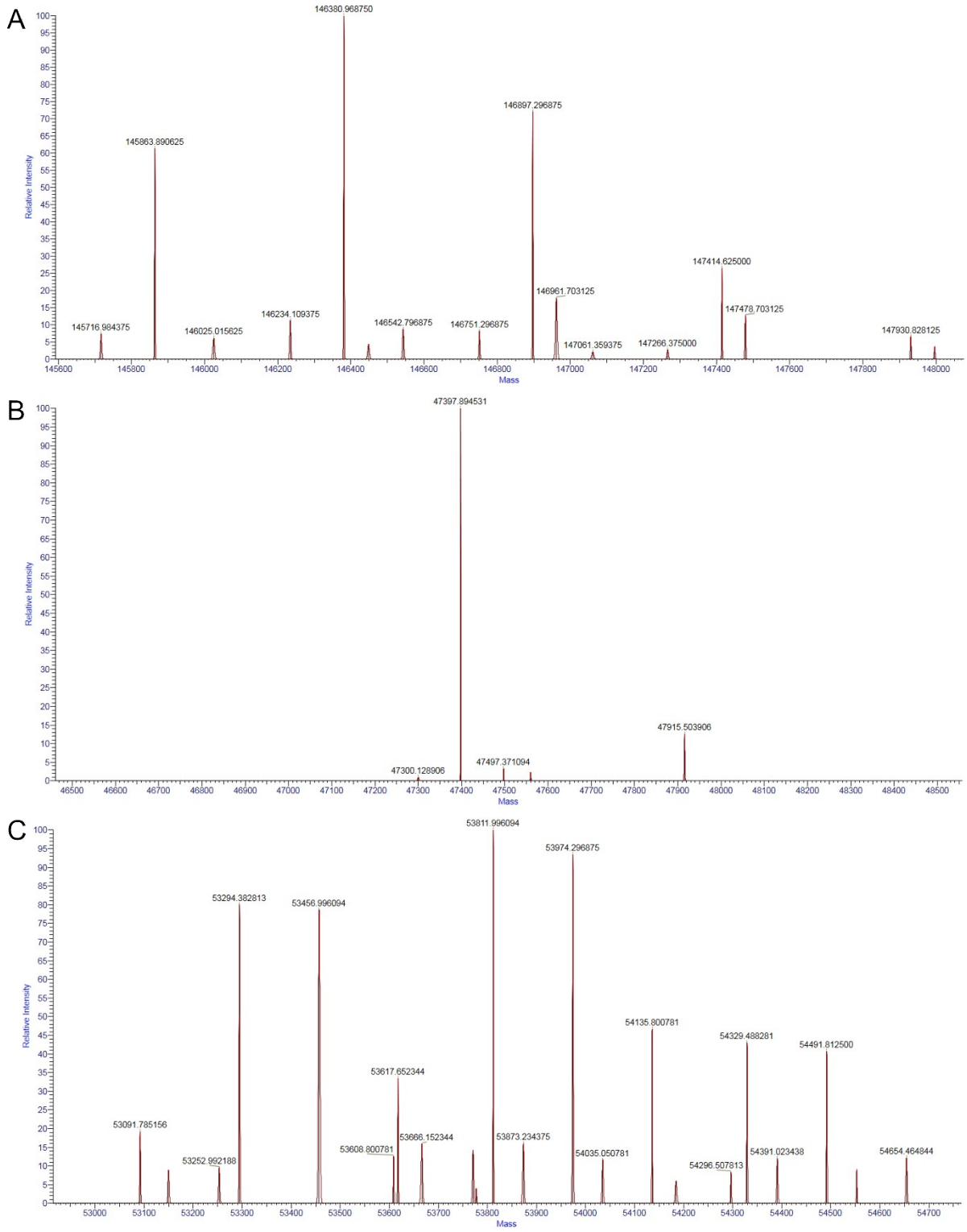


Figure S12. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG_{12}-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

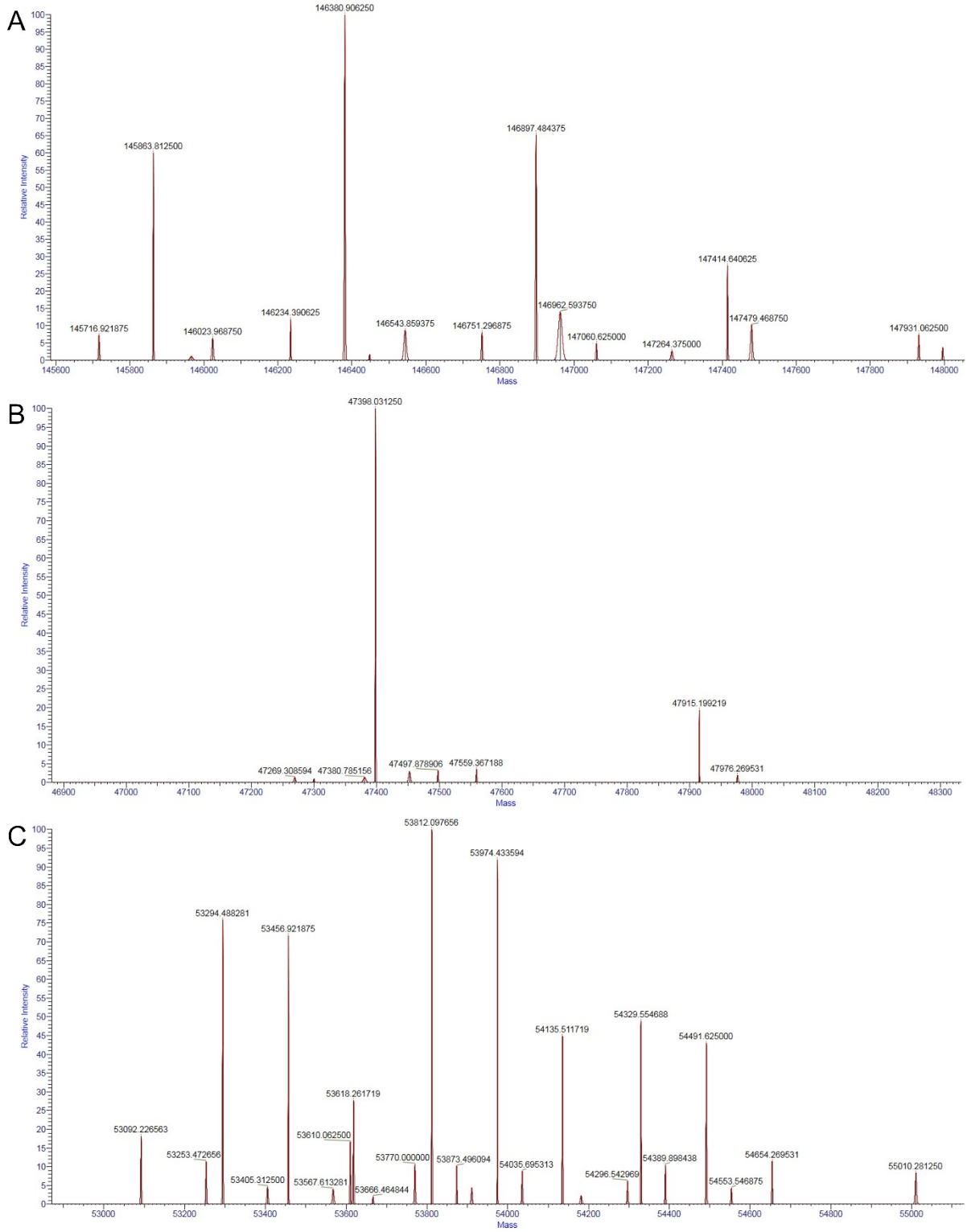


Figure S13. Deconvolved intact mass spectra of trastuzumab-DOTA (after the reaction of DOTA-PEG_{36}-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

Figure S14. Middle-down fragmentation maps of the IgG-Fc/2 subunit after labeling with DOTA-PEG$_{10}$-Fc-III demonstrating identified main conjugation sites: for one DOTA molecule being K81 (A) and for two DOTA molecules being K81 and K90 (B). Obtained sequence coverage for Fc/2-DOTA(s) subunits was 40% (A) and 37% (B), respectively. The payload conjugation site K (addition of 517.2384 Da) and glycans attachment site N (presence of 1444.5339 Da, G0F) are highlighted in yellow, identified b- and y-fragment ions – in blue. The DOTA labeling at position K81, and K90 within Fc/2 correspond to K317 and K326 in an intact Hc, respectively.

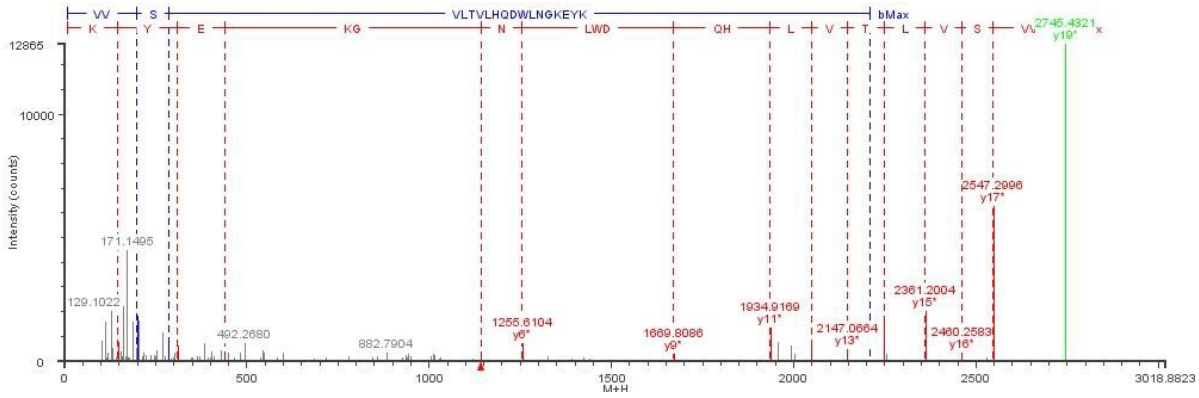


Figure S15: MS^E fragmentation data generated by BiopharmaLynx software for peptide HC302-320 (302VVSVLTVLHQDWLNGKEYK320) showing detected b and y" fragment ions. The fragment ions marked with an asterisk (*) carry the DOTA payload and allow to identify the antibody conjugation site. This data was generated by M-Scan.

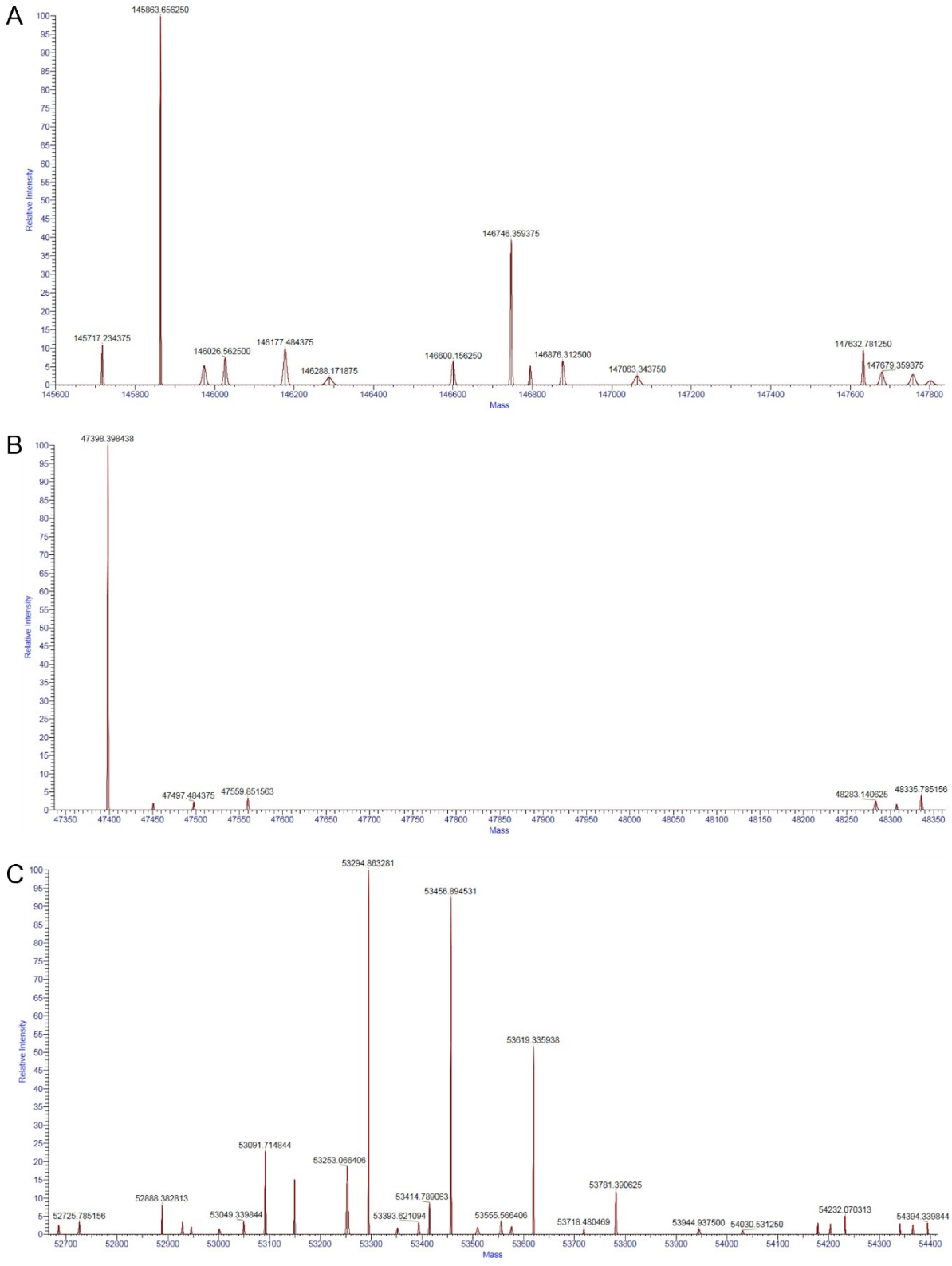


Figure S16. Deconvolved intact mass spectra of trastuzumab-DFO (after the reaction of DFO-PEG_{10}-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

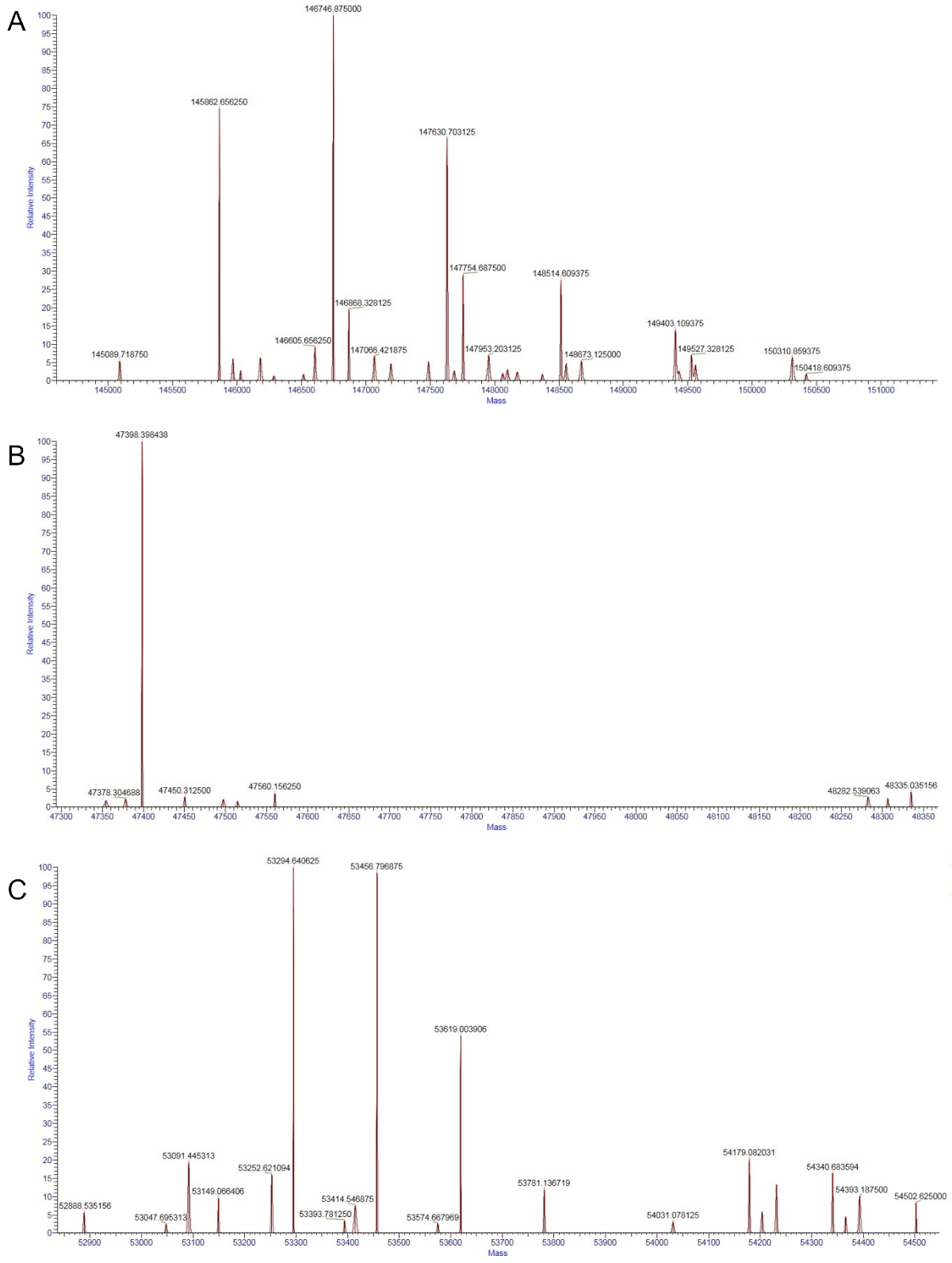
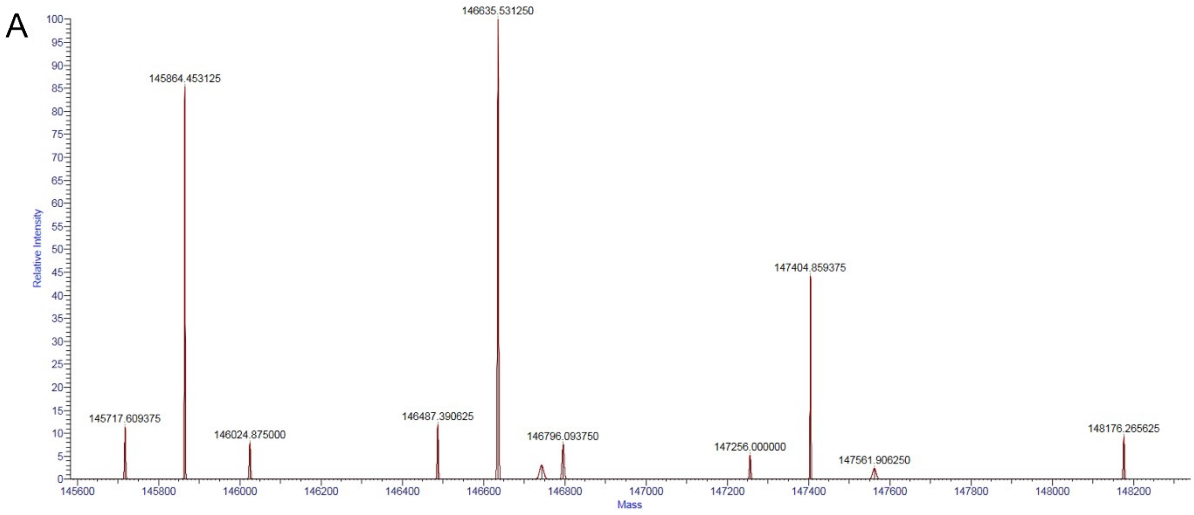
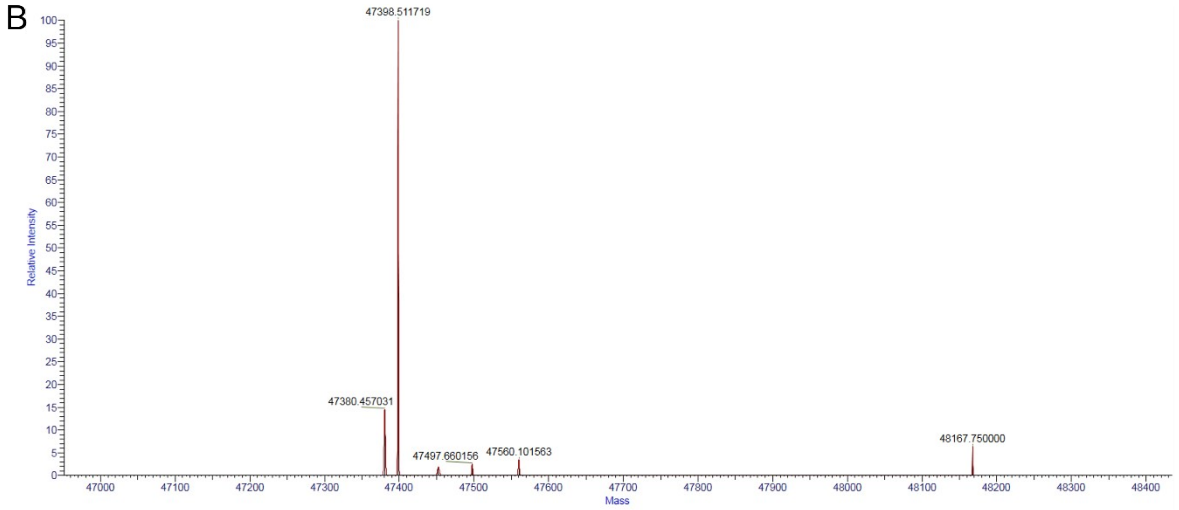


Figure S17. Deconvolved intact mass spectra of trastuzumab-DFO (after the reaction of 6 eq of DFO-PEG_{10}-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C).

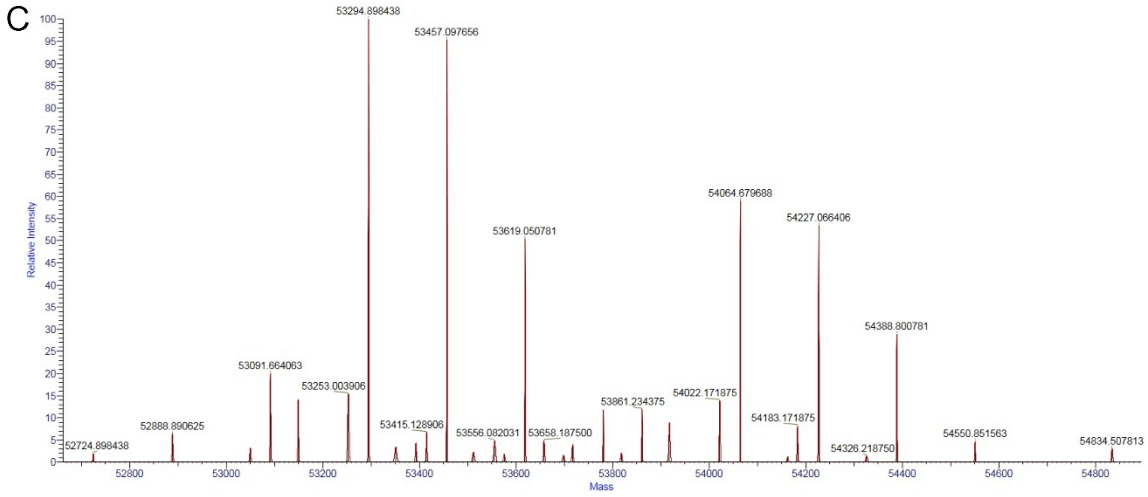
A


146635.531250
145864.453125
147404.859375
145717.609375
146024.875000
146487.390625
146796.093750
147256.000000
147561.906250
148176.265625
Relative Intensity
Mass

B


47398.511719
47380.457031
47497.660156
47560.101563
48167.750000
Relative Intensity
Mass

C


53294.898438
53457.097656
54064.679688
54227.066406
53619.050781
54388.800781
53091.664063
53253.003906
53861.234375
54022.171875
54183.171875
52888.890625
53415.128906
53556.082031
53658.187500
54326.218750
54550.851563
54834.507813
52724.898438
Relative Intensity
Mass

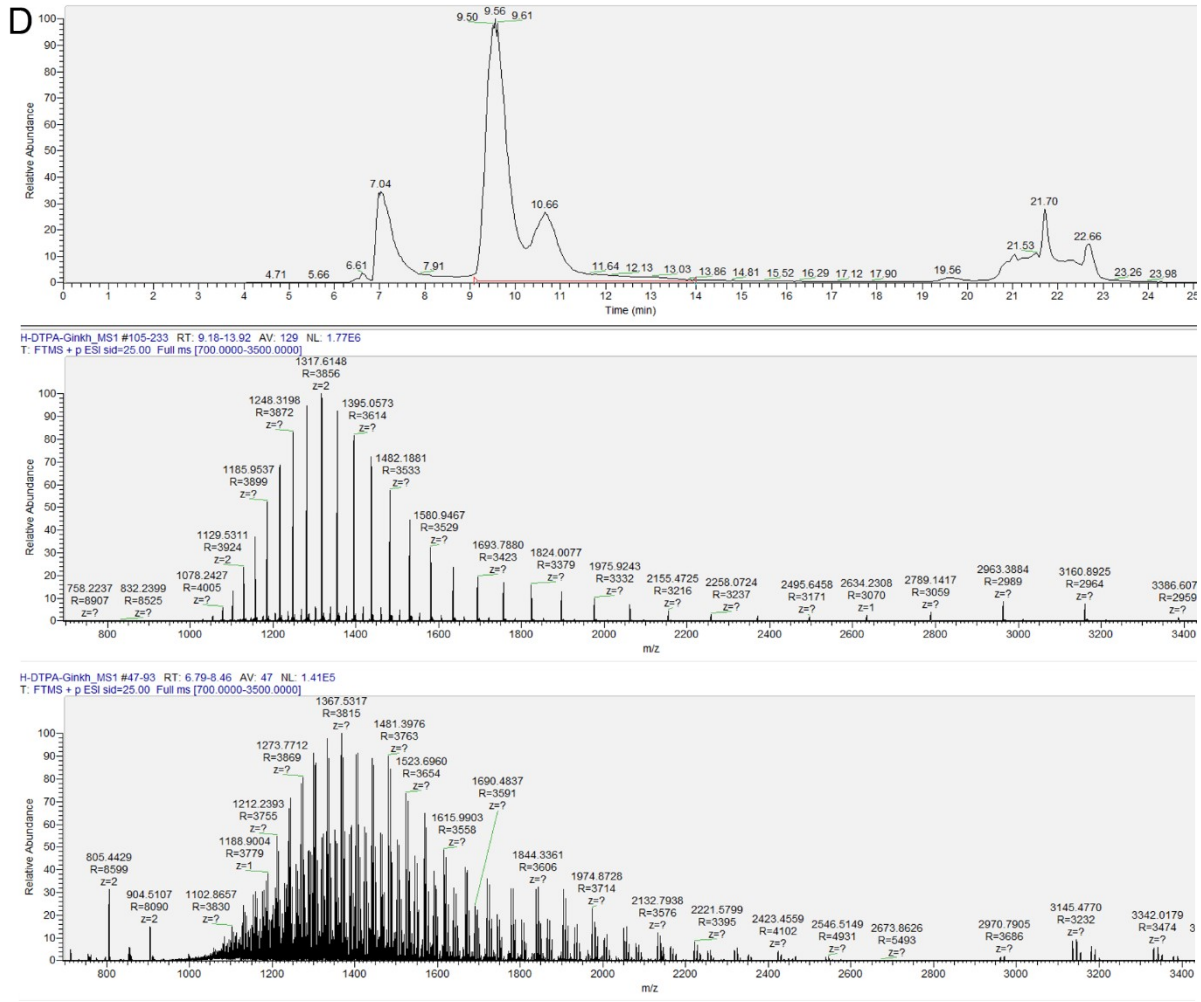
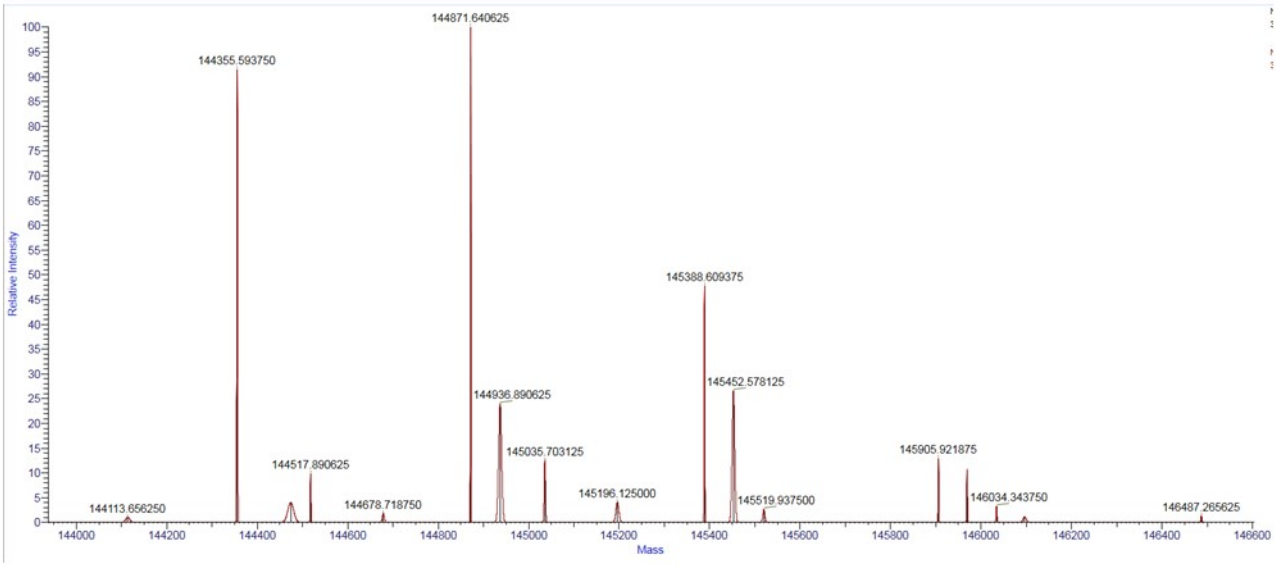
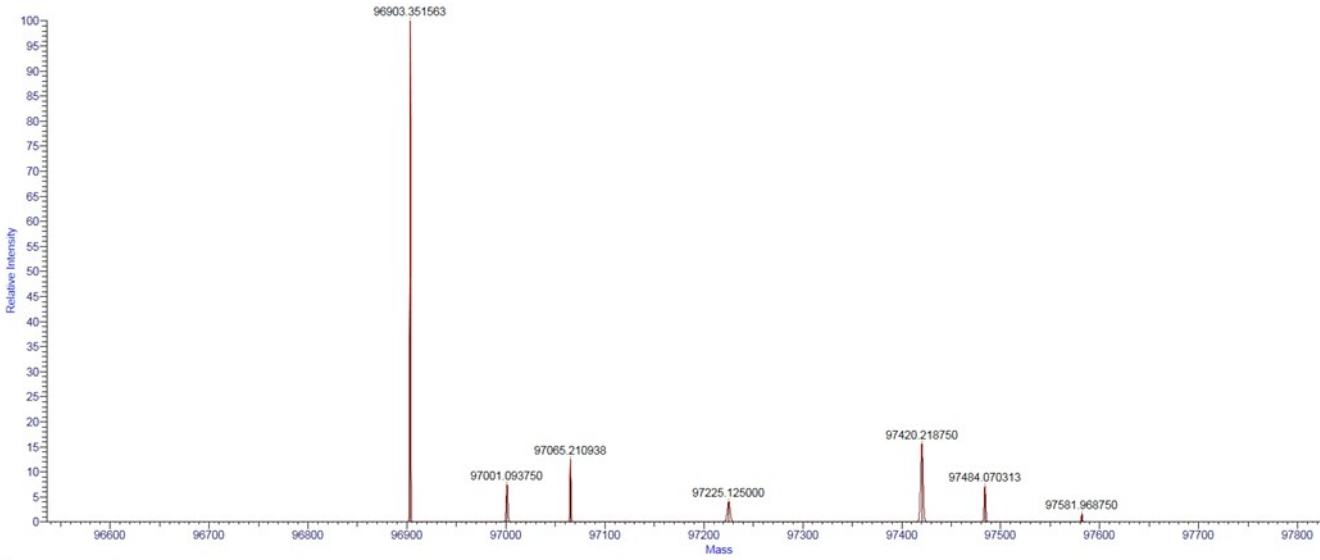


Figure S18. Deconvolved intact mass spectra of trastuzumab-DTPA (after the reaction of DTPA-PEG$_{10}$-Fc-III with trastuzumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into Fab (B) and Fc (C). Raw mass spectrum of cleaved trastuzumab-DTPA into Fc and Fab (D).

A


144871.640625
144355.593750
145388.609375
144936.890625
145452.578125
145035.703125
144517.890625
145905.921875
145196.125000
144678.718750
145519.937500
146034.343750
144113.656250
146487.265625
Relative Intensity
Mass

B


96903.351563
97420.218750
97065.210938
97001.093750
97484.070313
97225.125000
97581.968750
Relative Intensity
Mass

C

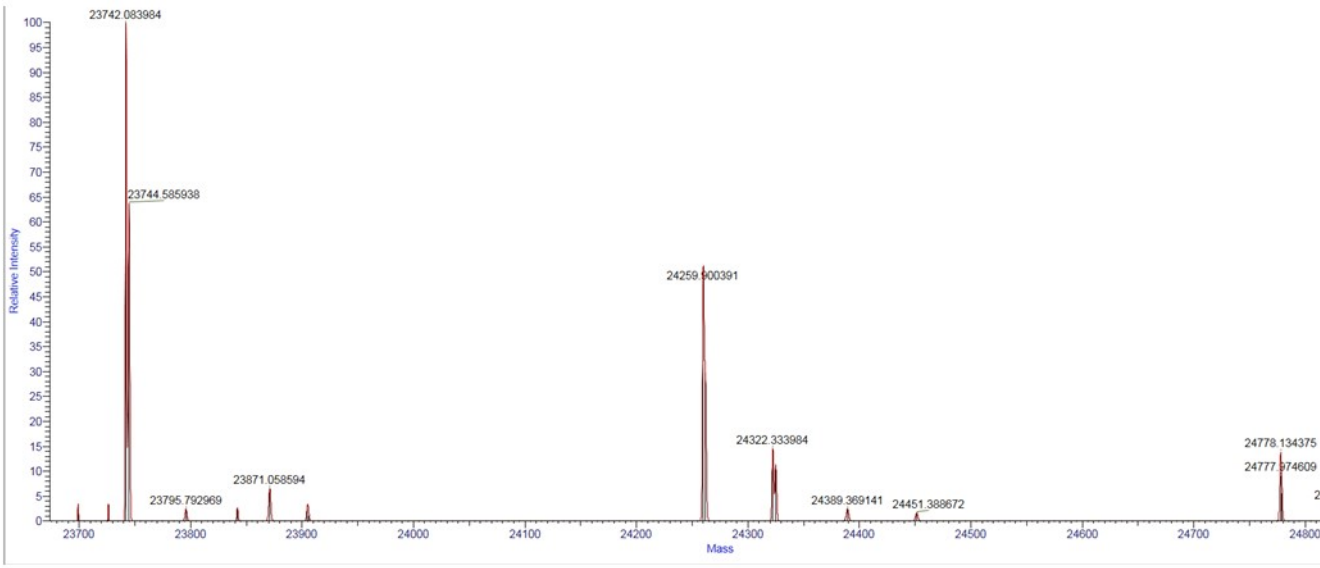

23742.083984
23744.585938
24259.900391
24322.333984
24778.134375
24777.974609
23871.058594
23795.792969
24389.369141
24451.388672
Relative Intensity
Mass

Figure S19. Deconvolved intact mass spectra of atezolizumab-DOTA (after the reaction of DOTA-PEG_{10}-Fc-III with atezolizumab): deglycosylated intact antibody conjugate (A); antibody conjugate cleaved into $F(ab')_2$ (B) and Fc/2 (C).

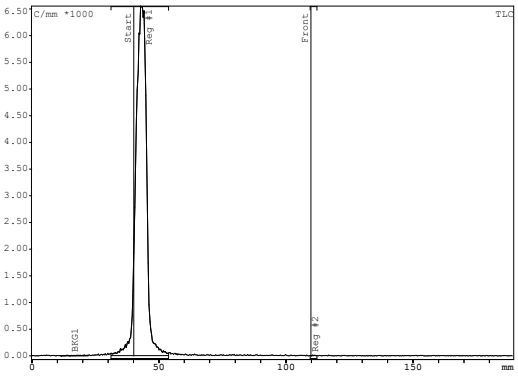


Figure S20. iTLC radiochromatograph of the unpurified [^{111}In]In-DTPA-trastuzumab which remain at $R_f = 0$ while the unbound ^{111}In radiochemical species migrate to the solvent front, indicating the radiochemical purity of >99%.

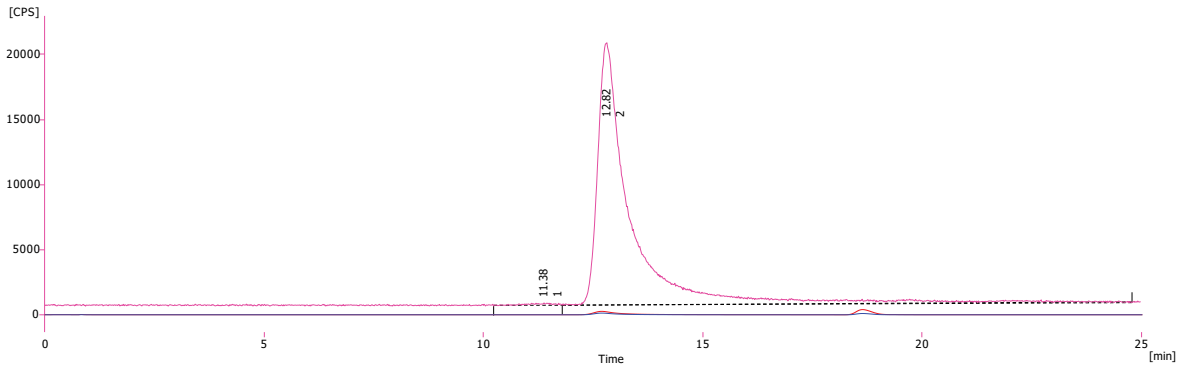


Figure S21. Radio-SEC profile of [^{111}In]In-DTPA-trastuzumab obtained after radiolabeling, confirming the iTLC results. The chromatogram shows <1% of aggregation.

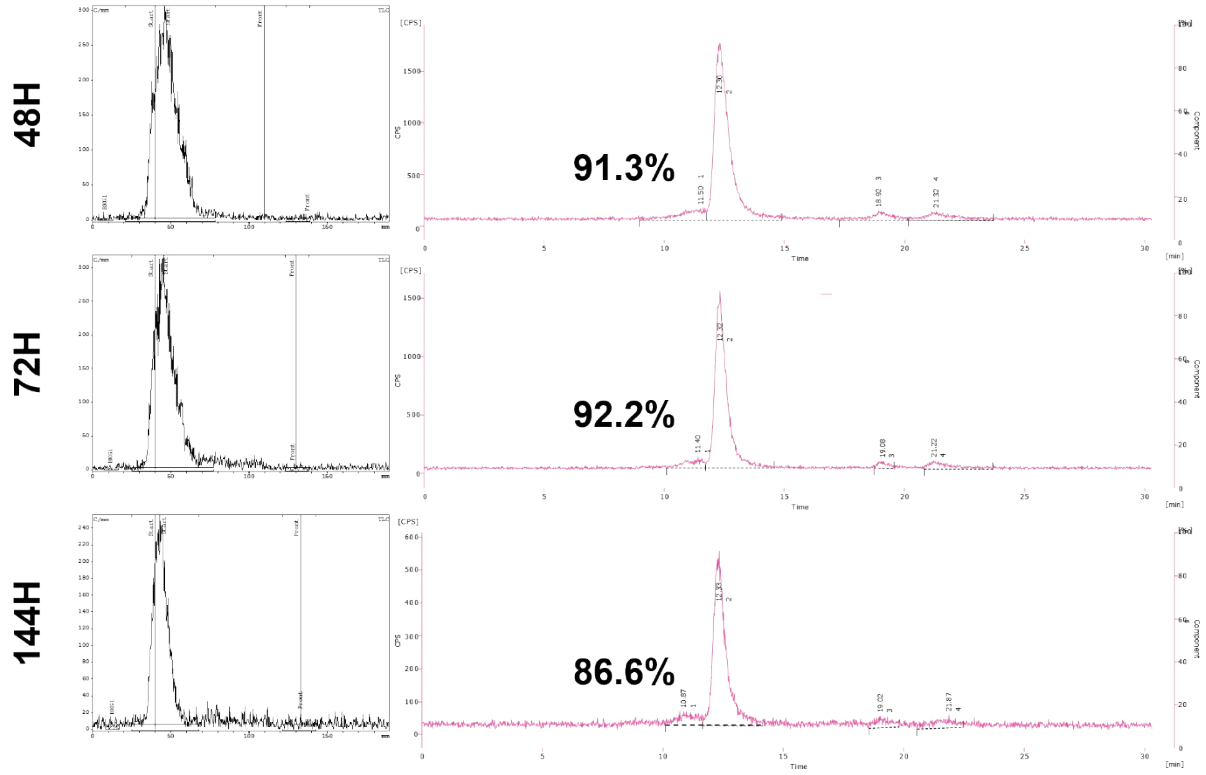


Figure S22. The stability studies performed by iTLC and radio-SEC of [^{111}In]In-DTPA-trastuzumab incubated in human serum for 48, 72, and 144 h. A decrease of the amount of [^{111}In]In-DTPA-trastuzumab in human serum was observed from 91.3% at 48 h to 86.6% at 144 h. The percentage of [^{111}In]In-DTPA-trastuzumab after serum incubation was calculated as a ratio of the area under the peak of [^{111}In]In-DTPA-trastuzumab to the sum of the areas of [^{111}In]In-DTPA-trastuzumab and unbound ^{111}In.